Fetal and Neonatal Alloimmune Thrombocytopenia: evidence based screening

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evidence based screening

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Voor mijn ouders

Voor Wouter

Table of contents

Chapter 1

General introduction and scope of this thesis

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Fetal and Neonatal Alloimmune Thrombocytopenia

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is the most important and most frequent cause of fetal and early neonatal thrombocytopenia in term-born infants.[1] During normal fetal life, the platelet count progressively increases, and reaches a constant level by the end of the first trimester. As in adults, normal fetal and neonatal platelet counts range from 150 $\times 10^9$/L to 450 $\times 10^9$/L. Thrombocytopenia is defined as a platelet count below 150 $\times 10^9$/L.[2,3] The degree of the thrombocytopenia can be further classified into either mild (100 – 150 $\times 10^9$/L), moderate (50 – 100 $\times 10^9$/L), severe (< 50 $\times 10^9$/L) or very severe (< 20 – 30 $\times 10^9$/L). Of all term-born infants, 2% have platelet counts below 150 $\times 10^9$/L and 0.1 – 0.2% suffer from a severe thrombocytopenia.[4] Approximately half of the cases with severe neonatal thrombocytopenia are caused by FNAIT.[1,5] Other conditions that are associated with fetal/neonatal thrombocytopenia can be divided into causes that lead to an increased destruction or a decreased production of platelets (Table 1.1).

Table 1.1 – Causes of fetal and early neonatal thrombocytopenia

Increased destruction
Immune thrombocytopenia
Maternal autoimmune (ITP, SLE)
Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT)
Severe fetal hemolytic disease due to red cell alloimmunization
Alloimmune drug-induced (penicillin, anti-epileptica, quinidine, indomethacin)
Peripheral consumption
Hypersplenism
Kasabach-Meritt
Disseminated intravascular coagulation (DIC)
Thrombosis (e.g. aortic, renal vein)
Decreased production
Genetic disorders (TAR syndrome, trisomy 13,18,21, triploidy, Turner's syndrome, amegakaryocytosis, Wiskott-Aldrich, May-Hegglin, Bernard-Soulier, Alport syndrome)
Bacterial infection (GBS, E.Coli, Listeria, Syphilis)
Viral infection (CMV, parvo, rubella, HIV, HSV)
Parasite infection (toxoplasmosis)
Asphyxia
Placental insufficiency (pre-eclampsia, IUGR, diabetes, premature birth)

GBS, group-B Streptococcus; CMV, cytomegalovirus; HIV, human immunodeficiency virus; HSV, Herpes Simplex Virus; ITP idiopathic thrombocytopenia; IUGR, intrauterine growth retardation; SLE, systemic lupus erythematosus; TAR, thrombocytopenia-absent radii syndrome.

Pathophysiology

FNAIT caused by alloantibodies against the foreign, paternally derived, fetal human platelet antigens (HPAs, figure 1.1). Consecutive conditions to be fulfilled are an incompatibility between mother and fetus, maternal alloantibody formation, active placental transport of antibodies into the fetal circulation and destruction of fetal cells. Exposure to the foreign, fetal HPA can occur physiologically as well as in pathological conditions. Fetal blood cells enter the maternal circulation, a phenomenon called fetomaternal hemorrhage (Figure 1.1B). This can occur spontaneously and often asymptomatically in healthy pregnancies, during or after delivery, as a result of invasive procedures or after abdominal trauma. In addition, the maternal circulation is exposed to the fetal placental tissue, in particular to the syncytiotrophoblast cells, which express the integrin β3 containing various HPA epitopes. Then, the exposure to the incompatible platelet antigens needs to be followed by a maternal immune response to result in the formation of platelet-specific alloantibodies (Figure 1.1C). Lastly, these alloantibodies, of the immunoglobulin G (IgG) subclass, can get in contact with the fetal HPA again, primarily by entering the fetal circulation through active transport across the placenta, by the neonatal Fc-receptor (FcRn, figure 1.1D). After entering the fetal circulation, these alloantibodies can cause destruction of fetal platelets and potentially damage other fetal cells containing the specific antigen involved.

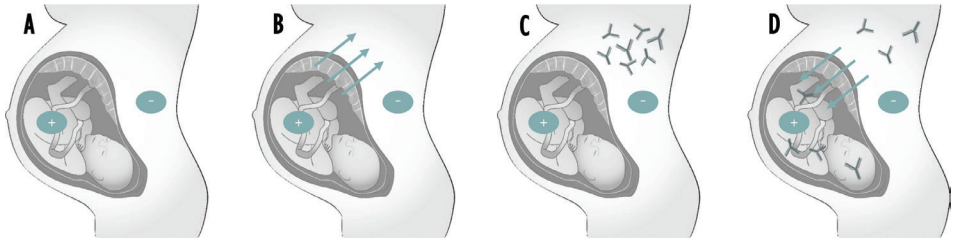

Figure 1.1 – Pathophysiology alloimmunization during pregnancy

A. Incompatibility between mother and fetus. **B.** Maternal exposure to fetal, paternally derived, antigens through fetomaternal transfusion. **C.** Maternal alloimmunization, formation of specific IgG alloantibodies. D. Transplacental transport of specific alloantibodies through FcRn into the fetal circulation.

Platelets

To date, 37 different HPAs have been identified and known to cause FNAIT. Various HPA epitopes are created by single nucleotide polymorphisms (SNPs) that result in small changes in the glycoprotein (GP) structure through an amino acid change.[6] The six different glycoprotein (GP) complexes (IIb/IIIa, Ib/IX, Ia/IIa and CD109), containing these 37 epitopes, are located on the platelet membrane. Twelve high-frequency HPAs are clustered into six biallelic groups; HPA-1, 2, 3, 4, 5 and 15 (Table 1.2).

The majority of HPAs are localized on GPIIb/GPIIIa (or integrin αIIbβ3), which is the most abundant membrane protein complex on the surface of platelets. In FNAIT in the Caucasian population, HPA-1a is the most targeted antigen, responsible for approximately 80% of the cases, followed by HPA-5b, which accounts for circa 10% of the cases (Table 1.3).[7,14-16] Genetic differences between ethnic populations lead to a variance in distributions of these incidences. For example, in the Asian population, anti-HPA-4b is the most frequently involved antibody, followed by anti-HPA-3a and anti-HPA-21b.[17-19] Furthermore, antibodies targeted against glycoprotein IV (also called CD36) are rarely seen in Caucasians (< 0.3% of cases), but are more frequently involved in FNAIT in African (7 – 8% of cases) and Asian (3 – 11% of cases) population.[13,20]

Endothelial cells

Glycoproteins containing the epitopes of HPAs are not solely present on platelets. Glycoprotein IIIa or integrin β3, containing the most HPAs, including HPA-1a, is expressed on the membranes of platelets in a heterodimer with GPIIb (integrin αIIb). In addition, integrin β3 can form a heterodimer with αV as well. This αVβ3 complex, still carrying the HPA-1a epitope, is scarcely expressed on platelets, but prominently present on the membrane of endothelial cells.[22-24] This raises an interesting dynamic: considering the fact that the pathogenic mechanism resulting in devastating intracranial hemorrhage (ICH) in FNAIT has never been adequately understood. Generally, alloantibodies are thought to enter the fetal circulation and cause bleeding complications and thrombocytopenia through destruction of fetal platelets. A theory that is not exactly airtight, given that severe bleedings have been described in only moderate thrombocytopenia and only a small proportion of severely thrombocytopenic new-borns actually suffer from bleeding complications. Additionally, research shows that mice that are completely lacking circulating platelets, survive in utero and do not bleed.[25] This combination of unexplained pathogenic bleeding mechanisms and the fact that the most involved antigen in FNAIT is present on endothelial cells has led to new insights. First, *in vitro* studies illuminated the direct interaction between anti-HPA-1a and human umbilical vein endothelial cells (HUVECs), demonstrated by a decreased HUVEC spreading as well as a decreased integrity of their monolayer in electric cell-substrate impedance sensing (ECIS) assays.[26] Second, a large *in vivo* study with both active and passive murine models of anti-HPA-1a mediated FNAIT showed that ICHs in these mice occurred regardless of platelet count. Also, HPA-1a antibodies inhibited angiogenic signaling, induced endothelial cell apoptosis and decreased vessel density in affected brains as well as retinas.[27] Lastly, a recent study with human sera of women with HPA-1a alloantibodies that caused FNAIT, suggested a correlation between the specific interaction and binding of the antibodies with αVβ3 only and whether or not an ICH had occurred in these pregnancies.[28]

Table 1.2 – Human platelet antigens and their prevalence

Antigen	Gene, chromosome, nucleotide change	Amino acid change	Allele or *phenotype*
HPA-1	*ITGB3*, 17, 196T>C rs5918	L33P	HPA-1a
			HPA-1b
			HPA-1b/b
HPA-2	*GP1BA*, 17, 482C>T rs6065	T145M	HPA-2a
			HPA-2b
			HPA2b/b
HPA-3	*ITGA2B*, 17, 2621T>G rs5911	I843S	HPA-3a
			HPA-3b
			HPA-3b/b
HPA-4	*ITGB3*, 17, 506G>A rs5917	R143Q	HPA-4a
			HPA-4b
			HPA-4b/b
HPA-5	*ITGA2,* 5, 16000G>A rs10471371	E505K	HPA-5a
			HPA-5b
			HPA-5a/a
HPA-15	*CD109*, 6, 2108C>A rs10455097	S682Y	HPA-15a
			HPA-15b
			HPA-15b/b
CD36, GPIV[¥]	CD36, 7, *variable*	*variable*	*GPIV negative*

GP, glycoproteint; HPA, human platelet antigen. Source: Immuno Polymorphism Database, https://www.ebi.ac.uk/ipd/hpa/freqs_2.html.

¬ Caucasian (French) population, n = 525-6135 7; ^North-African (Moroccan Berber) population, n = 104-112 8; °African (Egyptian) population, n = 367 9; ¤Sub-Sahara African (Congo), n = 125 10; ǂ African-American population (USA), n = 100 11; *Asian (Chinese Han) population. n = 1000 12; ¥ All phenotype numbers extracted from Curtis et al.13.

	Frequencies				
	Caucasian¬	North-African^	Sub-Sahara African¤	African-American‡	Asian*
	0.848	0.748	0.904	0.92	0.994
	0.152	0.252	0.096	0.08	0.006
	0.02	0.084	0.008	0	0
	0.92	0.818	0.776	0.82	0.952
	0.08	0.182	0.224	0.18	0.049
	0.006	0.028	0.040	0.03	0.001
	0.62	0.616	0.596	0.63	0.595
	0.38	0.384	0.434	0.37	0.406
	0.15	0.125	0.168	0.15	0.169
	1	1	1	1	0.996
	0	0	0	0	0.005
	<0.001	<0.001	0	0	0
	0.874	0.902	0.732	0.79	0.986
	0.126	0.098	0.268	0.21	0.014
	0.813	0.732	0.944	0.62	0.973
	0.455	0.861	0.701	-	0.532
	0.545	0.139	0.299	-	0.468
	0.23	0.221°	0.094	-	0.217
	-	<0.004	0.08	0.02	0.11

Table 1.3 – Alloantibodies involved in FNAIT

Authors	Number of patients	Alloantibody detected	Frequency	Alloantibody detected	Frequency
Mueller-Eckhart, 1989[15]	106	anti HPA-1a	90%	anti-HPA-3a	0.8%
		anti HPA-5b	8%	anti HPA-1a + 5b	0.8%
		anti HPA-1b	0.8%	anti-B	0.8%
Porcelijn, 2004	217	anti HPA-1a	73.7%	anti HPA-1b	1.4%
		anti HPA-5b	14.7%	anti HPA-15a	0.5%
		anti HPA-3a	4.6%	anti HPA-15b	0.5%
		anti Priv Ag	1.5%	anti A or anti B	2.8%
Davoren, 2004[16]	1162	anti HPA-1a	79%	anti GPIV (CD36)	0.4%
		anti HPA-5b	9%	anti HPA-4a	0.1%
		anti HPA-1b	4%	anti HPA-4b	0.1%
		anti HPA-3a	2%	anti HPA-6bw	0.1%
		anti HPA-5a	1%	Combinations	3.1%
		anti HPA-3b	0.8%		
Knight, 2011[21]	151	anti HPA-1a/b	81%	anti HPA-1a + 5b	5%
		anti HPA-5a/b	7%	Other	7%

FNAIT, fetal and neonatal alloimmune thrombocytopenia; HPA, human platelet antigen.

Placental function

In addition to platelets and endothelial cells, αVβ3 is also expressed on placental tissue by syncytiotrophoblast cells. Though there is no direct evidence, it has been suggested that anti-HPA-1a might induce placental insufficiency through interaction with these syncytiotrophoblast cells, possibly demonstrated by an association with intrauterine growth restriction (IUGR), as well as cases of intrauterine fetal demise (IUFD) in absence of bleeding problems.[29] Another correlation that has been suggested is one between FNAIT and miscarriages.[30] In addition, the expression of HPA-1a on placental tissue might lead to increased and early exposure to the fetal HPA-1a and might be a possible explanation for the high proportion of affected first pregnancies and first-born children in FNAIT.

Incidence

In the absence of population based screening, incidence and prevalence numbers have to be extracted from large prospective and preferably non-intervention studies. Such studies can be performed either postnatally, first screening for neonatal platelet count, followed by platelet-specific antibody testing, or antenatally, first screening for maternal HPA-type, then testing for antibody formation followed by assessment of neonatal outcome.

Postnatal estimation. When combining results from postnatal screening studies in all new-borns (n = 59,425), the incidence of severe FNAIT (platelet count < 50 × 10^9/L) was 0.04%, corresponding to 1 in 2500 new-borns, which led to an ICH in 25% of these cases.[31]

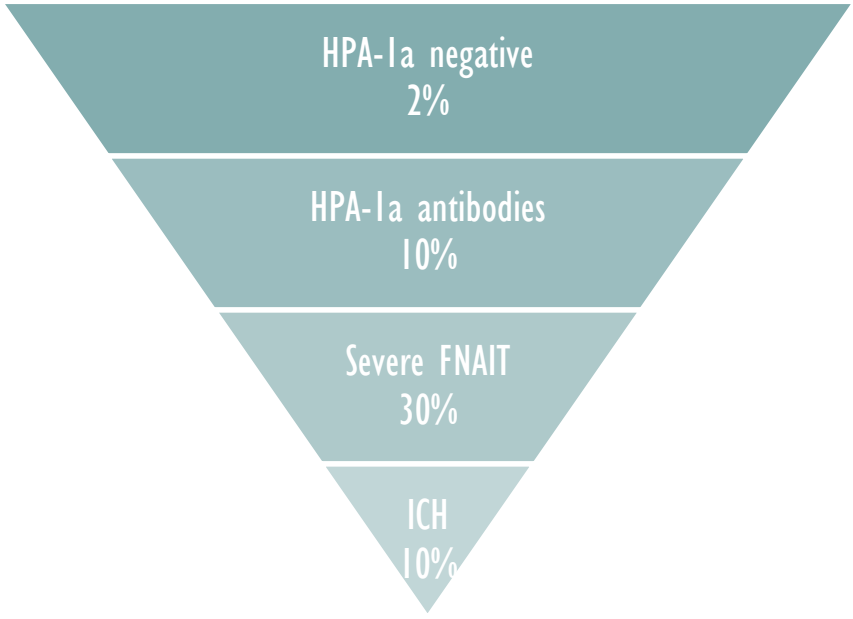

Figure 1.2 – Incidence of HPA-1a mediated FNAIT

FNAIT, fetal and neonatal alloimmune thrombocytopenia; HPA, human platelet antigen; ICH, intracranial hemorrhage.

Antenatal estimation. Antenatal cohort studies evaluating FNAIT incidence focus mainly on the predominant cause of FNAIT, HPA-1a alloimmunization. The largest prospective screening study, including 100,448 pregnant women in Norway, reported an incidence of HPA-1a negative women of 2.1%, leading to HPA-1a alloimmunization in 10.7%.[32] All alloimmunized women underwent an elective cesarean section at 2 – 4 weeks before term, so neonatal platelet counts and the incidence of bleeding problems are potentially an underestimation. They reported 58% of alloimmunizations to result in FNAIT, 33% in severe FNAIT and 2% of all alloimmunizations suffered from an ICH. A systematic review including ten prospective HPA-1a screening studies (n = 176,084 pregnant women), concluded that HPA-1a alloimmunization occurred in 9.7% of pregnancies at risk, leading to severe FNAIT in 31% of the cases and to perinatal ICH in 10% of the severe FNAIT cases (Figure 1.2).[33] While this review combined the largest screening studies performed thus far, only four of the ten studies did not perform an antenatal intervention. This leaves a total of 52,994 women observed, in which only a single ICH occurred. Therefore, no conclusions on natural history of the disease can be made and the above-mentioned estimates

are likely an underestimation of the true incidence of FNAIT caused by HPA-1a. The suggestion of underestimation was also put forth by an Irish study, that compared the diagnosed cases of FNAIT to the suggested number from the above mentioned screening studies.[34] Within eight years, 27 cases of FNAIT were diagnosed, all had bleeding manifestations and 20 were severely thrombocytopenic, which corresponds to 1 case of FNAIT in 16,500 live births. They compared this to estimated incidence in screening studies of 1 in 1,000 – 2,000 and therefore strongly suggested that FNAIT is currently underdiagnosed.

Clinical characteristics

FNAIT can have various clinical presentations. First, an asymptomatic thrombocytopenia might be detected as a chance finding without other signs of FNAIT. In these cases, FNAIT is usually only suspected after exclusion of other causes of fetal and early neonatal thrombocytopenia (Table 1.1). Second, mild bleeding symptoms might be present. These children might experience hematomas, petechiae, or small visceral bleeding. Also, transient hematuria or bloody stools might be seen.[15] Lastly, FNAIT can present with severe bleeding complications. Of these, an intracranial hemorrhage (ICH) is the most feared, due to its associated risk of lifelong disability and mortality.[35] An analysis of the short-term outcome of 43 cases of ICH showed that more than one-third (35%) resulted in perinatal death within four days of life and of the surviving children 82% suffered neurological disabilities.[35] Another cohort of consecutive cases of ICH at a single tertiary center showed an even higher mortality rate of 48%.[36] ICHs are estimated to originate before birth in over 80% of the cases, 67% of these bleedings started before 34 weeks' gestation and over half (54%) even before 28 weeks' gestation.[35,36] Also, the analysis by Tiller and colleagues showed that of the 43 cases of ICH, 23% occurred in first pregnancies, and 63% affected the first-born child.[35]

Diagnosis

In the absence of routine antenatal screening, suspicion of platelet alloimmunization leading to FNAIT usually arises in case of a clinically affected newborn (Figure 1.3A, 1.3B). Therefore, in the majority of the cases, diagnostic work-up is performed postnatally. However, antenatal suspicion and subsequent diagnostic work-up may be performed as well. This can be the case in antenatal ultrasound detection of fetal brain abnormalities, (Figure 1.3C, 1.3D) or because a sister of the pregnant women had a pregnancy complicated by FNAIT.

First, when HPA-alloimmunization FNAIT is suspected, diagnostic work-up should include HPA-typing of mother, father and child.[37] This way, possible HPA incompatibilities can be established. Second, an antibody screening should be performed to identify maternal platelet-specific alloantibodies, preferably using the MAIPA assay.[38] Additionally, tests for maternal autoantibodies, antibodies against private antigens expressed by the paternal platelets and Tpo levels can be part of the diagnostic work-up. Regulation of platelet production strongly

depends on the free plasma Tpo levels .[39-43] Therefore, plasma Tpo levels might be used to discriminate thrombocytopenia caused by megakaryocyte and platelet production failure (highly elevated Tpo levels) from thrombocytopenia caused by elevated platelet destruction as in ITP and FNAIT (normal or only slightly elevated Tpo levels).[44-48] FNAIT can be confirmed in case of a maternal-neonatal or maternal-paternal HPA incompatibility combined with the detection of alloantibodies for this specific HPA.

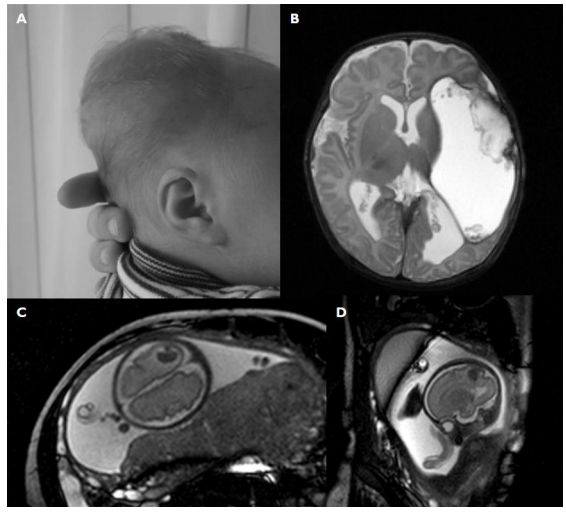

Figure 1.3 – Clinical aspects of FNAIT

A. Postnatal detection of FNAIT, large cephalic hematoma after vacuum assisted delivery, picture taken at 2 days of age. **B.** Postnatal detection of FNAIT, large intraparenchymal intracranial hemorrhage, Axial T2-weighted image of MRI obtained at 9 days of age showing porencephalic cysts both left parietal and right temporal. **C+D.** Antenatal detection of FNAIT, large intraparenchymal intracranial hemorrhage. T2-weighted image of MRI obtained at 28 weeks gestational age showing a hemorrhage left parietal.

Obstetric management

In current practice, preventive measures are virtually only available for subsequent pregnancies in women with known alloimmunization and diagnosed FNAIT due to a previously affected child. A rare exception concerns cases in which diagnostic work-up for FNAIT was performed following a sister with an affected child. Pregnancies at risk for FNAIT are best managed in a tertiary center with both obstetric and neonatal expertise in this disease. First, paternal genotype should be considered to assess the risk of an incompatible pregnancy. In case of paternal homozygosity, every next pregnancy for this couple will be incompatible by definition. In case of paternal heterozygosity, however, there is a 50% chance that the fetus is compatible with the mother and the pregnancy is not at risk to be complicated by FNAIT. In these cases, fetal genotype has to be determined to assess the need for monitoring and potential preventive treatment. For HPA-1a, the predominantly involved alloantibody, fetal status can be determined using non-invasive

testing of cell-free placental DNA in maternal plasma.[49] In recent years also non-invasive tests for other HPAs, based on massive parallel sequencing[50,51] or on digital droplet polymerase chain reaction (PCR, Hyland CA, personal communication) have been developed.

Risk assessment and monitoring

Once incompatibility between mother and fetus is confirmed, close ultrasound monitoring, specifically of the fetal brain, should be performed every 2-4 weeks. At this stage, clinicians should ideally be able to evaluate and monitor fetal disease severity as well as predict the occurrence of severe bleeding. Unfortunately, unlike in hemolytic disease of the fetus and newborn (HDFN), the red cell counterpart of FNAIT, there are no antenatal non-invasive diagnostic tests available to assess disease severity before severe bleeding complications occur. The only possibility is to assess fetal platelet count by fetal blood sampling (FBS), which means puncturing the umbilical cord. Besides the fact that this procedure is risky, in particular when platelets are low, platelet counts are not linearly correlated to disease severity. Because of this lack of reliable non-invasive diagnostic tools to guide obstetric management and treatment, several possible markers to select pregnancies at high risk have been suggested.

Antibody level. In some centers, antibody levels and titers are monitored by titration and quantification. While high titers do seem to be correlated to the severity of FNAIT, this is not a consistent relationship, and there are cases of severe hemorrhages with barely detectable antibody levels.[52-55] Therefore, monitoring antibody titers, if performed at all, is currently mostly in research setting and rarely influences obstetric treatment.

*HLA-DRB3*0101*. The HLA-DRB3*0101 genotype is positively correlated with the occurrence of alloimmunization in HPA-1a incompatible pregnancies.[56,57] However, besides this correlation to immunization, evidence on additional association with disease severity is inconsistent.[58-60]

Glycosylation. Another proposed predictive laboratory factor is the glycosylation pattern of the Fc-part of alloantibodies. Antibodies vary in glycosylation pattern, which influences the affinity and binding to Fc-receptors.[56,61] In FNAIT, a decreased fucosylation and increased galactosylation are reported to correlate to neonatal platelet counts and disease severity.[57]

Next to these laboratory parameters, clinical characteristics have been evaluated as well.[62] So far, the only clinical parameter directly correlated to disease severity is the occurrence of an ICH in a previous affected pregnancy. Estimated recurrence rate of an ICH, without the administration of preventive antenatal treatment, is as high as 79%.[63] Therefore, the only parameter that can guide the antenatal treatment regime is the occurrence of an ICH in a sibling.

Antenatal treatment

In current practice, without any tools to assess which alloimmunized pregnancies are at truly high risk for bleeding complications, preventive antenatal treatment is initiated in all incompatible pregnancies with known platelet-specific alloantibodies and an antigen-positive fetus. The preventive toolkit in these pregnancies consists of invasive and non-invasive treatment options.[64,65]

Fetal blood sampling – intrauterine platelet transfusion. The first prenatal strategy was adapted from the successful, and still routinely applied treatment of fetal anemia. In 1984, Daffos was the first to perform ultrasound-guided FBS followed by an intrauterine platelet transfusion (IUPT).[66] This strategy allowed both the assessment of fetal platelet count and the ability for direct treatment if necessary. Compared to serial intrauterine red blood cell transfusions as treatment for HDFN, there are two major differences to its application in FNAIT. First, the half-life of platelets is a few days, which is considerably shorter than that of red blood cells.[67] This results in the need for at least weekly fetal platelet transfusions. And even after a week, pre-transfusion platelet counts are often well-below 50×10^9/L, indicating that even weekly transfusions will not be enough to maintain safe platelet counts.[21,68,69] Second, cordocentesis in a thrombocytopenic fetus introduces a high risk of complications.[70,71] These complications include a high risk of bleeding, including exsanguination, due to this thrombocytopenic status. Also, fetal bradycardia is more often noted, which might possibly be attributed to the higher plasma volume transfused.[68]

Intravenous immunoglobulins. Endeavoring to replace this risky strategy with a safer non-invasive alternative, Bussel was the first, in 1988, to report the effect of maternal intravenous immunoglobulins (IVIg) in a pregnancy complicated by FNAIT.[67] The treatment, as well as the dose of 1.0 g per kg maternal body weight per week (g/kg/wk), was adapted from the treatment of idiopathic thrombocytopenic purpura (ITP), caused by platelet autoantibodies. Different strategies with regard to this dose (reduced to 0.5g/kg/wk or increased 2.0g/kg/wk) and timing of treatment have been investigated since.[72-74] Although there are several theories, the exact working mechanism of IVIg remains unsolved.[75] One theory states that the presence of IVIg might dilute and lower HPA-alloantibody levels in maternal serum, and would therefore result in a lower titer and level of antibodies. Another theory proposes that IVIg might compete with HPA-alloantibodies for FcRn on the placenta, leading to a lower number of antibodies transported into the fetal circulation. Further, this concept of competition might occur in the fetal circulation and spleen as well, leading to fewer antibodies binding to fetal platelets or fewer platelets destroyed in the spleen.[75-77] Despite IVIg being common practice in most specialized fetal therapy centers, officially, the use of IVIg in pregnancies at risk for FNAIT is still off-label. This might be because of uncertainty on the possible, long-term immunostimulatory or immunosuppressive effects of IVIg to the maturing fetal immune system. The only knowledge on neurodevelopmental outcome of children after intrauterine IVIg exposure during fetal life, reported no clinically apparent adverse effects in early childhood in 37 children.[76]

Corticosteroids. Another non-invasive treatment is the administration of corticosteroids. These can be administered either as single therapy or, more often, in addition to IVIg. When comparing IVIg to corticosteroids, both applied as singular treatment, corticosteroids are less efficient.[78,79] As an addition to IVIg, corticosteroids are thought to reduce possible headache complaints, an IVIg side effect, and support its efficiency. This strategy of adding steroids to IVIg treatment was first described by Bussel and colleagues.[80] They started with dexamethasone 1.5mg/kg – 5mg/kg, but because of limited beneficial effects and significant side effects such as oligohydramnios, dexamethasone was replaced by prednisone, which seemed to have less side effects at a dose of 0.5 mg/kg/day.[81] Evidence on the efficiency of adding corticosteroids to IVIg treatment seems inconsistent and no clear conclusions can be reached so far.

Mode and timing of delivery

The final part of antenatal management comprises the mode of delivery. A planned, near-term, cesarean section is often performed, in order to reduce the birth trauma with risk of bleeding problems. However, evidence for this rationale is lacking. Firstly, specific intrapartum risk of bleeding has never been proven and, in a small cohort analysis, vaginal delivery was not associated with the occurrence of ICH.[82] Second, in the analysis of 43 cases of ICH no intrapartum bleeding was detected and only 3/43 ICHs were thought to have occurred after delivery.[35] The majority of women are multiparous and a non-traumatic vaginal delivery is usually expected. So, in women with a previous vaginal delivery, without a sibling that suffered from ICH, a planned induction of labor at term can be considered. In contrast to women that previously delivered a child that suffered from ICH, a near-term planned delivery or CS can be offered. In case of vaginal delivery, it is recommended to avoid any potential traumatic events such as scalp electrode, scalp blood sampling or assisted vaginal delivery.

Neonatal management

Neonatal management is aimed at reducing bleeding tendency by increasing platelet counts.[83] Initial neonatal evaluation should always include clinical assessment, platelet count and cranial ultrasound. The combination of clinical and laboratory parameters determines the need for treatment. Various national guidelines differ in threshold for the start of treatment, 20×10^9/L, 30×10^9/L or 50×10^9/L.[2,84,85] A recent study on management of thrombocytopenia in preterm children demonstrated that a lower transfusion threshold was associated with better outcome.[86] Consensus does exist on the first choice of treatment when platelet count drops below threshold, is a platelet transfusion. Ideally, the transfused product contains platelets that lack the involved HPA (HPA-compatible or HPA-matched transfusion). An alternative can be a platelet transfusion with random-donor platelets. Kiefel and colleagues[87] showed in a small cohort that multiple random platelet transfusions can be sufficient in increasing platelet counts. IVIg can be administered as well, although its efficacy in treatment of neonatal thrombocytopenia is not clearly proven.[88,89]

Prevention

Primary prevention - prophylaxis

In HDFN, the red cell counterpart of FNAIT, the implementation of anti-D prophylaxis has led to a great decrease of mortality and morbidity caused by RhD immunization.[90] Historically, RhD, like HPA-1a in FNAIT, was the most frequently involved antigen of severe HDFN.[91,92] The possibility of immunoprophylaxis for HPA-1a immunization in FNAIT, as a prophylactic equivalent to anti-D, is debated for years and is an important focus for research. In vivo animal studies have reported that antibody mediated immune suppression can also occur in FNAIT mouse models.[93] In these murine studies, β3 integrin-deficient (β3-/-) mice are used to mimic HPA-1a negativity. After injection with HPA-1a/a or HPA-1a/b platelets to these β3-/- female mice, the administration of human anti-HPA-1a strongly reduced the β3 antibody response. Besides a drop in β3 antibody level of 90%, there were fewer miscarriages, fewer stillborn pups, fewer pups with ICH and the pups had significantly higher platelet counts.[94,95] Nevertheless, a few obstacles still have to be taken. First, without population-based screening, it is currently impossible to identify women that will benefit from a potential anti-HPA-1a prophylaxis. A collaboration between nine North-European institutions, PROFNAIT project, aims to develop such a prophylaxis. They have already collected plasma of HPA-1a immunized women and announced a collaboration for the manufacturing of NAITgam from these plasmas, to develop the first drug for the prevention of FNAIT. After successful development, a phase 1-2 study of NAITgam to demonstrate efficacy and safety of the drug will be planned.[96]

Secondary prevention – screening

Without the availability of a prophylaxis, disease burden caused by FNAIT, can only be prevented by timely detection of alloimmunization and treatment in pregnancies that are at high risk for developing bleeding complications. Because FNAIT can occur in first pregnancies, the only way to adequately identify all pregnancies is through prenatal population-based screening programs.

1. **The condition sought should be an important health problem**
2. **There should be an accepted treatment for patients with recognized disease**
3. **Facilities for diagnosis and treatment should be available**
4. **There should be a recognizable latent or early symptomatic stage**
5. **There should be a suitable test or examination**
6. **The test should be acceptable to the population**
7. **The natural history of the condition, including development from latent to declared disease, should be adequately understood**
8. **There should be an agreed policy on whom to treat as patients**
9. **The cost of the case-finding program (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole**
10. **Case-finding should be a continuing process and not a "one-time" project**

Figure 1.4 – Principles of early disease detection - Wilson & Jungner criteria[97]

Aim and outline of the thesis

Population-based screening in order to timely detect and prevent or treat FNAIT has been a debated topic over the past decades.[98-102] Similarities to its red cell counterpart, HDFN, together with the availability of an effective non-invasive preventive therapy, make it seem logical and desirable to instate such a screening program. However, differences to HDFN together with critical missing knowledge, hamper nationwide implementation. Despite efforts from various countries in performing large prospective studies, nationwide population-based screening for FNAIT has not been implemented thus far. The research presented in this thesis is aimed at gaining the evidence necessary to answer this decade-long question on the feasibility and efficacy of population-based screening to prevent morbidity and mortality caused by FNAIT. To guide this quest ten criteria or principles of screening or early disease detection, defined by James Wilson and Gunner Jungner (W&J), were used (Figure 1.4).[97] These ten criteria, that were published in a report in 1968 by the World Health Organization (WHO), became a 'public health classic' and still remain the gold standard for assessing the usefulness of population-based screening. Later, in 2008, driven by the development of new genetic screening possibilities, Andermann and colleagues[103] state 10 criteria as a synthesis of 50 lists of screening criteria that have been proposed (Figure 1.5). These, partly overlapping and mainly complementary, criteria were published in the bulletin of the WHO. Whereas they state themselves, that the value of the W&J criteria remains undisputed, we primarily focus on these. In this thesis, we will evaluate the existing evidence, formulate research questions based on missing knowledge and evidence, and contribute to the fulfilling of these ten screening criteria (Table 1.4).

Table 1.4 – Research questions and outline of this thesis

W&J	Research questions	Chapter
1	What is the incidence of the disease?	2, 9
	What is the long-term follow-up after ICH?	3
	Severe hemorrhage besides ICH?	4
2	What is the optimal antenatal management?	5
	What is the optimal postnatal management?	6
3	Are there facilities for diagnosis and treatment in place?	1
4	Is there a recognizable latent stage?	1
5	Is there a suitable test for HPA-typing?	7
6	Is screening acceptable for the population?	8
7	What is the natural history of the disease?	9
8	Is there effective treatment to halt pathologic changes?	5
	Is there a clear policy whom to treat or follow-up?	10
9	Is screening cost-effective?	1
10	Continuity of the screening process	1

HPA, human platelet antigen; ICH, intracranial hemorrhage; W&J, Wilson & Jungner.

1. *Important health problem*
   A health problem might be important from the community perspective (e.g. disease with a high prevalence) or from the individual perspective. The latter includes relatively rare diseases with very severe consequences. In *chapter 3* severe consequences that FNAIT can have besides the well-known and described ICH. Besides the high mortality and short-term morbidity caused by severe bleeding complications in FNAIT, these hemorrhages impact long-term outcome as well. In *chapter 4* we are the first to evaluate the short- and long-term outcomes with a neurodevelopmental follow-up study of cases of ICH.
2. *Accepted treatment for patients with recognized disease*
   In *chapter 5 and 6* we describe both the optimal postnatal and antenatal treatment in cases of FNAIT. *Chapter 5* is a systematic review of all available evidence on antenatal treatment strategies. In *chapter 6* we performed a nationwide cohort study to evaluate postnatal management and outcome of all newly detected cases.
3. *Facilities for diagnosis and treatment*
   In current practice, there is a lot of experience with diagnosing the disease, for which specialized laboratories exist. Additionally, in subsequent pregnancies with known immunizations facilities are instated to monitor the disease and administer preventive treatment.
4. *Recognizable latent or early symptomatic stage*
   An incompatible pregnancy with known alloimmunization, in which the fetus has no signs of bleeding, is a easily recognizable and latent stage of FNAIT.
5. *Suitable test or examination*
   In terms of screening there has to be a first-line test that identifies a population at risk for the disease that is screened for. In *chapter 7* we describe the development and validation of an HPA-1a ELISA, that can be used for high-throughput, low-cost, serological HPA-1a typing in a general pregnant population.
6. *Acceptable for the population*
   With increasing consumerism in current health care this sixth screening criterion is getting more and more important. In *chapter 8* we describe the results of a cross-sectional questionnaire study, using a validated model for assessing informed decision making to assess women's attitude towards a potential HPA-screening in pregnancy.
7. *Natural history should be understood*
   Despite a couple of prospective cohort studies, knowledge on the natural history of the disease is still lacking. Due to the intervention performed in most of the large prospective studies, no conclusions can be drawn from these results. Therefore, we designed and started the HIP-study (HPA-screening In Pregnancy): a large, nationwide, observational, prospective, cohort study. The study-protocol is described in *chapter 2*. Results of a 1-year interim analysis are described in *chapter 9*.

8. *Agreed policy whom to treat as patients*
   Despite a recognizable latent stage, that is alloimmunization, it is not feasible to treat all alloimmunized women as patients. This would probably lead to a major over-treatment. With the HIP-study we are able to collect data and plasma samples from immunized pregnancies with and without disease. The latter being a unique group, necessary for development of a risk assessment tool. In *chapter 10* we describe the effect of interaction between endothelial cells and disease severity.
9. *Costs should be economically balanced*
   Obviously, an adequate estimation on costs can only be performed when the design of the screening program in the Netherlands is known and an adequate estimation of the natural history of the disease can be made. Indeed, natural history represents the costs that can be prevented. Despite this missing information, several studies have estimated cost-effectiveness as accurately as possible and concluded population-based screening and intervention programs are likely to be cost-effective. It was stated that a potential national screening program as described by Kjeldsen-Kragh *et al*[32], which included performing a near-term cesarean section in all alloimmunized pregnancies, would save up to 210 – 230 quality-adjusted life years and could reduce health care costs by €1.7 million per 100,000 pregnant women.[104]
10. *Continuous process*
    In the era of the development of the W&J criteria were developed was that screening should be a continuous process and not a one-time exercise that would minimize the yield of the program. In current health care and practice, the doctrine of starting population-based screening is by definition a continuous process, until new insights prove otherwise.

1. **The screening program should respond to a recognized need**
2. **The objectives of screening should be defined at the outset**
3. **There should be a defined target population**
4. **There should be scientific evidence of screening program effectiveness**
5. **The program should integrate education, testing, clinical services and program management**
6. **There should be quality assurance, with mechanisms to minimize potential risks of screening**
7. **The program should ensure informed choice, confidentiality and respect for autonomy**
8. **The program should promote equity and access to screening for the entire target population.**
9. **Program evaluation should be planned from the outset**
10. **The overall benefits of screening should outweigh the harm**

Figure 1.5 – Synthesis of emerging screening criteria from over the past 40 years[103]

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Chapter 2

HIP-study (HPA-screening In Pregnancy): Protocol of a nationwide, prospective and observational study to assess incidence and natural history of fetal/neonatal alloimmune thrombocytopenia and identifying pregnancies at risk

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Abstract

Introduction. Fetal and neonatal alloimmune thrombocytopenia (FNAIT) may lead to fetal or neonatal brain damage or perinatal death. Maternal alloantibodies, targeted against the foreign, paternally derived, human platelet antigens (HPAs) on fetal platelets, can result thrombocytopenia and bleeding complications. In pregnancies with known immunisation, fetal bleeding is effectively prevented using maternal intravenous immunoglobulin (IVIg) infusions. In the absence of population-based screening, immunisation is only detected after birth of an affected infant. Therefore, prevention with antenatal IVIg treatment is only available in subsequent pregnancies. Preventing all bleeding complications, including first affected children, requires population-based screening. With anti-HPA-1a being the most commonly involved antibody, responsible for approximately 90% of all cases of FNAIT resulting in intracranial haemorrhage (ICH), this is the target for potential screening. To guide consideration of the efficacy of such a program, the World Health Organization published ten screening criteria, originally developed by Wilson and Jungner. Following these criteria, we identified that knowledge on incidence, natural history and identification of pregnancies at high risk of anti-HPA-1a-mediated FNAIT is incomplete. We designed a study aimed to obtain this missing knowledge.

Methods and analysis. The HIP-study (HPA-screening In Pregnancy) is a nationwide, prospective and observational cohort study, aimed to assess incidence and natural history of FNAIT, as well as identifying pregnancies at high risk for developing HPA-mediated bleeding complications. Pregnant women that are RhD or Rhc-negative and therefore take part in the Dutch population-based prenatal screening program for erythrocyte immunisation, are eligible for enrolment. Serological HPA-1a typing is performed with left-over material and a luminex-based multiplex assay will be performed in HPA-1a negative samples for the detection of anti-HPA-1a antibodies. Results will not be communicated to patient or caregivers. Clinical data of HPA-1a negative women and a HPA-1a positive control group will be collected after birth. Samples of HPA-1a immunised pregnancies with and without signs of bleeding will be compared to identify (laboratory) parameters for identification of pregnancies at high risk for developing bleeding complications.

Ethics and dissemination. Ethical approval for this study has been obtained from the Committee of Medical Ethics (CME) from the Leiden University Medical Centre (P16.002). Study enrolment began in March 2017. All pregnant women have to give informed consent for testing according to the complete protocol. Results of the study will be disseminated through (international) congresses and publication in relevant peer-reviewed journals.

Strengths and limitations of this study

- The HPA-screening In Pregnancy (HIP) study is the first prospective and completely non-interventional screening study with a large cohort that enables assessing the true natural history of fetal and neonatal alloimmune thrombocytopenia (FNAIT).
- The unique infrastructure in the Netherlands with one national referral laboratory for FNAIT (Sanquin, Amsterdam) collaborating with the national fetal therapy centre (LUMC, Leiden) will result in complete data and focus on both laboratory and clinical parameters.
- A limitation of the study is that we rely on the clinical judgement of bleeding tendency after birth, and do not obtain cord blood platelet counts or perform routine neonatal cerebral ultrasounds. Therefore, we may still underestimate disease prevalence due to subclinical cases.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is the most frequent cause of severe thrombocytopenia in term born infants.[1,2] FNAIT is caused by the production of maternal alloantibodies against the paternally derived, fetal human platelet antigens (HPAs). Clinical consequences can vary from an asymptomatic thrombocytopenia to minor skin haemorrhage, such as hematoma or petechiae, or ultimately severe internal organ and intracranial haemorrhage (ICH).[3,4] Bleeding complications that, in subsequent pregnancies can be effectively prevented by weekly administration of intravenous immunoglobulins (IVIg) to the mother.[5] The vast majority of cases with (severe) clinical consequences are caused by maternal alloantibodies targeted against fetal HPA-1a.[6-8] FNAIT is considered to be the platelet counterpart of haemolytic disease of the fetus and the new-born (HDFN) because of their similar pathophysiologic fundaments. In this comparison, HPA-1a, that causes 90% of the ICH caused by FNAIT, is regarded to be the equivalent of RhD of the red blood cell (RBC) in HDFN.[8] Important differences, however, exist as well. First, whereas RhD is only expressed on red blood cells, the HPA-1a epitope expressed on platelets is also present on the membrane of endothelial cells and syncytiotrophoblast cells.[9,10] Second, whereas RhD is mainly a problem of second or subsequent incompatible pregnancies, more than half of the severe cases of HPA-1a-mediated FNAIT already occur in first-born children.[4,11] For decades, the possibility of prevention of FNAIT by population-based screening for HPA-1a is discussed, in analogy to the RhD prophylaxis and erythrocyte immunisation screening.[12-14]

Careful evaluation of the feasibility, benefits and harms and cost effectiveness of a possible FNAIT screening program showed that knowledge is missing on different aspects of the disease, needed before the introduction of a screening program. First, despite a couple of large

prospective cohort studies, no data exist on the natural history of the disease. Most of the large prospective, screening studies performed, were not only observational, but included some kind of intervention, thereby making it impossible to draw any firm conclusion on the natural history of FNAIT.[15-19] Further, more accurate estimates of incidence and prevalence of the disease in the Dutch population need to be known. One of the most important differences, making it hard to implement a program similar to the antenatal screening program for erythrocyte immunisation, is the lack of tools to identify pregnancies at high risk for developing bleeding complications. Detecting HPA-1a negative women and further HPA-1a alloimmunised pregnancies can be easily done. When alloimmunisation is detected in HDFN several parameters, laboratory as well as clinical, are available to assess disease severity and to predict which cases would benefit from treatment. For example, RBC alloantibody titre and functional assays such as an antibody-dependent cellular cytotoxicity assay can be performed, followed in pre-selected cases by estimation of fetal anaemia by Doppler-based assessment of flow velocity in the middle cerebral artery of the fetus. In this way, high risk cases are identified that most likely benefit from fetal blood sampling (FBS), followed by an intrauterine transfusion.[20] Treating all HPA-alloimmunised pregnancies with IVIg would lead to a considerable and undesirable overtreatment. So, identification of HPA-alloimmunised pregnancies at high risk for disease, like in HDFN, would be preferable as well. FBS to determine fetal platelet count and if necessary administer intrauterine platelet transfusion, can be performed in these pregnancies as well. However, in potentially thrombocytopenic fetuses this is a risky procedure with a high rate of associated complications. Unfortunately, no non-invasive laboratory or clinical diagnostic tests to select HPA-alloimmunised pregnancies that would benefit from treatment are applicable in a clinical setting.

To obtain information necessary to judge the effectiveness and feasibility of a potential population-based screening, we designed the HIP (HPA-screening In Pregnancy) study. With the HIP-study we aim to collect data on the incidence of HPA-1a alloimmunisation and clinically relevant FNAIT in the Netherlands. The study will be completely observational. This way we will be able to conclude on the natural history of FNAIT. Ultimately, by comparing test characteristics of blood samples from pregnancies with and without clinical manifestations of bleeding we aim to develop on or more diagnostic tools, allowing more effective and personalised management by selecting pregnancies at high risk for bleeding complications that have the highest chance to benefit from antenatal preventive treatment with IVIg. This would not only be desirable in current management of FNAIT but especially in potential future screening setting.

Methods and analysis

Study objectives

The primary objective of this study is to determine incidence of HPA-1a alloimmunisation and the incidence of clinically relevant HPA-1a-induced FNAIT in the Netherlands. Clinically relevant FNAIT will be defined as minor bleeding (hematoma, bruising, petechiae or small visceral bleeding) and severe bleeding (ICH or internal organ haemorrhage) with the presence of an anti-HPA-1a alloantibody. Additionally, as secondary objective, we aim to collect a set of blood samples that can contribute to the development of a risk assessment model to be used as a diagnostic tool enabling the identification of alloimmunised pregnancies that are at high risk of developing bleeding complications.

Study design

The HIP-study is a nationwide prospective and observational cohort study, conducted in all settings of obstetric care in the Netherlands, for a period of two and a half years.

Patient and public involvement

In 2008, the ministry of Health, Welfare and Sport (In Dutch: Ministerie van VWS) gave instructions to investigate preventive interventions for 27 significant health problems that could be cost-effective. As a result the National Institute for Public Health and the Environment (in Dutch: RIVM) published a report stating that antenatal screening for FNAIT would be cost-saving, but they advised that more knowledge on natural history of the disease and treatment of detected cases should be obtained to support possible implementation of screening.[21] Also, the RIVM was involved in the design of the study. There was no further involvement of patients or public in the recruitment or the conduct of the study.

Study population

For logistic purposes, RhD or Rhc negative pregnant women were selected for enrolment in the HIP-study. As part the Dutch prenatal screening program for infectious disease and erythrocyte immunisation (in Dutch: PSIE), these women are offered a free of charge red cell antibody screening and/or fetal RHD typing at 27 weeks' gestation. For this, nine ml ethylenediamine tetra-acetic acid (EDTA) anticoagulated blood is drawn by their midwife or at certified, local laboratories all over the Netherlands ($n = \pm 90$) and transported to Sanquin laboratory in Amsterdam by regular surface mail or private courier service. The program has a voluntary participation grade of 99%.[22,23] With approval of the RIVM, that organises this population screening program, left-over material can be used for the HIP-study for HPA-1a typing and stored for further antibody testing after informed consent.

Inclusion criteria

Prior to enrolment, participants have to fulfil these following criteria:

- Pregnant women participating in the currently implemented prenatal screening program for erythrocyte immunisation and who are typed RhD or Rhc negative.
- Ability to make an informed decision on participating in the population screening program as well as in the HIP-study.

Exclusion criteria

- Cases with insufficient material to perform HPA-1a typing by enzyme-linked immunosorbent assay (ELISA)
- Cases with known HPA-1a alloimmunisation

Participating centres

All obstetric care centres, hospitals, midwifery practices as well as general practices that provide obstetric care, in the Netherlands are able to enrol pregnant women to participate in the HIP-study. In order to ensure that obstetric caregivers were equipped to inform and counsel pregnant women, communicatory symposia were organised at six locations all over the Netherlands. Additionally, an informational leaflet was produced in different languages (Dutch and English on paper; Spanish, Arabic, Turkish and Polish digitally available; supplemental material). Two informational videos were made informing on FNAIT as well as the HIP-study. Lastly, a website was created containing news and information about the HIP-study (www.HIPstudie.nl).

Study outcomes

The main study parameters / primary endpoints are:

- Incidence of HPA-1a negativity in the RhD or Rhc-negative pregnant population in the Netherlands
- Incidence of HPA-1a alloantibodies in the tested population
- Incidence of clinically relevant HPA-1a-mediated FNAIT; classified into as mild or severe FNAIT
    - Severe FNAIT
        - ICH
        - internal organ haemorrhage
    - Mild FNAIT
        - Neonatal bleeding signs other than ICH or internal organ haemorrhage: hematoma, bruising, petechiae, purpura, mucosal or visceral bleeding
        - Thrombocytopenia for which treatment was administered (platelet transfusion or IVIg) or for which clinical observation was performed

Our secondary study parameters / endpoints are:

- Neonatal treatment for thrombocytopenia: platelet transfusion (with random-donor platelets versus compatible platelets), IVIg, red blood cell transfusion
- Neonatal morbidity: infection, hours/days in hospital (NICU versus Medium Care), need for additional treatment, congenital abnormalities, other causes causing increased bleeding tendency
- Neonatal laboratory findings: platelet count, haemoglobin, CRP

HIP-Study procedure

As part of the prenatal screening program for erythrocyte immunisation, an EDTA tube of blood of RhD and Rhc negative pregnant women will be sent in to Sanquin at 27 weeks' gestation. These women are eligible for enrolment in the HIP-study and will be informed about the study and asked for consent by their obstetric care givers. This consent or decline of participation is added to the regular laboratory request form for the 27th week assessment, that is already sent to Sanquin with each tube of blood. No additional blood will be drawn for the HIP-study. Once the tubes of blood are sent to Sanquin the consent is either received digitally or on paper, depending on the route and location (various hospitals, midwifery practices and local laboratories).

The procedures that are performed after consent and enrolment in the HIP-study can be divided into four separate phases, depending on the time in and after pregnancy (Figure 2.I).

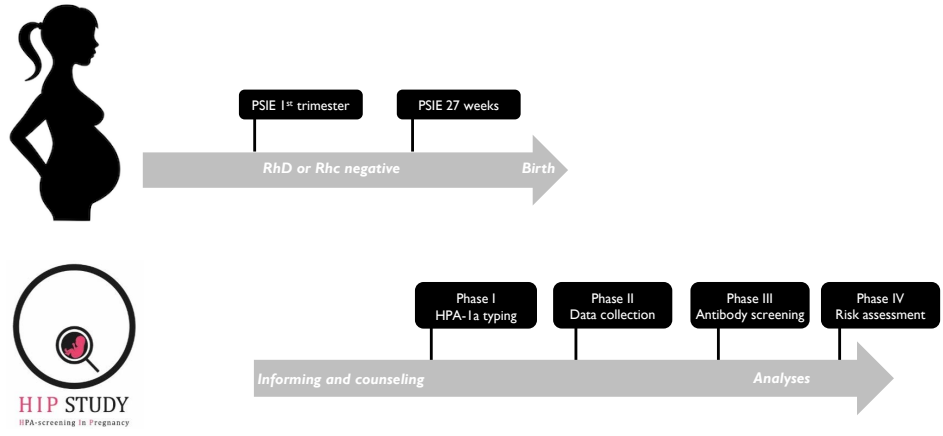

Figure 2.I – Schedule of selection, enrolment and tests in the HIP-study

HPA, human platelet antigen; PSIE, prenatal screening of infectious diseases and erythrocyte immunisation; RhD, rhesus D; Rhc, rhesus c.

Phase I. After regular screening, authorisation and correspondence of the results for the prenatal screening program for erythrocyte immunisation, the tubes are made available for the HIP-study. For the HIP-study the platelet containing plasma of the stored blood tubes is serologically typed for HPA-1a, using a sandwich ELISA.[24] In short, 20 µL of plasma containing platelets will be automatically pipetted into microtiter plates that have been coated with a monoclonal antibody CLBthromb/1 (C17) directed against glycoprotein IIIa, at a concentration of 3 µg/mL to capture all platelets from the plasma. Then HRP-conjugated B2G1, an antibody targeting HPA-1a, will be added and plates will be centrifuged and incubated for 45 minutes. Lastly, after washing of the plates, HRP-substrate solution will be added for 15 minutes and after stopping of this reaction the reactions will be quantified using an ELISA reader ((Biochrom Anthos, Cambridge, United Kingdom). This HPA-1a ELISA was specifically designed for the HIP-study, thus for quick and high-throughput screening. All samples with an ELISA value below a defined optic density (OD) are called HPA-1a negative. The HPA-1a typing result is supported with an allelic discrimination polymerase chain reaction (PCR) assay. Plasma and buffy-coat of samples that are typed HPA-1a negative will be stored at -20°C, using only a study number. Additionally, for each HPA-1a negative case, material of one HPA-1a positive control will be stored simultaneously.

Because this first phase comprises serological HPA-1a typing, which is performed with fresh material, and a delay in the arrival of consent forms might exist, this phase is performed with all samples from pregnant women who did not decline participation for the HIP-study. All consecutive phases, such as antibody screening, risk-assessment development and clinical data retrieval, are solely performed in case of informed consent for the HIP-study.

Phase II. Of all samples stored with consent, obstetric care givers will be contacted to obtain clinical information. An overview of these clinical parameters is provided in figure 2.2. The clinical data will be stored in a secured digital database, designed by the LUMC, called ProMISe. First, study numbers of HPA-1a negative cases and HPA-1a positive controls with corresponding obstetric care givers are entered into the database. Then, for each case, ProMISe randomly generates a code. Thereafter obstetric care givers will receive a secured digital invitation to add clinical data to a digital case report form (CRF) for the cases from their practice. This secured invitation contains the initial personal data for the sample sent in for erythrocyte immunisation screening program together with the code generated by ProMISe. In the digital CRF they fill in this code and the clinical data. Clinical data is stored in ProMISe, only by anonymous study codes. This way, no personal information is being transferred or entered in our database, nor is the obstetric care giver in possession of a key that links the anonymous study number to personal information, nor does the care giver know whether their patients or clients are HPA-1a negative or positive.

Medical history:	known with immune thrombocytopenic purpura (ITP)
Obstetric history:	previous pregnancies, deliveries, miscarriages (spontaneous as well as pregnancy terminations) or intra-uterine fetal demises.
Pregnancy:	gestational diabetes, hypertensive disorders (pregnancy-induced hypertension, pre-eclampsia), intrauterine growth restriction (IUGR).
Perinatal:	gestational age at delivery, mode of delivery, Apgar score, birth weight
Neonatal:	gender, chromosomal disorder, laboratory assessment (CRP or platelet count), consultation of pediatrician, admission to the neonatal care unit, mortality
FNAIT-related:	hematomas, petechiae, visceral bleeding, internal organ haemorrhage, intracranial haemorrhage, platelet count (if tested), treatment for thrombocytopenia

Figure 2.2 – Clinical parameters

Phase III. The next step is to evaluate the incidence of alloimmunisation. Of all HPA-1a negative women that gave consent for the HIP-study, we will use the stored left-over plasma to screen for HPA-1a alloantibodies. For antibody screening the Pak Lx assay, a qualitative immunoassay, will be used, according to the manufacturer's' recommendations (LIFECODES Pak Lx Assay, Immucor GTI Diagnostics, Norcross, United States of America). In short, plasma samples are incubated with reconstituted beads and for the removal of unbound antibodies, the beads are washed. Next a conjugate (anti-human immunoglobulin G antibody conjugated to phycoerythrin) is added and incubated with the sample for 30 minutes at room temperature. Lastly, the Luminex 200 instrument is used to analyse the data. The advantage of this assay is that it is quick and uses only a small amount of plasma so there will be enough left-over for further testing in phase IV.

Phase IV. Combining the results from phase II and phase III, will enable us to select cases of alloimmunisation with and without clinical manifestations of FNAIT to identify possible parameters to predict the development of (severe) bleeding complications. For this we will be testing different laboratory parameters as well as clinical parameters (Box I). Laboratory parameters that will be tested to assess risk at bleeding complications are: HLA-DRB3*0101 status, antibody level, Fc-core glycosylation and FcgRIII-binding index, endothelial cell binding, endothelial cell function.[25-28]

Sample size calculation

The HIP study is designed to assess the incidence of clinical relevant FNAIT in pregnant women in the Netherlands. Therefore, the incidence of ICH in HPA-1a immunised cases was compared with HPA-1a positive women. The estimated risk of ICH in immunised cases was 3%, for our power calculation we took a marge of 1% on the estimated incidence of ICH in FNAIT.[19,29] In our control group we assumed a risk on symptomatic ICH of 4.9 in 10.000 (0.05%).[30] To achieve a power of 80% at an alpha level of 5%, we calculated that a total study population of 2,400 pregnant women is needed. Within this calculation, we took into account the unequal distribution

between HPA-1a positive controls and immunised cases. We considered 5% of our total study population to consist of immunised cases, which means that we need to include 120 immunised cases. Calculations were performed using logistic regression model making use of PASS 11.

Each year, approximately 60,000 RhD or Rhc negative pregnant women will participate in the prenatal screening program for erythrocyte immunisation and are therefore eligible for enrolment in the HIP-study. To include 120 immunised cases, we need to include 1,200 HPA-1a negative women (immunisation rate of approximately 10%).[29] Because 2.1% of the Caucasian population is HPA-1a negative, the total study population should exist of 60,000 pregnant women (Table 2.1). Based on previous experience with the OPZI-study and the highly positive attitude toward potential HPA-screening in pregnancy women expressed in our previous study, the expected enrolment was 50%.[31,32] This would correspond with a study period of two years.

Table 2.1 – Estimated cases in HIP-study

	%	Incidence	Cases in the Netherlands *Total pregnancies n = 170,000*	Cases during study period *Total included n = 60,000**
HPA-1a negative	2.1	1 : 50	3,570	1,260
HPA-1a antibodies	10	1 : 400	428	126
Severe FNAIT	30	1 : 1,300	129	36
ICH	10 – 30	1 : 12,500	13	3-4

* Assuming 50% enrolment of the 60,000 RhD/Rhc negative women each year, for two years

FNAIT, fetal and neonatal alloimmune thrombocytopenia; HIP, HPA-screening in pregnancy; HPA, human platelet antigen; ICH, intracranial haemorrhage.

Statistical analysis

Clinical data will be entered into a validated data capture system, provided and designed by the LUMC. The system is protected by password and contains internal quality checks to identify inaccurate or incomplete data. Laboratory data will be entered in a separate password protected database by independent technicians, inaccessible to the researchers. Both clinical and laboratory data will be combined and further data management and analysis will be performed using SPSS (version 23.0) and Graphpad (version 8.0). An interim-analysis after 1-year will be performed.

Ethics and dissemination

Patient recruitment started in March 2017 and the study is planned to close to recruitment on the spring/summer of 2019. However, to ensure the inclusion of 1,000 – 1,500 HPA-1a negative women the inclusions period might take longer. Accurate predictions on the duration of the study will be made after interim-analysis at 1 year. Results will be published in relevant scientific journals and be disseminated in international conferences.

Discussion

FNAIT can cause severe bleeding complications in fetuses and neonates, with a high risk of associated morbidity and mortality.[33] A preventive antenatal treatment, that effectively prevents these bleeding complications from occurring, is available.[5] In current practice, this prevention is only available in pregnancies with known alloimmunisation, usually after a previously affected child. To prevent these first cases as well, timely detection by prenatal and population-based screening is necessary.

Current lack of prospective non-interventional studies providing data on natural history of the disease as well as a reliable risk assessment tool to identify alloimmunised pregnancies that are at high risk for developing bleeding, complicates the implementation of such population-based screening. The aim of the HIP-study is to gather this missing knowledge necessary to adequately evaluate the potential efficacy and feasibility of prenatal population-based screening in order to timely detect and prevent FNAIT-related complications. With the current study design and logistics, making use of the current national screening program for red blood cell immunisation with a participation grade of 99.1%, we expect our results to give an adequate representation of the Dutch population of pregnant women.

A potential limitation of this study protocol is the lack of routine determination of neonatal platelet counts. However, the goals of potential screening and prevention of FNAIT is not to prevent a low platelet count as reflected as a laboratory result, but to prevent symptomatic disease, mainly ICH, with associated morbidity caused by FNAIT. However, routine neonatal cerebral ultrasound is not performed either. Therefore, cases of subclinical ICH without symptoms (such as convulsions or reduced consciousness) or additional bleeding manifestations might be missed, although in theory these might lead to developmental problems later in life. However, major ICHs detected in prospective studies that did perform routine cerebral ultrasound, were cases that were symptomatic as well.[19,34]

Further underestimation might occur due to the fact that we will perform only a single screening for anti-HPA-1a alloantibodies, that is at 27 weeks' gestation. Immunisations that occurs later in pregnancy or after delivery will not be detected. Also, immunisations that will result in complications and termination of pregnancy or IUFD before 27 weeks' gestation will not be identified. However, in terms of assessing feasibility and cost-effectiveness of population-based screening, a slight underestimation is unquestionably preferred to an overestimation.

Overall, to our knowledge, the HIP-study will be the first study to prospectively and observationally collect data on incidence and natural history of FNAIT by including this large amount of pregnant women without performing any kind of intervention. Additionally, it will be

the first study to be able to identify a unique study group, that is immunised pregnant women without disease and without intervention. This is the pre-eminent group to be used for the development of a risk-assessment platform in order to select immunised pregnancies that are at high risk to develop bleeding complications and would therefore benefit from antenatal preventive measures, such as IVIg treatment.

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Supplemental material

HIP-study

In the Netherlands all pregnant women are screened for antibodies against red blood cells as these antibodies can lead to anemia or jaundice in the newborn. In the blood of pregnant women, antibodies against platelets may also be present, these platelet antibodies can lead to bleeding problems in the newborn.

Can we prevent bleeding problems caused by antibodies against platelets? To answer this question we first need to know the following:

- How often are antibodies against platelets present in the blood of pregnant women?
- If these antibodies are present how often do they cause bleeding problems in newborn babies?
- Which newborns are at high risk of suffering from bleeding problems?
- Can we predict bleeding problems by testing the blood of pregnant women?
- How can we prevent these bleeding problems in newborn babies?

The HIP-study can provide answers to these questions. To answer all these questions , we need the help of midwives, gynaecologists and pregnant women. By agreeing to participate, you can make an important contribution to the HIP-study.

More information

For further information, please check
www.HIPstudie.nl
Or scan this QR-code to watch our informative video.

Please do not hesitate to consult your obstetric caregiver with any further questions you may have.
Or send an email to: **info@HIPstudie.nl**

Principal investigator:
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Independent medical doctor:
Dr. M. Sueters, gynecologist, LUMC

Research study on platelet antibodies in pregnancy

HIP STUDY
HPA-screening In Pregnancy

Sanquin LUMC

Why do you receive this flyer?

In the first trimester of your pregnancy, your red blood cell type was tested. The results showed that your red blood cells are RhD or Rhc negative. All RhD and Rhc negative pregnant women in the Netherlands are tested again at 27 weeks gestation by Sanquin in Amsterdam. We would like to perform an extra test, for the HIP-study, with the remainder of this blood sample. Here we ask your consent to perform this extra test.

What does this mean for you?

Participation is completely voluntary and will have no further consequences for the care you receive during your pregnancy. If you participate, no additional actions are necessary. No extra blood is drawn nor will you be personally contacted. You and your baby will be unaware of participation.

It is important to know that there is no personal gain in participating. However, your participation will contribute to improving knowledge on diseases caused by antibodies against platelets and will help to improve treatment during pregnancy in the future.

Which tests and actions are part of the HIP-study?

If you consent to participate in the HIP study, we will perform an extra test on the blood sample that has been already drawn. With this test we will determine the blood type of your platelets. This blood type is called HPA-1a. If you don't have this blood type, you are HPA-1a negative (1 in 50 women). If you are HPA-1a negative, we will store the tested blood sample. We will also store the blood sample from a small number of HPA-1a positive women as part of the so called 'control-group'.
After the expected delivery date all stored blood samples will be tested for antibodies against platelets . A member of the study team will contact your obstetric care giver to enquire on the health of your child during the first hours-days of life. All results and data will be anonymized and saved in a secured database. Results will not be reported to you or your obstetric care giver. The study results cannot be requested by third parties.

Participate?

Your obstetric care giver will ask you if you are willing to participate in the HIP-study.
Your answer will be indicated on the HIP-study special check box on the blood collection form.

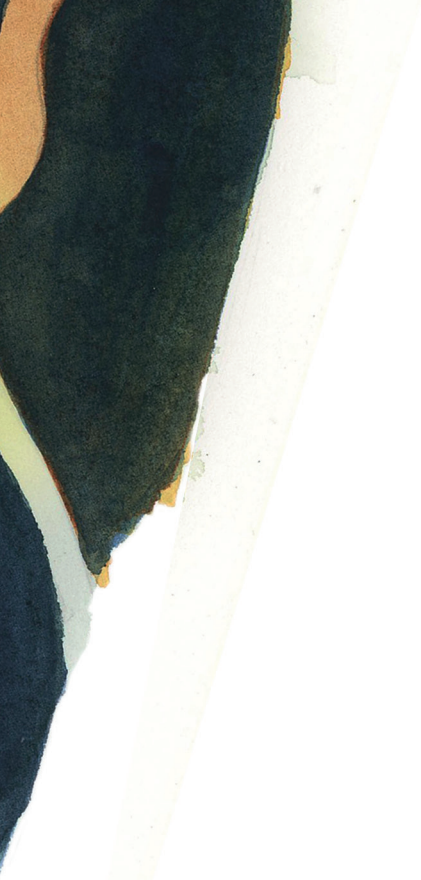
Background information

Our blood contains billions of cells. For example: red blood cells, white blood cells and platelets. All these cells express characteristics that are called blood types. Our body can produce antibodies against these blood types. During pregnancy a pregnant women can produce antibodies against the blood type of her child. Sometimes these antibodies are able to destroy the blood cells of the child. This can lead to disease and the need to start timely treatment.

'HIP' in HIP-study stands for:
HPA-screening In Pregnancy

HPA is an abbreviation for a blood type on platelets and means: Human Platelet Antigen.

Supplemental figure S2.1 – Flyer HIP-study



Chapter 3

Perinatal outcome and long-term neurodevelopment after intracranial hemorrhage due to fetal/neonatal alloimmune thrombocytopenia

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Abstract

Objectives. To evaluate perinatal and long-term neurodevelopmental outcome in a cohort of children with an intracranial hemorrhage (ICH) due to fetal and neonatal alloimmune thrombocytopenia (FNAIT) and clearly outline the burden of this disease.

Subjects and methods. We performed an observational cohort study and included all consecutive cases of ICH caused by FNAIT from 1993 to 2015 at Leiden University Medical Center. Neurological, motor and cognitive development were assessed at a minimum age of one year. Primary outcome was adverse outcome, defined as perinatal death or severe neurodevelopmental impairment (NDI). Severe NDI was defined as any of the following: cerebral palsy (Gross Motor Function Classification System (GMFCS) ≥ 2), bilateral deafness, blindness, severe motor and/or cognitive developmental delay (< -2 SD).

Results. In total, 21 cases of ICH due to FNAIT were included in the study. Perinatal mortality rate was 10/21 (48%). Long-term outcome was assessed in ten children (n = 1, lost-to follow-up). Severe NDI and moderate NDI were diagnosed in 6/10 (60%) and 1/10 (10%) of surviving children. Overall adverse outcome, including perinatal mortality or severe NDI, was 16/20 (80%).

Conclusions. The risk of perinatal death or severe NDI in children with ICH due to FNAIT is high. Only screening and effective preventive treatment can avoid this burden.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is one of the leading causes of thrombocytopenia in otherwise healthy newborns.[1,2] Maternal alloantibodies are formed after exposure to the incompatible, paternally derived human platelet antigen (HPA) on fetal platelets. In FNAIT, these alloantibodies are predominantly targeted against HPA-1a, in approximately 80% of cases.[3,4] When these antibodies enter the fetal circulation, they can destroy fetal platelets and damage endothelial cells.[5] Hence, FNAIT presents as an (asymptomatic) thrombocytopenia or results in bleeding complications. The most feared complication is an intracranial hemorrhage (ICH), due to its associated risk of lifelong handicaps and neurological sequelae.[6,7] In addition, ICHs caused by FNAIT have a high recurrence rate, in up to 79% of subsequent pregnancies.[8] Therefore, in the absence of population-based screening for FNAIT, current management is focused on preventing the occurrence of bleeding complications and ICHs in subsequent pregnancies through antenatal treatment with intravenous immunoglobulin (IVIg).[9] ICH is estimated to occur in 1 out of 25,000 pregnancies and in 1 out of 10 cases of severe FNAIT.[10]

Despite the fact that ICH caused by FNAIT is often more severe compared to ICH from other causes, leading to a high mortality and handicap rate, no detailed long-term follow-up studies have been published so far. [8,11,12] Long-term follow-up data are necessary for performing adequate evidence-based counseling of parents, and for professionals involved in guiding these children. Even more, in light of potential future implementation of population-based screening, knowledge on the long-term implications of these ICHs is indispensable.

We evaluated the perinatal and long-term neurodevelopmental outcome in a cohort of children with ICH due to FNAIT and clearly outlined the burden of this disease in survivors in the current era of fetal medicine and neonatal intensive care treatment possibilities.

Subjects and methods

Study population

The Leiden University Medical Center serves as the national center of expertise for FNAIT in the Netherlands. From 1993 to 2015 all consecutive cases with ICH due to FNAIT were identified and eligible for inclusion. We identified through women that were counselled, diagnosed, or treated at our center, either in the pregnancy of interest or during a subsequent pregnancy. These cases were cross-checked with Sanquin, the national reference laboratory for FNAIT, where the diagnosis FNAIT was confirmed in case of incompatibility between maternal and paternal/fetal HPA type in combination with the detection of maternal anti-HPA antibodies.

Outcomes

The primary outcome was perinatal death and/or severe neurodevelopmental impairment (NDI). Severe NDI was defined as any of the following: severe cerebral palsy (Gross Motor Function Classification System [GMFCS] score ≥ 2), a cognitive and/or motor test score <70 (< -2 SD), bilateral blindness, or bilateral deafness requiring amplification. The secondary outcome was moderate NDI defined as cerebral palsy GMFCS < 2 or mild-to-moderate motor and/or cognitive developmental delay (< -1 SD and > -2 SD).

The following ante- and neonatal data were retrieved from the medical files: antenatal treatment, gestational age at birth, mode of delivery, birth weight, platelet count at birth, clinical course and cerebral imaging. When available, neuroradiological images were reviewed by an experienced neonatologist (S.J.S.) to confirm the presence of ICH and to classify the type of bleeding. When original images from another hospital were unavailable for review, written reports by other experienced radiologists were obtained. Hemorrhages were classified as subdural, subarachnoid, cerebellar, intraventricular or intraparenchymal with a separate notion for unilateral or bilateral occurrence and the extent of lobar involvement (frontal, parietal, occipital or temporal).[13] Cases with no imaging or no classification of imaging performed elsewhere available were excluded.

Neurological, motor and cognitive development was assessed at a minimum of 1 year of age. The following standardized psychometric tests, appropriate for age, were used: the Bayley Scales of Infant and Toddler Development third edition (Bayley-III), the Wechsler Preschool Primary Scale of Intelligence third edition (WPPSI-III), and the Wechsler Intelligence Scale for Children third edition (WISC-III).[14-16] Bayley-III, WPPSI-III, and WISC-III scores follow a normal distribution curve with a mean of 100 and a standard deviation of 15. A cognitive test score, i.e., a Bayley-III cognitive composite score, WPPSI-III or WISC-III total IQ score < 70 (< -2 SD) indicates severe cognitive delay. Scores < 85 (< -1 SD) indicate mild-to-moderate cognitive delay. Children with severe cognitive impairments (with scores < 50) or who were unable to participate in standardized testing due to severe cognitive impairment were assigned a score of 49 in the database. Testing was either performed by our specialized medical psychologist at our outpatient clinic or results were requested at their institution of care.

Cerebral palsy was defined according to the European Cerebral Palsy Network and classified as diplegia, hemiplegia, quadriplegia, dyskinetic, or mixed. Subsequently, cerebral palsy was scaled according the gross motor function classification system (GMFCS) in level I-V varying from decreased speed, balance and coordination at level I to impaired in all motor functions, cannot sit, stand, walk independently and has physical impairments that restrict voluntary control of movement and the ability to maintain head and neck position against gravity at level V.[17]

Statistical analysis

All data were analyzed with SPSS software (version 18.0 SPSS Inc., Chicago, Illinois, USA), using descriptive statistics. Categorical data are presented as numbers and percentages. Continuous variables are presented as median with range or mean with standard deviation.

Results

Study population

Between 1993 and 2015 a total of 31 cases with an ICH due to FNAIT were identified (Figure 3.1). Of these, 7 (23%) were excluded because there were no clinical data available on either short- or long-term outcome. Three additional cases were excluded because there were no images or reports of the ICH available. The remaining 21 children with ICH were included and assessed for short-term outcome. Perinatal death was reported in 10 (48%) cases because of fetal demise at 19-22 weeks gestation ($n = 3$), death during labor after drainage of post-hemorrhagic hydrocephalus ($n = 1$) and neonatal death related to severe ICH ($n = 6$).

Obstetric history revealed a previous miscarriage in 10 (48%) cases (Table 3.1). HPA-1a was the predominantly involved alloantibody, in 18 (86%) cases. In 5 (24%) cases the ICH occurred in the first pregnancy and in 12 cases the ICH affected the first-born child. The lowest platelet count in all cases not treated antenatally with IVIg was below 30×10^9/L, with a median of 11×10^9/L.

Short-term outcome

Antenatal treatment

In 11 (52%) cases, the ICH was already detected antenatally, and in 4 of these pregnancies antenatal treatment was administered. One mother (#17) had a previous child with FNAIT without ICH, which led to the proposed plan of antenatal treatment with IVIg from 28 weeks of gestation. Just before the start of treatment a hemorrhage was detected during fetal cranial ultrasound. IVIg was started as planned. The second case concerned a dichorionic twin pregnancy (#16) of which one suffered from ICH. Maternal HPA-5b antibodies were found and IVIg was started to protect the co-twin from bleeding and to prevent worsening of bleeding of the affected fetus. In the other 2 cases, ICH was detected during routine ultrasound at 20 weeks of gestation. In one of these cases the mother had a previous child with ICH, presumed to be caused by birth trauma. In this subsequent pregnancy HPA-5a antibodies were detected and FNAIT was diagnosed. In 19 (90%) cases, it was clear that the ICH occurred antenatally. In the other two cases the exact timing of the ICH was not reported.

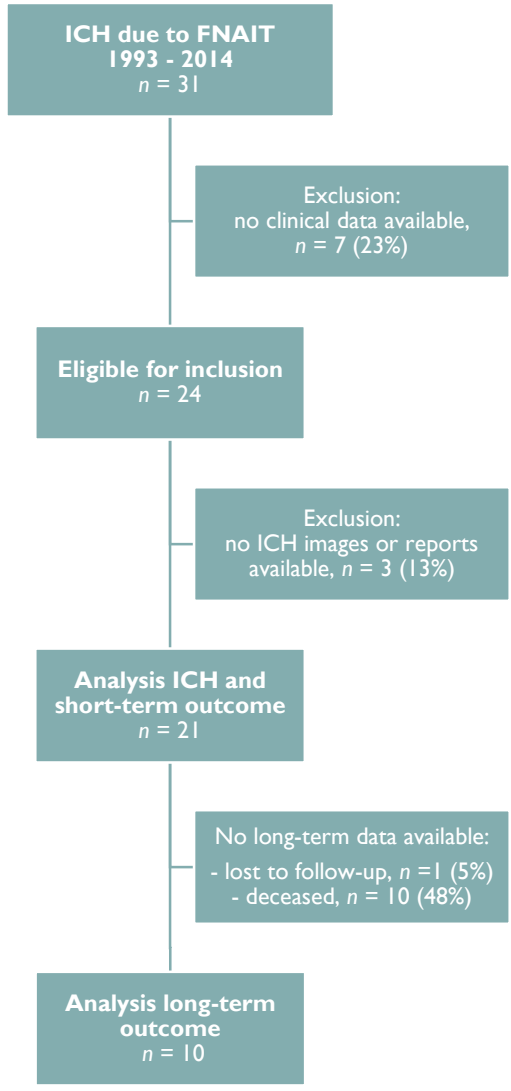

Figure 3.1 – Flow chart study population

Neuroimaging examinations of ICH

Type and localization of ICH of the 21 children with ICH are reported in table 3.2. From 8 (38%) children MRI images were available for review; the other 13 (62%) could be classified using written reports. Nineteen (91%) children had intraparenchymal hemorrhage. In 8 cases there was also intraventricular bleeding and in 2 cases subarachnoidal bleeding. Eight cases had bilateral hemorrhage. Eleven cases were complicated by posthemorrhagic hydrocephalus, of whom 6 developed a porencephalic cyst, resulting in 5 of these children requiring a ventricular peritoneal shunt.

Table 3.1 – Demographic characteristics of study population

Maternal age (years)	30 (21-41)
Obstetrical history	
Sibling with ICH	1 (5)
Sibling with FNAIT	2 (10)
Miscarriage	10 (48)
HPA type	
HPA 1a	18 (86)
HPA 5b	2 (10)
HPA 5a	1 (5)
Obstetrical characteristics	
Singleton pregnancy	20 (95)
First pregnancy	5 (24)
First born child	12 (57)
Antenatal treatment,	4 (19)
IVIg 1 gr/kg/week after antenatal detection ICH	
Gestational age at delivery*, weeks^{+days}	36^{+0} (30^{+0} – 41^{+6})
Delivery*	
Vaginal	6 (33)
Ventouse	3 (17)
Caesarean section	9 (50)
Neonatal characteristics	
Male sex	13 (62)
Birth weight, gram*	2408 (1178-4080)
Platelet count, $\times 10^9$/L *	
without antenatal IVIg	158 (133-164)
with antenatal IVIg	11 (6-29)

Data are presented as *n* (%), mean (SD) or median (IQR). FNAIT, fetal and neonatal alloimmune thrombocytopenia; HPA, human platelet antigen; ICH, intracranial hemorrhage; IVIg, intravenous immunoglobulin.

* Terminations of pregnancy excluded ($n = 3$).

Long-term neurodevelopmental outcome

In total, 10 surviving children with ICH were included for long-term follow-up (Table 3.3). Long-term outcome could not be assessed in 1 child (5%) due to loss of contact information.

Neurodevelopment was already assessed elsewhere (rehabilitation clinic or pediatric department) in 6 cases, using developmental tests adapted to their cognitive, motor and/or visual impairments (i.e. Snijders Oomen Nonverbal Intelligence Test and Kent Infant Development Scale). Two children were evaluated by the medical psychologist at our center. Two children could not be assessed with psychometric tests due to very severe cognitive and motor impairment and were assigned a score of 49. Children were tested at a median age of 7.5 years (range 1 – 23). Overall adverse outcome, including perinatal mortality or NDI, was 16/20 (80%).

Table 3.2 – Intracranial hemorrhage characteristics and short-term outcome

Child #	GA at birth	IVIg antenatal	Location ICH
1	33+4	No	extensive subarachnoid and unilateral parenchymal frontal/temporal/occipital
2	35+0	No	unilateral intraventricular and parenchymal and parenchymal
3	31+5	No	bilateral parenchymal
4	36+5	No	extensive bilateral parenchymal.
5	38+1	No	extensive bilateral parenchymal
6	22+0	No	bilateral parenchymal
7	32+2	No	extensive subarachnoidal
8	30+0	No	bilateral intraventricular and parenchymal
9	19+0	No*	extensive bilateral parenchymal
10	19+4	No	unilateral parenchymal and intraventricular
11	38+1	No	unilateral parenchymal, occipital
12	36+0	No	unilateral parenchymal, temporal
13	35+0	No	bilateral parenchymal, temporal
14	36+1	No	bilateral parenchymal, temporal and occipital
15	35+3	Yes, from 30 weeks	extensive bilateral intraventricular, parenchymal and cerebellar hemorrhage
16	36+0	Yes, from 28 weeks*	unilateral parenchymal, occipital and cerebellar
17	40+6	No	bilateral parenchymal, parietal, temporal and occipital
18	41+3	No	unilateral parenchymal, fronto-temporal
19	37+6	Yes, from 20 weeks	unilateral parenchymal, intraventricular and bilateral cerebellar
20	41+5	No	bilateral frontal parenchymal and intraventricular
21	37+0	No	extensive bilateral intraventricular

APLA, abortus provocatus lege artis; GA, gestational age; ICH, intracranial heamorrhage; IVIg, intravenous immunoglobulins; TOP, termination of pregnancy; VPD, ventriculoperitoneal drain.

	Associated lesions	Mortality	Obstetric history mother	
	-	Yes, neonatal	G1P0	
	hydrocephalus	Yes, neonatal	G2P1	healthy child
	-	Yes, neonatal	G1P0	
		Yes, neonatal	G3P1	healthy child, miscarriage
	hydrocephalus	Yes, fetal during labor	G2P0	miscarriage
	hydrocephalus	Yes, TOP	G2P0	miscarriage
		Yes, neonatal	G2P1	child with trisomy 21
	hydrocephalus	Yes, neonatal	G3P0	miscarriage, one abortion
	-	Yes, TOP	G4P3	two healthy children, one child with FNAIT
	-	Yes, TOP	G3P1	healthy child, miscarriage
	-	No	G3P0	two miscarriages
	hydrocephalus, VPD	No	G1P0	
	porencephalic cyst hydrocephalus, VPD	No	G2P0	miscarriage
	porencephalic cyst hydrocephalus, VPD	No	G2P0	miscarriage
	bilateral porencephalic cyst, cerebel-lar destruction hydrocephalus, VPD	No	G2P1	healthy child
	-	No	G4P1	two miscarriages, one child with FNAIT
	bilateral porencephalic cyst hydrocephalus, VPD	No	G1P0	
	-	No	G1P0	
	hydrocephalus, unilateral porencephalic cyst	No	G4P2	immature delivery at 17 weeks, child ICH, abortion
	hydrocephalus, bilateral porencephalic cysts	No	G2P0	molar pregnancy
	-	No	G2P1	healthy child

Table 3.3 – Intracranial hemorrhage and long-term outcome

Child #	Associated lesions	Age at evaluation	Cerebral palsy
11	None	8 year	-
12	Hydrocephalus, VPD	2, 8 and 14 years	spastic tetraplegia GMFCS V
13	Porencephalic cyst hydrocephalus, VPD	20 year	spastic tetraplegia GMFCS V
14	Porencephalic cyst hydrocephalus, VPD	23 year	spastic tetraplegia GMFCS V
15	Bilateral porencephalic cyst, cerebellar destruction hydrocephalus, VPD	3 year	spastic diplegia GMFCS IV
16	None	5 year	-
17	Bilateral porencephalic cyst hydrocephalus, VPD	1 year	spastic hemiplegia GMFCS IV
18	None	7 year	
19	Hydrocephalus, unilateral porencephalic cyst	5 year	spastic hemiplegia GMFCS I
20	Hydrocephalus, bilateral porencephalic cysts	8 year	spastic diplegia GMFCS II
21	None	None	*Loss of contact information, no long-term follow-up available*

ADHD, attention deficit hyperactivity disorder; Bayley-III, Bayley Scales of Infant and Toddler Development third edition; GMFCS, Gross Motor Function Classification System; KID-N, Kent Infant Development Scale; NDI, neurodevelopmental impairment; SON, Snijders-Oomen Nonverbal Intelligence Test; VPD, ventriculoperitoneal drain; WISC-III, Wechsler Intelligence Scale for Children third edition; WPPSI-III, Wechsler Preschool Primary Scale of Intelligence third edition.

Severe NDI in the studied cohort was found in 6/10 cases (60%). Cerebral palsy was diagnosed in 7 cases (70%). One child had moderate NDI due to spastic hemiparesis with a GMFCS score of I. Severe cognitive delay was detected in 6 children (60%) and severe motor delay in 6 children (60%). Three children were blind (30%) and 1 child was diagnosed with severe visual impairment. Epilepsy was reported in 4 (40%) children. One child was diagnosed with attention deficit hyperactivity disorder; 1 child had problems with behavior and attention regulation, but was too young to be already diagnosed with attention deficit hyperactivity disorder.

Developmental test	Total IQ	Long-term outcome	Severe NDI
WISC	86	ADHD	no
Bayley/BSID;Reynell-Zinkin;KID-N	49	bilateral blindness, severe cognitive and motor delay, epilepsy	yes
not tested due to severe impairment	49	bilateral blindness, severe cognitive and motor delay, epilepsy	yes
not tested due to severe impairment	49	bilateral blindness, hearing impairment, severe cognitive and motor delay	yes
SON	60	severe cognitive and motor delay	yes
WPPSI	110		no
KID-N	49	visual impairment, severe cognitive and motor delay, epilepsy	yes
WISC	112		no
WPPSI	85	problems with behaviour and attention-regulation	no
SON	50	severe cognitive and motor delay, epilepsy	yes

Discussion

This study shows that ICH caused by FNAIT is associated with a high risk of perinatal death and lifelong neurological sequelae in survivors. Of the 10 surviving infants, 6 had severe NDI and 2 had moderate NDI. Therefore, only 2 of the 10 survivors were completely free of long-term neurodevelopmental sequelae. Cerebral palsy was diagnosed in 70% and severe cognitive delay in 60%. In addition, 40% of the children had severe visual impairment and 40% was diagnosed with epilepsy. Our findings stress the severity and implications of major and permanent life-long impairments associated with FNAIT, particularly in case of ICH.

The vast majority (90%) of the ICHs occurred antenatally, which is in line with a previously published report on the short-term outcome of 43 ICHs due to FNAIT.[7,18] Like in this previously reported series, most ICHs were parenchymal hemorrhages, with the majority complicated by hydrocephalus and/or porencephalic cysts. In our cohort, cases with hydrocephalus and porencephalic cysts were more likely to result in severe NDI (6/7 and 5/6, respectively). Due to the relative small sample size, no correlation could be identified between localization (frontal/temporal/occipital or parenchymal, intraventricular, cerebellar) or extent (uni-/bilateral) and long-term outcome.

Obviously, our study does not match the true prevalence of ICH in our country, whereas it is a single center study and there is a considerable amount of cases with missing clinical information (7/31, 23%). This might have resulted in an overrepresentation of the more severe cases of ICH. For example, we report a rate of perinatal death of 48% (10/21), which is somewhat higher than the previously reported rate of 35% (15/43).[7] Also, because many women were identified because they were treated or counselled at our center in subsequent pregnancies, we might have found a higher rate of primigravid women and first-born children. Furthermore, cultural differences or legal restrictions in administration of intensive neonatal care may have influenced the outcome in this cohort. It is plausible that withholding or withdrawing neonatal intensive care treatment in cases with poor prognosis, may have led to a higher perinatal mortality and therefore to a lower number of survivors with poor neurodevelopmental outcome. However, cases were not selected because of behavioral or developmental problems, so it is not likely that cases with better developmental outcome were missed. Undoubtedly, there is heterogeneity in developmental testing performed, adapted to the age as well as to the severity of impairment of the children included for follow-up. This severity limited our ability to perform standardized psychometric testing in all children.

Despite these limitations, this is an unique study that focuses on long-term outcome of ICH due to FNAIT, clearly outlining the burden of this disease in survivors. One of the strengths of this study is that we used standardized psychochometric tests. Moreover, we were able to do long-term follow-up at a median age of 7.5 years. Previously, follow-up at one year was analyzed in newly detected FNAIT cases, with various clinical presentations, by Knight and colleagues.[19] They reported death or disability in 9 out of 88 cases, 2 infants died, 2 infants had severe global developmental delay, 4 infants had motor and visual impairment and 1 infant had only visual problems. However, no standardized tests were reported and classification of impairment was not further specified.[19] Lastly, we were able to focus on a clear and homogenous group consisting of children with ICH solely due to FNAIT. Earlier, cohorts of ICHs have been described, of which the largest series of intraventricular hemorrhage (IVH) in full term newborns was reported by Mao and colleagues.[11] They analyzed a total of 36 newborns and found a low mortality rate and, generally, a favorable outcome, with 63% of

all cases having no or only mild impairment. In contrast, they found FNAIT to be the single most important cause of adverse outcome. Out of 9 cases, 3 children died and 6 were severely impaired. Jocelyn and Casiro studied a cohort of 15 intraventricular hemorrhage cases in full term newborns, of which three were caused by FNAIT.[12] Of these 3 patients, 2 survived and were both severely impaired. Both studies are limited by the small number of patients as well as by their selection of cases. Whereas both studies selected newborns with diagnosis of intraventricular hemorrhage, there might be an underrepresentation of (minor) ICHs caused by FNAIT.

In the absence of screening programs for FNAIT, the disease is almost always detected after birth of an affected child, and preventive measures with antenatal IVIg can only be taken in the following pregnancies. Implementation of routine HPA-typing, primarily for HPA-1a, and consequent antibody screening in the near future would strongly reduce the burden associated with this disease. However, before such screening can be implemented, costs and potential benefits should be weighed carefully. So far, several attempts to estimate cost-effectiveness reached the same conclusion, namely that such programs are likely to be cost-effective.[10,20,21] This study, as it is the first one to provide detailed long-term follow-up data of children that suffered ICH due to FNAIT, provides essential knowledge for this debate. In addition, prospective studies including general screening for FNAIT and long-term follow-up are needed to learn more about the pathophysiology of this disease, including establishing if there is also a milder phenotype of ICH with discrete symptoms and better outcome. Furthermore, whereas only a proportion of alloimmunized pregnancies will result in devastating ICH as described in this study, research is needed to establish diagnostic tools to identify pregnancies that are at high risk for these bleeding complications and that would benefit from antenatal intervention and treatment.

Conclusion

This is the first study focusing and reporting on the long-term neurodevelopmental outcome of children suffering from ICH caused by FNAIT, using standardized psychometric measures. In the vast majority of cases, ICH leads to either perinatal death or, in survivors, severe impairment. These long-term sequelae can only be avoided by screening and effective preventive treatment.

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Chapter 4

Severe bleeding complications other than intracranial hemorrhage in neonatal alloimmune thrombocytopenia: a case series and review of the literature

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Abstract

Background. The most feared bleeding complication in fetal and neonatal alloimmune thrombocytopenia (FNAIT) is an intracranial hemorrhage (ICH). However, FNAIT may also lead to other severe bleeding problems. The aim was to analyze this spectrum and evaluate the occurrence of severe hemorrhages other than ICH in fetuses or neonates with FNAIT.

Study design and methods. A retrospective chart analysis of cases of FNAIT presenting with severe bleeding complications other than ICH at our institution from 1990 to 2015 was conducted. Additionally, a review of the literature was performed to identify case reports and case series on FNAIT presenting with extracranial hemorrhage.

Results. Of 25 fetuses or neonates with severe bleeding due to FNAIT, three had isolated severe internal organ hemorrhage other than ICH; two pulmonary hemorrhages and one gastrointestinal hemorrhage. Two of these three neonates died due to this bleeding. Eighteen cases of extracranial bleeding complications as a first presentation of FNAIT were found in the literature, including ocular, gastrointestinal, spinal cord, pulmonary, renal, subgaleal, and genitourinary hemorrhages.

Conclusion. Bleeding complications other than ICH may be more extensive, and the presentation of FNAIT may have a greater spectrum than previously described. A high index of suspicion on the possible diagnosis of FNAIT with any bleeding complication in a fetus or neonate may enable adequate diagnostics, adequate treatment and appropriate follow-up in future pregnancies, as is especially relevant for FNAIT.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by human platelet antigen (HPA) incompatibility between mother and child. Alloantibodies produced by the mother can cross the placental barrier and lead to destruction of fetal platelets (PLTs) as well as compromised vascular integrity.[1] FNAIT is the main cause of severe thrombocytopenia in term neonates.[2] Low PLT count in FNAIT correlates with an increased risk of bleeding complications, of which the most feared is an intracranial hemorrhage (ICH). The incidence of FNAIT-related ICH or perinatal death is estimated to be at least 1:11,000 fetuses or neonates; this, however, is likely an underestimation.[3] ICH is a devastating complication, which may lead to death or permanent neurological impairment. Outcomes are often more severe than for neonatal ICH from other causes.[4] Consequently, most publications and clinical guidelines of FNAIT are focused on the occurrence and prevention of ICH. The same trend can be observed in clinical practice, where the occurrence of ICH in an otherwise healthy infant is immediately associated with FNAIT. This is in contrast to other bleeding problems, where the proper diagnosis might be delayed or even missed, denying these women adequate care in future pregnancies.[5,6] In this study, we evaluated the occurrence of severe hemorrhage other than ICH, in neonates with FNAIT, and present a review of the literature.

Materials and methods

We conducted a retrospective study to evaluate the incidence and clinical course of fetuses and neonates presenting with severe extracranial hemorrhage due to FNAIT. We searched the FNAIT registry of our tertiary center for cases presenting with severe hemorrhage from 1990 to 2015. Clinical and demographic data were retrieved from medical charts. The diagnosis FNAIT had to be confirmed by demonstrating HPA incompatibility together with the presence of maternal alloantibodies. An extracranial hemorrhage was defined as an internal organ hemorrhage other than ICH or cutanomucosal bleeding.

Additionally, we reviewed and summarized the literature for cases of extracranial hemorrhage due to FNAIT. Relevant publications up to August 2015 were identified by searching MEDLINE, Embase and the Cochrane Library databases, using a combination of the keywords stated in figure 4.1. No restriction of language or type of publication was applied. Authors were contacted if additional data were needed. We focused on information on initial clinical presentation, immunohematologic evaluation and treatment provided.

Results

During the study period, from 1990 to 2015, a total of 25 index cases of severe bleeding complications could be extracted from our own FNAIT registry. Of these cases, 22 children suffered from ICH, of which eight died. We identified three neonates with severe extracranial hemorrhage.

Case report #1

A 29-year old healthy women gave birth to her second child after an uneventful pregnancy at 39+4 weeks' gestation. The first pregnancy and delivery were uneventful as well. A boy was born after an uncomplicated spontaneous delivery, birth weight 3955 g, and Apgar scores of 8 and 9 at 1 and 5 minutes. Two hours after birth the boy developed respiratory distress, for which continuous positive airway pressure treatment was started. Chest X-ray revealed diffuse consolidation of the right hemithorax. Due to persistent respiratory distress, intubation and mechanical ventilation were required. At intubation a massive amount of blood in nasopharynx and trachea was detected, severely hampering visualization. The infant was transported to our neonatal intensive care unit. On examination, diffuse petechiae were detected on the infant's chest and abdomen. Laboratory evaluation showed a very severe thrombocytopenia (PLT count 5×10^9/L). In absence of signs of placental insufficiency, asphyxia, perinatal infection or maternal autoimmune diseases there was a high suspicion of FNAIT. HPA-1a negative PLTs were ordered immediately. Meanwhile, a random PLT transfusion was administered, followed directly by matched PLTs. PLT count after these two transfusions was 216×10^9/L. The mother was found to be HPA-1a negative and the child was HPA-1a positive. Maternal HPA-1a alloantibodies confirmed the diagnosis FNAIT. Besides slightly abnormal brainstem-evoked response audiometry patterns, implicating damage caused by postnatal hypoxia, a full recovery was achieved.

Case report #2

A healthy 28-year old women gave birth at 33^{+6} weeks' gestation. Obstetric history revealed a curettage because of a missed abortion at 12 weeks. This second pregnancy was complicated by premature contractions, occurring at 21 weeks, leading to admission and tocolytic treatment. At 33^{+5} weeks' gestation, membranes ruptured spontaneously, followed by contractions and preterm birth. The girl had a birth weight of 1630 g. There were no signs of perinatal asphyxia or a neonatal infection. Shortly after delivery the clinical condition worsened progressively and a massive lung bleeding was discovered. Resuscitation was to no avail and the infant died a couple of hours after birth. Because of a severe thrombocytopenia (PLT count 40×10^9/L), immunohematologic investigation was performed and showed a HPA-1a mismatch mother and father together with maternal HPA-1a alloantibodies. In retrospect, the diagnosis FNAIT was stated as the most likely cause of this pulmonary hemorrhage and neonatal death.

Case report #3

A healthy 36-year-old woman, G2P0, gave birth at 39^{+3} weeks' gestation. Obstetric history included a miscarriage at 10 weeks. After an uneventful pregnancy, delivery occurred at home and was uncomplicated, with a birth weight of 2810 g and Apgar scores of 10 and 10 at 1 and 5 minutes. A couple of hours after delivery the infant was brought to the hospital because of petechiae and hematomas. Laboratory examination revealed a very severe thrombocytopenia (PLT count 2×10^9/L), which did not improve after emergency transfusion with random platelets. The evaluation presented no evidence for neonatal infection. The clinical situation worsened, demonstrated by the occurrence of large amount of bloody stool and acute abdominal distension. A laparotomy was performed, which demonstrated extensive gastrointestinal and intra-abdominal hemorrhage. After surgery, the clinical condition remained unstable with hypotension, metabolic acidosis and progressive abdominal distension, leading to a second look surgery involving extensive lavage and abdominal drain placement. Eventually, at 4 days of age, an irreversible shock resulted in neonatal death. Immunohematologic evaluation confirmed the presumed diagnosis FNAIT caused by HPA-1a and HPA-5b alloantibodies.

Review of literature

Our initial search yielded a total of 1,240 publications. Figure 4.1 shows a flowchart of the complete search strategy. After full-text screening, 16 articles containing 18 cases of neonates or fetuses with isolated severe extracranial bleeding complication caused by FNAIT, in absence of ICH, were identified (Table 4.1).

Genitourinary hemorrhage

Baber and colleagues[7] described a case of post circumcision bleeding at 2 days of age. After a neonatal circumcision, uncontrolled bleeding occurred. PLT count was 5×10^9/L and the diagnosis FNAIT was made. After treatment with intravenous immunoglobulins (IVIg) and PLT transfusions the bleeding stopped on Day 2. The infant made a complete recovery. As part of a retrospective cohort study, describing 20 cases of FNAIT, Cook and colleagues[8] reported a case of scrotal bleeding caused by HPA-1a alloimmunization. The infant's PLT count was 7×10^9/L. Unfortunately, despite platelet transfusion the hemorrhage led to the loss of a testicle.

Subgaleal hemorrhage

Two cases of subgaleal hemorrhages in neonates with FNAIT have been published.[9,10] Borensztajn and colleagues[9] reported a 2-day-old infant with petechiae and a subgaleal bleeding after vacuum extraction. Only after an unfavorable response to a random PLT transfusion (PLT count 13×10^9/L), FNAIT was suspected. After transfusion with HPA-1a negative platelets there was a full recovery. Davoren and coworkers[10] described a neonate with subgaleal bleeding as part of a case control study of 27 FNAIT cases. In this case, severe hypotensive shock occurred, requiring cardiorespiratory resuscitation. Eventually, a complete recovery was achieved.

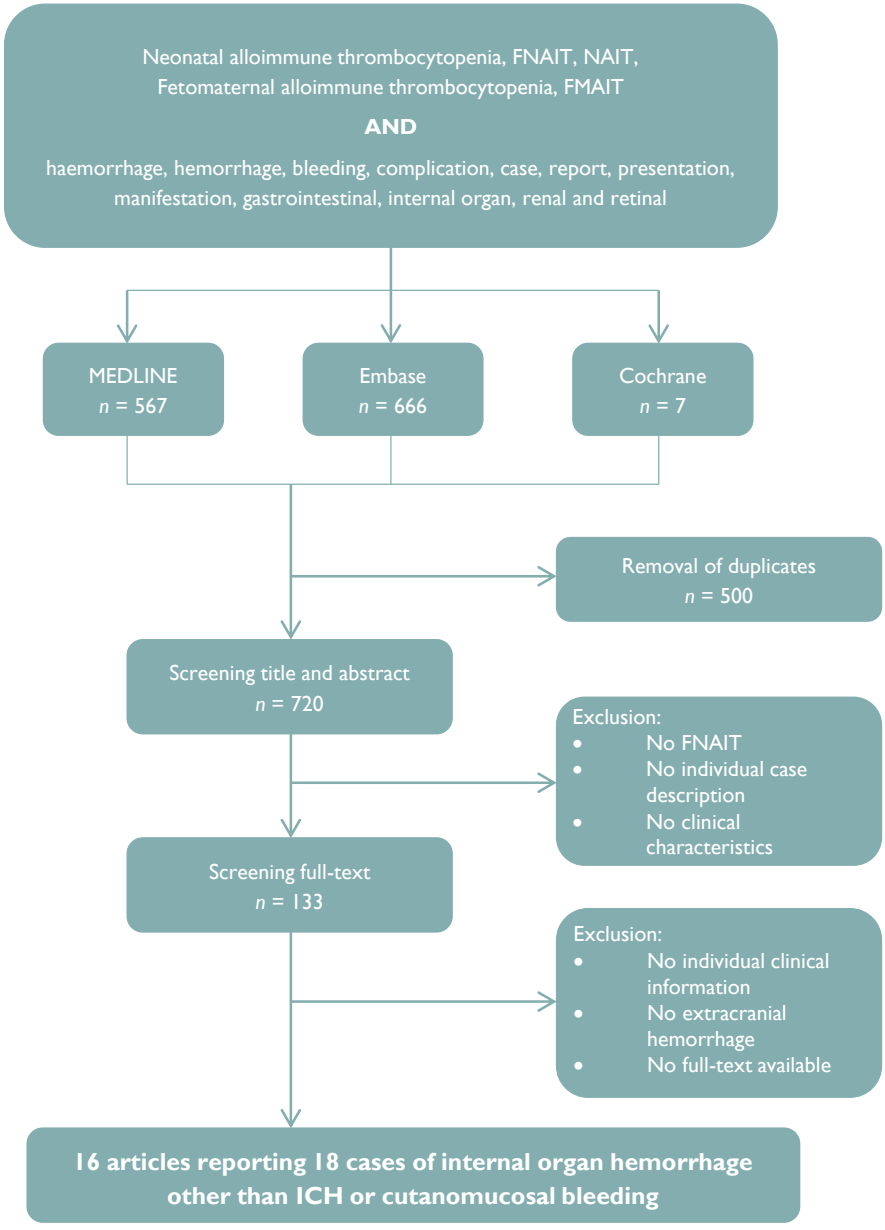

Figure 4.1 – Flow-chart of search strategy

Table 4.1 – Reports of extracranial hemorrhage caused by FNAIT

First author, year	Hemorrhage	Skin manifest-tations	Lowest platelet count $\times 10^9/L$	HPA	Treatment
Baber, 2015	Post circumcision	No	5	Unknown	IVIg, PTx
Jerónimo, 2014	Intraocular	No	27	HPA-1a	IVIg, PTx
Cook, 2012	Scrotal	Yes	7	HPA-1a	PTx
Borensztajn, 2010	Subgaleal	Yes	13	HPA-1b	RBC transfusion, PTx
Nomura, 2010	Retinal	Yes	42	HPA-5b	Expectative
Ghevaert, 2007	Pulmonary	No	23	HPA-5b	Unknown
Paladini, 2007	Renal	Na	Na	HPA-5b	Termination of pregnancy
Rousseau, 2004	Gastrointestinal	Unknown	Unknown	HPA-1a HPA-5b	Unknown
Abel, 2003	Cervical spinal cord	Yes	2	HPA-1a	IVIg, PTx
Davoren, 2002	Retinal	Unknown	Unknown	HPA-1a	IVIg, PTx
	Subgaleal	No	14	HPA-1a	Steroids, RBC transfusion, PTx
Tomicic, 2001	Gastrointestinal	Yes	29	HPA-1a	Steroids
Kankirawatana, 2001	Gastrointestinal	Yes	15	NAK	IVIg, steroids, PTx
Mokhtari, 1997	Gastrointestinal	Yes	8	HPA-3a	Exchange transfusion, PTx, IVIg, steroids
Allen, 1992	Gastrointestinal	Yes	36	HPA-5b	None
Puig, 1993	Gastrointestinal	Yes	9	HPA-4b	PTx
Kaplan, 1991	Gastrointestinal	Yes	10	HPA-5b	RBC transfusion, PTx
	Pulmonary	Yes	80	HPA-5b	IVIg, steroids

FNAIT, fetal and neonatal alloimmune thrombocytopenia; HPA, human platelet antigen; IVIG, intravenous immunoglobulins; Na, not applicable; PTx, platelet transfusion; RBC, red blood cell.

Ocular hemorrhage

Ocular bleeding sites were illustrated by Davoren and colleagues[10] as well as Jeronimo and Nomura and colleagues[11]. The first described a retinal bleeding occurring at the first day of life. After treatment with IVIg and a PLT transfusion the infant made a complete recovery.[10] Jeronimo and coworkers[11] described a premature neonate, born at 29 weeks' gestation, with proptosis and hyphema, along with a retinal bleeding (PLT count 27×10^9/L). A random PLT transfusion was administered with a favorable response. However, PLTs dropped again after 5 days, resulting in suspicion of FNAIT. HPA-1a alloimmunization was discovered, and after two additional HPA-1a negative PLT transfusions and a single dose of IVIg, PLT count increased and the intraocular hemorrhage decreased.

Intra-abdominal hemorrhage

Eight cases of severe intra-abdominal hemorrhage were described as a presentation of FNAIT, of which seven were gastrointestinal bleedings.[12-19] For the gastrointestinal hemorrhages a variety in accountable alloantibody as well in treatment was observed (Table 4.1). A relatively mild case was reported by Allen[16]; after an uneventful pregnancy and delivery, the infant showed rectal bleeding at 2 days of age and the infant's PLT count was 41×10^9/L. A diagnosis of FNAIT due to HPA-5b alloimmunization was stated and without any treatment the hemorrhage ceased and PLT count increased. In contrast, Mokhtari and coworkers[14] describe a more severe case of HPA-3a alloimmunization in which extensive treatment was necessary. Eventually, resulting in a normal platelet count after 5 days. The other intra-abdominal hemorrhage was described by Paladini and coworkers[19], a case of severe bilateral renal hemorrhage discovered on ultrasound in a fetus at 22 weeks' gestation. A broad panel of blood tests revealed HPA-5b alloimmunization. After extensive counseling the patient decided to terminate the pregnancy.

Pulmonary hemorrhage

Two pulmonary hemorrhages were reported, both part of larger cohort studies.[17,20] Ghevaert and coworkers[20] identified 123 new cases of FNAIT in their prospective cohort, including three cases of internal organ hemorrhages; gastrointestinal, retinal and pulmonary. Only the pulmonary hemorrhage, caused by HPA-5b alloimmunization, occurred in absence of ICH. Kaplan and coworkers[17] present the second case of pulmonary hemorrhage, part of a retrospective cohort of 39 HPA-5b alloimmunizations, occurring in a premature infant (delivered at 33 weeks' gestation). The outcome was favorable after treatment with red blood cell (RBC) and PLT transfusion together with IVIg and corticosteroids.

Spinal cord hemorrhage

Finally, a cervical spinal cord hemorrhage caused by FNAIT was described by Abel and colleagues[21] in a full term infant, born after cesarean section because of a non-reassuring fetal heart rate pattern with vacuum assistance for delivering the head. A PLT count of 2×10^9/L together with skin manifestations implied FNAIT. Because of hypotonic upper extremities and severe head lag, magnetic resonance imaging was performed, revealing a hemorrhage in the medulla extending inferiorly into the spinal cord. After PLT transfusion and IVIg, PLT count increased. Unfortunately, at 6 weeks' follow up, the infant continued to have upper extremity weakness and hypotonia.

Discussion

Although ICH is the most feared and well-known bleeding complication, FNAIT can have a wide variety of presentations. Symptoms can differ from isolated minor skin manifestations such as petechiae or purpura to massive and possible life-threatening organ hemorrhages. The most reported severe bleeding complication is ICH; hence the brain seems to be the most susceptible organ. Although the pathogenic mechanism is not fully understood, recently published data suggest that impairment of angiogenesis rather than thrombocytopenia causes these ICHs, possibly explaining the vulnerability of the fetal brain.[22] Though seldom described in guidelines or review articles on FNAIT, the site of bleeding can conceptually be in all kinds of organs.

To our knowledge, we present the first case series and review of literature of severe internal organ hemorrhages other than ICH caused by FNAIT. A total of 21 cases, including three cases from our center, were identified. The overall incidence of extracranial hemorrhages per 100,000 live births cannot be calculated based on these numbers, retrospectively acquired from a selected population. The relative frequency of extracranial hemorrhage in relation to ICH found in this study is 0.12 (3/25). Therefore, either the proportion of bleedings in the brain in FNAIT is much larger than at other sites in the body, or bleeding in other organs is underreported. Our series shows that the consequences of bleeding in other organs than the brain can lead to severe morbidity and even mortality. Obviously, our study design does not permit any conclusion on prevalence. Underestimation, in terms of both numbers and severity, seems to be likely. To gain more insight into incidence numbers and severity of bleeding complications in FNAIT a nationwide prospective cohort study is being set up by our center.

Clinicians need to be aware that FNAIT can also present as an extracranial hemorrhage in fetuses and neonates. Not only will this lead to a quicker diagnosis and targeted treatment, it will also lead to the ability of preventing FNAIT and bleeding complications in a possible subsequent pregnancy. This rationale is underlined by Fontano-Wendel and colleagues[5] as well as Madani and coworkers[6]. The latter reported a delayed or missed diagnosis in 15% of first affected children, retrospectively, in 26 FNAIT cases that were treated during a subsequent pregnancy. Fontano-Wedel and coworkers[5] described a case of a firstborn with petechiae and a PLT count of 8×10^9/L, discharged without a diagnosis. A year later, preconceptional advise yielded immunohematologic evaluation, which showed a HPA-1a mismatch between mother and father, leading to adequate monitoring and treatment in the subsequent pregnancy.

Knowledge of a history with confirmed FNAIT not only provides the opportunity to apply additional diagnostics during the subsequent pregnancy, for example determining fetal HPA genotype in case of paternal heterozygosity and monitor the pregnancy and fetal brain by ultrasound. It also presents the ability to administer adequate treatment during pregnancy, to

prevent the development of ICH and likely other bleeding. This is even more relevant now that the antenatal treatment strategy over the past years has changed from invasive to a safe and effective non-invasive weekly administration of IVIg.[23]

In conclusion, FNAIT is the most common cause of thrombocytopenia in term neonates. The most feared and well-known bleeding complication is an ICH. However, this is not the only possible presentation of this disease. We have described the first case series strengthened by an extensive review of the literature of severe bleeding complications other than ICH as a first presentation of FNAIT. A wider scope when dealing with bleeding problems in full-term newborns and adequate diagnostics will result in adequate treatment and appropriate follow-up as well as antenatal treatment with IVIg, when applicable, in future pregnancies.

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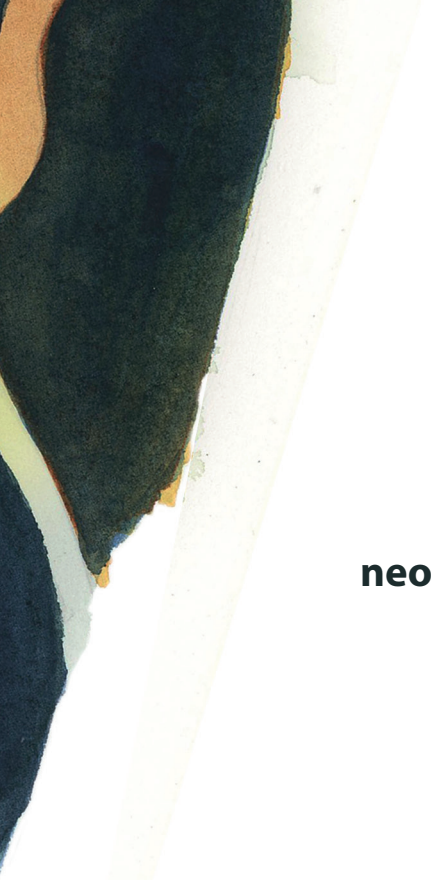

Chapter 5

Antenatal management in fetal and neonatal alloimmune thrombocytopenia: a systematic review

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Abstract

Several strategies can be used to manage fetal or neonatal alloimmune thrombocytopenia (FNAIT) in subsequent pregnancies. Serial fetal blood sampling (FBS) and intrauterine platelet transfusions (IUPT), as well as weekly maternal IV immunoglobulin infusion (IVIg), with or without additional corticosteroid therapy, are common options, but the optimal management has not been determined. The aim of this systematic review was to assess antenatal treatment strategies for FNAIT. Four randomized controlled trials and 22 nonrandomized studies were included. Pooling of results was not possible due to considerable heterogeneity. Most studies found comparable outcomes regarding the occurrence of intracranial hemorrhage, regardless of the antenatal management strategy applied; FBS, IUPT or IVIg with or without corticosteroids. There is no consistent evidence for the value of adding steroids to IVIg. FBS or IUPT resulted in a relatively high complication rate (consisting mainly of preterm emergency cesarean section) of 11% per treated pregnancy in all studies combined. Overall, noninvasive management in pregnant mothers who have had a previous neonate with FNAIT is effective without the relatively high rate of adverse outcomes seen with invasive strategies. This systematic review suggests that first line antenatal management in FNAIT is weekly IVIg administration, with or without the addition of corticosteroids.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) may lead to severe bleeding complications such as intracranial hemorrhage (ICH), in the fetus or newborn. Thrombocytopenia is caused by maternal alloantibodies against human platelet (PLT) antigens (HPAs) resulting from maternal alloimmunization after exposure to paternally derived antigens on fetal PLTs. The most commonly involved are HPA-1a alloantibodies, which are responsible for approximately 80% of FNAIT cases.[1,2] Not only do these maternal alloantibodies cause destruction and inhibit the production of fetal PLTs, they are also thought to affect vascular integrity and angiogenesis, resulting in an increased risk of intracranial and extracranial bleeding complications in fetuses and neonates and potentially intrauterine and perinatal death.[3-6]

In the absence of population-based screening programs, the diagnosis of FNAIT is usually made after an incidental finding of neonatal thrombocytopenia or because of bleeding complications ranging from bruising or petechiae to intracranial hemorrhage in the fetus or newborn.

Consequently, with an estimated recurrence rate of 79% of severe bleeding complications, the current challenge is to determine the best management strategy of subsequent pregnancies in women with a history of FNAIT with the goal of preventing these complications and avoiding maternal toxicities.[7] To avoid unnecessary interventions and anxiety, paternal genotyping should always be performed for the HPA involved in the preceding FNAIT. In case of paternal heterozygosity, maternal-fetal incompatibility should be determined either using amniocentesis or assessing cell-free fetal DNA, when HPA-1a is involved.

One of the first prenatal treatment strategies was ultrasound-guided fetal blood sampling (FBS) and intrauterine platelet transfusion (IUPT).[8] This technique, used for the treatment of fetal anemia, was applied to fetuses with thrombocytopenia and involved the transfusion of PLTs. Cordocentesis in the presence of thrombocytopenia may, however, lead to fetal bradycardia, tamponade of the cord and bleeding complications in the fetus including exsanguination. In addition, given the short life span of transfused PLTs, transfusions are needed regularly, increasing the overall risk of fetal loss.[9] The first non-invasive treatment, maternal infusion of intravenous immunoglobulin (IVIg) was reported in 1988, after which IVIg rapidly gained ground as a standard antenatal treatment strategy for FNAIT as have corticosteroids.[10] Prolonged use of IVIg and corticosteroids during pregnancy are associated with adverse effects as well. Although the side effects of IVIg are usually mild, hemolytic anemia, renal failure, aseptic meningitis and thrombotic complications may occur.[11,12] Corticosteroids are associated with hypertension and diabetes. Both agents can affect the quality of life of patients.[12]

No international consensus on the optimal antenatal management of FNAIT exists, and numerous strategies, non-invasive as well as invasive, are applied in different centers that specialize in antenatal therapy. Because FNAIT is a rare disease, systematically reviewing the literature to determine the evidence to support antenatal treatment options can inform practice. Hence, we performed a systematic review of all available literature on antenatal management strategies, to inform and assist in the development of guidelines.

Methods

Data Sources

This review was performed according to the PRISMA guidelines.[13] With the assistance of a medical research library specialist, an electronic search strategy was developed, and applied to databases Medline, EMBASE and Cochrane Library from 1946 to December 2015 (supplemental Appendix, available on the Blood Web site). Reference lists were cross-checked for relevant citations.

Study Selection and data extraction

Citations were reviewed by 2 reviewers to identify studies that met the following inclusion criteria: (1) original study; (2) included ≥5 pregnant women with pregnancies at risk for FNAIT or fetuses/neonates diagnosed with FNAIT; (3) treated with either IVIg, steroids or IUPT; (4) included any of the outcomes: intracranial hemorrhage and fetal/neonatal PLT count; and (5) published in the English language. When there was a disagreement, the full report was retrieved and independent assessment was repeated. Disagreements for inclusion were resolved by consensus. For articles that were published more than once and contained the same FNAIT population, only the study with the largest number of women and the most complete data extraction was included. Data extraction was performed by 2 authors according to a predetermined standardized format of study characteristics, outcome data and complications of interventions (Table 5.1).

Risk of bias was assessed according to The Cochrane Collaboration's tool.[14] for randomized studies, and Newcastle-Ottawa Scale (NOS)[15] for nonrandomized studies. The Newcastle-Ottawa Scale is based on 3 parameters: selection, comparability, and outcome (Table 5.2). For the parameter selection, we assessed whether the exposed cohort was representable for the FNAIT population (defined as HPA incompatible pregnancies), if the patient enrollment was consecutive, and if ICH was absent at start of the treatment. The parameter comparability was met if cohorts had a comparable proportion of siblings with ICH. For the parameter outcome of the Newcastle-Ottawa Scale, we assessed if the outcome of ICH was assessed by cranial ultrasound, if the follow-up was adequate (neonatal instead of fetal PLT count) and lastly, if neonatal PLT count and ICH data were available for all subjects.

Data Analysis

Due to considerable methodological heterogeneity of the studies, a descriptive review of all included studies was performed rather than a meta-analysis. In 2011, a Cochrane review of part of the included RCTs was performed by Rayment et al,[16] who also did not pool data.

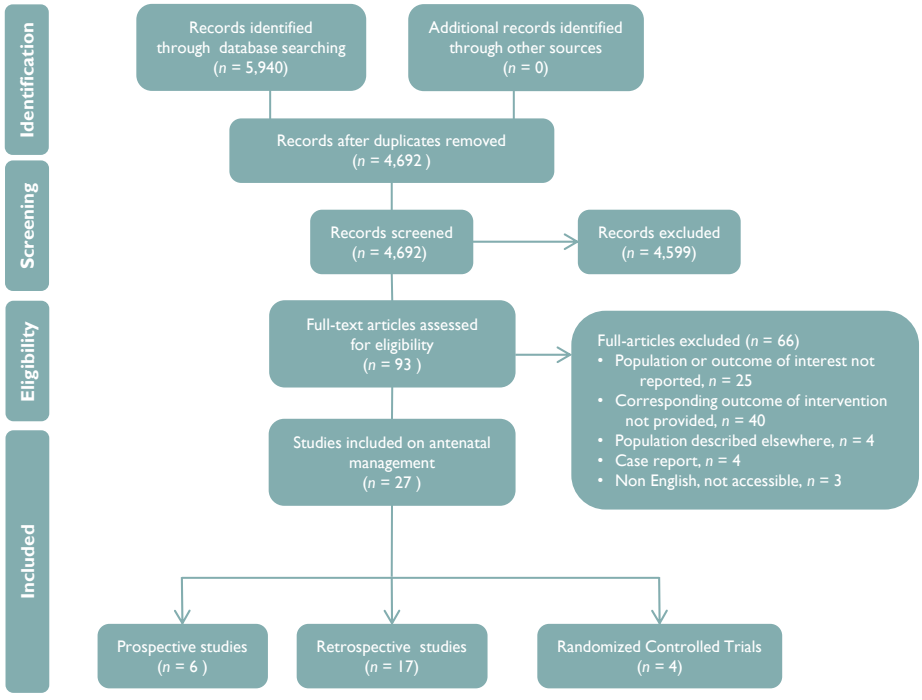


Figure 5.1 – Flowchart of search strategy

Table 5.1 – Study Outcomes

First author, year	**Study arms** (risk group in case of stratification)	***n***	**ICH in sibling** *n* (%)	**FBS** *n* (%)	**IUPT** *n* (%)
Randomized Controlled Trials					
Paridaans, 2015[30]	IVIG 0.5g	12	0	0	0
	IVIG 1g	11	0	0	0
Berkowitz, 2007[37]	IVIG 2g	37	0	All	0
	IVIG 1g + steroids	36	0	All	0
Berkowitz, 2006[35]	IVIG (all)	40	4 (10)	All	0
	IVIG + steroids (high)	19	3 (16)	All	0
	Steroids (standard)	20	0	All	0
Bussel, 1996[20]	IVIG§	28	6 (21)	All	0
	IVIG + steroids	26	4 (15)	All	0
Prospective Studies					
Kanhai, 2006[17]	IVIG ± IUPT	7	7 (100)	3 (43)	3 (43)
Bertrand, 2006[39]	IUPT pre-delivery	2	0	All	2 (100)
	IVIG ± IUPT	4	0	2 (50)	2 (50)
	IVIG + steroids	13	2 (15)	1 (8)	1 (8)
Radder, 2004[18]	IVIG ± IUPT	37	8 (19)	26 (70)	26 (70)
	FBS ± IUPT	13		All	9 (69)
Silver, 2000[23]	IVIG	8	3 (30)	All	NR
	Fetal IVIG	2		All	NR
Lynch, 1992[22]	IVIG	9	5 (56)	All	1 (11)
	IVIG + steroids	9	3 (33)	8 (89)	0
Retrospective Studies					
van der Lugt, 2015[31]	IVIG 1g (all)	5	2 (40)	0	0
	IVIG 0.5g (standard)	17	0	0	0
Bertrand, 2011[32]	IVIG	27		0	0
	IVIG + steroids	54	9 (14)	0	0
	Steroids	11		0	0
Mechoulan, 2011[24]	IVIG	17	5 (29)	9	0
	IVIG + steroids	6	0	0	0
Bussel, 2010[19]	IVIG 1g (high, very high)	5	5 (100)	All	1 (17)
	IVIG 1g + steroids (all)	19	19 (100)	All	0
	IVIG 2g (all)	4	4 (100)	All	0
	IVIG 2g + steroids (all)	9	9 (100)	All	0
Giers, 2010[21]	Fetal IVIG + IUPT	10	NR	All	10 (100)

	FBS/IUPT Related AE *n* (%)	**Duration IVIG in wks** mean (range)	**SE IVIG/ steroids** *n* (%)	**ICH** *n* (%)	**Mean PLTs** $\times 10^9$/L	**PLT <50 $\times 10^9$/L** *n* (%)	**Mortality** *n* (%)
	NA	10 (7-11)	0	0	81	3 (25)	0
	NA	11 (7-12)	0	0	110	4 (36)	0
	2 (5)	16 (11-20)	12 (32)	1	169	5 (14)	0
	2 (6)	16 (12-19)	13 (33)	1	134	4 (11)	0
		NR	NR		104	NR	
	11 (14)	NR	NR	3	99	NR	4 (5)
		NA	NR		108	NR	
	5 (9)	10	0	0	96	<30 6 (21)	5 (9)¶
		11	2 (8)	0	110	<30 5 (19)	
	NR	17 (8-21)	NR	0	28‡	7 (100)‡	0
	NR	NA	NA	0	210	0	0
	NR	17 (15-19)	NR	0	204	0	0
	NR	12 (5-20)	NR	0	118	4 (31)	0
	0	5 (2-15)	NR	0	67	17 (46)	0
	2 (22)	NA	NA	0	32	7 (54)	1 (8)
	2 (20)	12 (3-16)	NR	0	NR	1 (13)	0
		2 (1-2)	NR	0	NR	2 (100)	0
	NR	NR	NR	0	57	4 (44)	NR
	NR	NR	5 (56)	0	64	3 (33)	NR
	NA	NR	0	1*	63	8 (67)	0
	NA	NR	0	0	104	4 (33)	0
	NA	14	NR	0	89	12 (44)	0
	NA	14	NR	0	135	13 (27)	0
	NA	NA	NR	0	46	8 (73)	0
	1 (11)	12	NR	0	68	10 (59)	0
	NA	7	NR	0	78	4 (67)	0
		15 (7-25)	NR	1	165	0	0
	3 (8)	12 (5-25)	NR	3	85	6 (40)	2 (11)
		22 (18-25)	NR	0	112	0	0
		23 (18-27)	NR	1	135	0	0
	0	10 (6-14)	NR	0	189†	0	0

Table 5.1 – Continued

First author, year	Study arms (risk group in case of stratification)	*n*	ICH in sibling *n* (%)	FBS *n* (%)	IUPT *n* (%)
te Pas, 2007[25]	IVIG	13	5 (38)	NR	2 (15)
van den Akker, 2007[34]	IVIG (all)	53	5 (9)	0	0
	FBS + IVIG (all)	33	11 (33)	All	NR
	FBS + IUPT (standard)	13	0	All	13 (100)
Ghevaert, 2007[41]	IUPT ± IVIG ± steroids	40	NR	All	40 (100)
	IVIG and/or steroids	7	NR	NR	0
	No treatment	8	NR	NA	NA
Yinon, 2006[38]	IVIG	24	0	4 (17)	0
	No treatment	6	0	NA	NA
Tiblad, 2003[27]	IVIG §	9	5 (56)	0	0
	IUPT	3	0	All	3 (100)
	No treatment	6	2 (34)	0	0
Birchall, 2003[28]	IVIG ± IUPT§	18	6 (60)	All	6 (33)
	IUPT weekly	31	11 (42)	All	31 (100)
	FBS ± single IUPT	7	0	All	5 (71)
Sainio, 1999[42]	IVIG ± IUPT§	11	1 (9)	All	9 (82)
	IUPT	4	0	All	4 (100)
Kaplan, 1998[29]	IVIG	27	7 (26)	All	1 (4)
	Steroids	10	NR	All	NR
Kornfeld, 1996[33]	IVIG + IUPT	4	1 (25)	All	4 (100)
	IVIG	6	1 (17)	All	0
Murphy, 1994[40]	IVIG + IUPT ± steroids	8	6 (75)	All	6 (100)
	IUPT ± steroids	7	5 (71)	All	7 (100)
Wenstrom, 1992[36]	IVIG	2	NR	All	0
	IVIG + steroids	4	NR	All	0
Kaplan, 1988[25]	IUPT	4	1 (20)	All	5 (100)
	IVIG (5days) + IUPT	1	0	All	1 (100)

*** ICH occurred before start therapy; † Platelet count after IUPT; ‡ Platelet count before pre-delivery IUPT; § One or two patients also received steroids (supplementary table S5.1); ¶ Five fetuses exsanguinated and were excluded from analysis.**

FBS/IUPT Related AE *n* (%)	Duration IVIG in wks mean (range)	SE IVIG/ steroids *n* (%)	ICH *n* (%)	Mean PLTs $\times 10^9$/L	PLT <50 $\times 10^9$/L *n* (%)	Mortality *n* (%)
NR	NR	NR	0	83	6 (46)	0
NA	8 (2-24)	NR	0	125	10 (19)	0
3 (7)	6 (2-21)	NR	0	174	0	1 (3)
0	NA	NA	0	145	3 (23)	0
3 (8)	NR	NR	4	107	NR	6 (15)
1 (14)	NR	NR	0	7-219	NR	1 (14)
NA	NA	NA	0	6-84	NR	0
NR	15 (9-19)	NR	0	118	<30 2 (8)	0
NA	NA	NA	0	24	<30 4 (67)	0
NA	NR	NR	0	90	4 (44)	0
3 (100)	NA	NA	0	47	2 (100)	1 (33)
NA	NA	NA	1	9	4 (80)	1 (17)
3 (17)	9 (1-19)	1 (6)	1*	81	6 (33)	0
10 (30)	NA	NA	2*	NR	NR	3 (10)
2 (29)	NA	NA	0	NR	NR	0
2 (18)	6 (1-12)	1 (9)	0	109	5 (45)	0
2 (50)	NA	NA	0	76	2 (50)	0
NR	7 (2-15)	NR	2	69	13 (48)	2 (7)
NR	NA	NR	NR	NR	6 (60)	NR
1 (25)	NR	0	0	182	0	1 (25)
1 (17)	NR	0	0	98	2 (33)	1 (17)
1 (13)	9 (4-17)	NR	1*	340†	0†	2 (25)
0	NA	NR	2*	305†	0†	1 (14)
NR	14 (13-14)	0	0	60	1 (50)	0
NR	11 (5-20)	0	0	146	0	0
0	NA	NA	0	200	0	0
0	5	0	0	107	0	0

AE, adverse events; FBS, fetal blood sampling; ICH, intracranial hemorrhage; IVIG, intravenous immunoglobulins; IUPT, intrauterine platelet transfusion; NA, not applicable; NR, not reported; PLT, neonatal platelet count; SE, side effects.

Results

Study selection and characteristics

Our search strategy retrieved a total of 4692 single records that were screened for title and abstract, resulting in 93 full-text articles to be assessed for eligibility. Of those, 26 studies describing antenatal interventions in FNAIT were included (Figure 5.1), consisting of 4 RCTs, 5 prospective and 17 retrospective studies (Table 5.1; supplementary table S5.1).

Most studies included pregnancies at risk for FNAIT based on a history of FNAIT, additionally specified as with ICH,[17-19] PLT < 100×10^9/L,[20,21] PLT < 50×10^9/L[17,22] or signs of bleeding,[21,23,24] or based on another female family member with FNAIT[25] (1) or recurrent spontaneous miscarriages.[21] One study identified postnatal FNAIT patients from a population of thrombocytopenic neonates.[26] Five studies did not report testing for incompatibility between pregnant women and fetus as a condition for inclusion in their study.[21,22,27-29] FBS was performed in all but 3 studies.[30-32] The earliest that fetal blood was sampled was in gestational week 16,[33] but most commonly began in weeks 20 or 22. Of the 16 studies performing IUPT, 8 reported a fetal PLT count threshold to infuse PLTs.[17-19,21-23,28,34] HPA-1a was the predominant cause of FNAIT in all articles, ranging from 72 to 100% of reported patients.

The overall quality of the RCTs was considered adequate, with the lack of blinding presenting the highest risk of bias (Table 5.2). Comparing or pooling data from the nonrandomized cohort studies included in this review was hampered by differences in patient selection, in particular HPA type and severity of disease in the previous affected siblings.[21,24] In addition, relevant data were lacking in several studies, such as exclusion of ICH by ultrasound before starting treatment[35] and outcome data for all treatment arms (Table 5.2).

Antenatal management

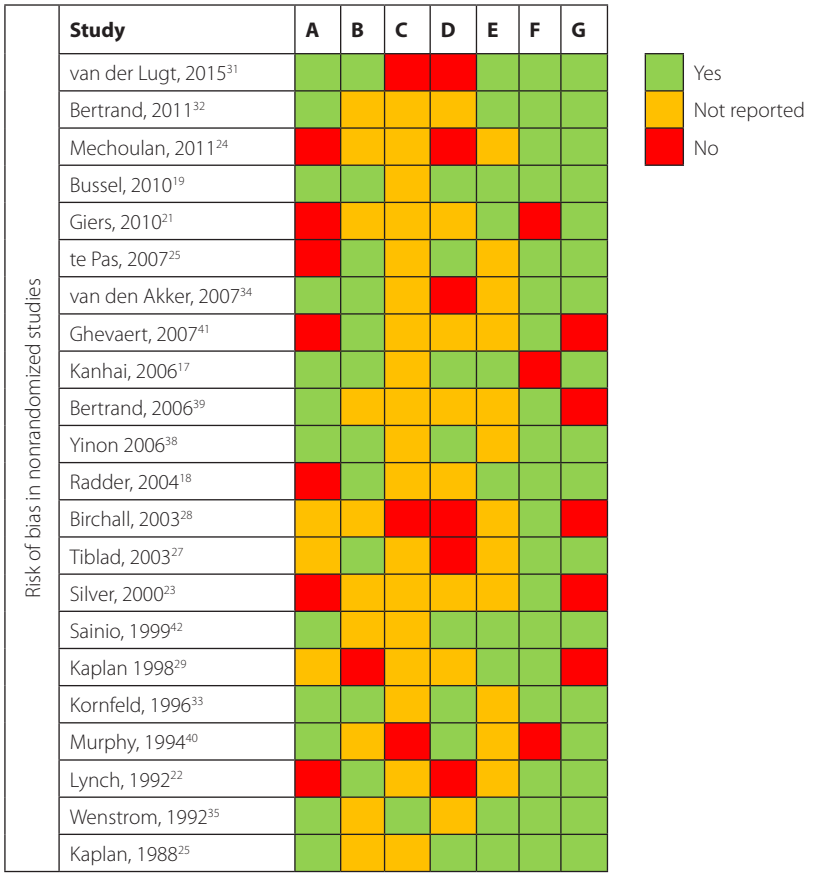
IVIg and corticosteroids

Of the 26 studies, 17 had a treatment arm with IVIg alone,[19,20,22-24,26,27,29-38] 3 studies with corticosteroids alone[29,35] and 11 had a study arm that combined IVIg and corticosteroid treatment.[19,20,22,24,32,35-37,39-41] There were 2 studies comparing all 3 arms.[35,39] In most studies, IVIg was administered at dose 1 g/kg/week.

Doses other than 1 g/kg/week in one or more cases were reported in 9 studies; 0.4 g/kg/day for 5 days,[25,40] 0.5 g/kg/week,[22,30,31] 0.8 g/kg/week,[28] 1g/kg/2weeks[24,32] and 2g/kg/week.[19,37] Two studies did not report the IVIg dose.[39,41] IVIg administration commenced as early as 10 weeks' gestation[19] and as late as 32 weeks gestation.[18] Two studies administered IVIg directly to the fetus.[21,23] Prednisone was used mainly at a dose of 0.5 mg/kg/day and dexamethasone at a dose of 1.5 mg/day. Specific dosages can be found in supplementary table S5.1.

Table 5.2 – Quality assessment of all 26 included studies

A. Random sequence generation (selection bias); **B.** Allocation concealment (selection bias); **C.** Blinding of participants and personnel (performance bias); **D.** Blinding of outcome assessment (detection bias); **E.** Incomplete outcome data (attrition bias); **F.** Selective reporting (reporting bias); **G.** Other bias.



A. Representative exposed cohort; **B.** Consecutive patient enrolment; **C.** Outcome absent at start study; **D.** Comparable proportion ICH in siblings; **E.** Outcome assessment; **F.** Adequate duration follow-up; **G.** Complete outcome data for all subjects.

FBS / IUPT

FBS was employed in 24 of the 26 studies. In 16 of these studies, FBS was combined with IUPTs. Five studies included a study arm with IUPT as sole treatment.[25,27,28,38,42] IUPT in combination with IVIg was used in three studies.[21,33,40] The remainder of IUPTs were performed in addition to a maternal therapy strategy of IVIg and/or steroids.[17-19,21,25,28,29,39,42] One study reported FBS and PLT transfusion in all fetuses prior to delivery.[21] Three studies did not report the number of IUPTs performed for their study groups.[22,29,34]

Risk stratification

Four studies stratified by risk group and altered interventions based on risk.[19,31,34,35] The stratification was either based on whether a sibling suffered an ICH[31,34,35], (high-risk), or on the timing of the ICH in the sibling (e.g. antenatal or postnatal) (supplementary table S5.1).[19]

Perinatal outcome

ICH

All but 1 study described the occurrence of ICH for all study arms.[29] In the 25 studies in which ICH was described, of the 839 pregnancies, a total of 24 ICHs were observed (3%). Seven of these occurred before treatment was started and 1 occurred in a group where no treatment was provided. Four ICHs were described by Ghevaert et al[41] as part of a large retrospective analysis of patients with suspected FNAIT investigated at a reference laboratory. Unfortunately, no additional information on previously affected pregnancies or on the patients themselves was provided. Of the remaining 12 patients, 5 were described by Bussel et al,[19] who reported different strategies of IVIg treatment in a high risk population (all siblings suffered from ICH). Three ICHs (2 grade III-IV hemorrhages resulting in fetal demise and 1 grade I hemorrhage) occurred after receiving 1 g/kg/week IVIg and 1 mg/kg/day prednisone, the fourth one was a grade II-III perinatal hemorrhage after delivery at 24 weeks' gestation and the last one was a grade I hemorrhage, both after a combination of 2 g/kg/week IVIg with 1 mg/kg/day prednisone. Furthermore, Berkowitz et al[35] described 2 neonates with ICHs that occurred in a low-risk population where none of the siblings suffered an ICH. Both ICHs were grade I subependymal hemorrhages, detected postnatally with normal neonatal PLT counts at birth (133 and 197 $\times$ 10^9/L) after treatment with 2 g/kg/week IVIg and 1g/kg/week IVIg with 1 mg/kg/day prednisone (treatment started at 20 weeks). Kaplan et al[29] described 27 pregnancies treated with 1 g/kg/week of IVIg, in which 2 fetuses had ICHs (one resulting in death and one resulting in neurological sequela), both in the group of nine patients with persistent low PLTs despite treatment. Lastly, Berkowitz et al[35] reported 3 ICHs, 2 grade I hemorrhages and 1 grade III ICH in a neonate that was delivered at 28 weeks' gestation because of persisting fetal bradycardia after FBS. Overall, no remarkable or significant differences could be identified in the occurrence of ICHs between various study arms.

Mortality

Two studies did not report mortality rates.[22,29] In the 24 remaining studies there was an overall mortality rate of 4% (30/821); of these, 17 were related to a FBS/IUPT (53%) and seven were due to ICH (22%). In 6 fetuses/neonates the cause could not be determined. Ghevaert et al[41] described a fetal loss due to acute amnionitis at 16 weeks gestation (not related to treatment) and Murphy et al[40] report a fetal loss after a severe fall of the mother on icy pavement.

Neonatal PLT Count

Twenty studies reported neonatal PLT counts. Of the other 6 studies, 1 study reported the fetal PLT counts before pre-delivery IUPT,[17] 2 studies reported fetal PLT counts after pre-delivery IUPT[21,40] and 3 studies did not provide the neonatal PLT counts for all study arms.[23,28,29] The mean neonatal PLT counts ($\times 10^9$/L), as well as the proportion of neonates with PLT counts below 50×10^9/L, varied widely between the studies ranging from 0% to 100%, regardless of the intervention.

Three studies compared IVIg treatment alone to corticosteroids alone.[29,32,35] Kaplan et al[29] found a higher proportion of neonates with a PLT count $<50 \times 10^9$/L in the group treated with steroids compared with IVIg only (60% vs 48%) as did Bertrand et al[32] (73% vs 44%). Berkowitz et al[35] found comparable mean PLT counts between those groups in patients; 104×10^9/L with IVIg only versus 108×10^9/L in the steroids only arm.

Three studies compared a non-invasive strategy (IVIg or IVIg and corticosteroids without FBS) to a strategy that included FBS and IUPT.[27,33,34] Kornfeld et al[33] showed that IVIg treatment alone improved neonatal PLT counts in 4 of 6 patients, however, only 1 pregnancy was high risk. Tiblad et al[27] reported a higher median PLT count of 90×10^9/L in the group treated with IVIg and a lower proportion of neonates with PLT counts below 50×10^9/L, 44% versus 100% in patients treated with IUPT. In addition, in the group treated with IVIg, 56% of the pregnancies were high risk, compared to 0% in the group treated with IUPT. Most recently, Van den Akker et al[34] compared 53 women treated with IVIg only to 13 women treated with IUPT only; median neonatal PLT counts were 125×10^9/L and 145×10^9/L, respectively.

Table 5.3 – Complications of antenatal treatment

First author, year	AE in FBS/IUPT *n*/N (%)*	Complications after FBS or IUPT (*n*)
Mechoulan, 2011[24]	1/9 (11)	Emergency CS due to fetal distress (1), <34 weeks (0)
Bussel, 2010[19]	4/37 (11)	Emergency CS or delivery (4), <34 weeks (NR) due to fetal distress (3), insertion bleeding (1)
van den Akker, 2007[34]	3/99 (3)	Perinatal death (1) Emergency CS due to fetal distress (3), <34 weeks (0)
Berkowitz, 2007[37]	4/74 (5)	Emergency CS (4), <34 weeks (3) due to fetal distress (2), ROM (2)
Berkowitz, 2006[35]	11/79 (14)	Fetal death (1). Neonatal death (1) Emergency CS or delivery (10), <34 weeks (NR) due to fetal distress (8), streaming (1), PROM (1)
Radder, 2004[18]	2/40 (5)	Neonatal death after fetal distress (1) Emergency CS due to exsanguination (1)
Birchall, 2003[28]	15/38 (39)	Fetal death (2), after exsanguination (1) Emergency CS/delivery (13), <34 weeks (6) due to fetal distress (6), infection (1), technical difficulties (3), cord spasm or thrombosis (2), placental artery bleeding (10)
Silver, 2000[23]	2/10 (20)	Emergency CS due to insertion bleeding (2), <34 weeks (1)
Sainio, 1999[42]	4/15 (27)	Emergency CS or delivery (4), <34 weeks (1) due to fetal distress (3) acute amnionitis after ROM (1)
Bussel, 1996[20]	5/59 (9) ‡	Fetal or neonatal death after exsanguination (5) ‡
Kornfeld, 1996[33]	2/10 (20)	Pregnancy loss at 16 weeks gestation (1) Neonatal death due to chorioamnionitis at 25 weeks (1)
Murphy, 1994[40]	1/15 (7)	Fetal death due to cord hematoma (1)
Lynch, 1992[22]	NR	NR

AE, adverse events; CS, cesarean section; CTG, cardiotocogram; FBS, fetal blood sampling; IVIg, intravenous immunoglobulins; IUPT, intrauterine platelet transfusion; SE, side effects; PROM, rupture of membranes.

	SE in IVIg *n*/N (%)*	**Reported side effects in IVIg treatment** (*n*)	**SE in steroids** *n*/N (%)*	**Reported side effects in steroid treatment** (*n*)
	NR	NR	NR	NR
	NR	NR	NR	NR
	NR	NR	NA	NA
	NR	Rash (1) discontinued IVIG Headache, fatigue	NR	Gestational diabetes (7) Insomnia, mood swings
	NR	NR	NR	NR
	NR	NR	NR	NR
	1/18 (6)	Headache and tachycardia (1), continued IVIG	NR	NR
	NR	NR	NA	NA
	1/11 (9)	Headache and tachycardia (1), continued IVIG	NR	NR
	0/54	None	2/26 (8)	Oligohydramnios (2) in - Dexamethasone 1.5mg - Dexamethasone 4.5mg
	0/10	None	NA	NA
	NR	NR	NR	NR
	NR	NR	5/9 (56)	Oligohydramnios (4) in - Dexamethasone 5mg

*** number of reported complications (*n*) versus the total number of patients treated with this specific strategy (N); † Number of side effects reported, the total number of patients that reported a side effect is unclear; ‡ The complications occurring during this study were reported in detail elsewhere[40].**

Of the 8 studies comparing IVIg only with IVIg and corticosteroids, Berkowitz et al[35] identified comparable platelet counts between groups treated with IVIg only and IVIg with steroids (104×10^9/L and 99×10^9/L respectively). The same group of investigators[19] described management in 37 high-risk pregnancies. Four regimens, based on the timing of a ICH occurring in a previous pregnancy, were compared (Table 5.1 and supplementary table S5.1). No differences in neonatal PLT count between the treatment groups were identified (Table 5.1). Although Bertrand et al[32] reported a significant difference in the number of neonates that needed postnatal treatment (26% in the group treated with IVIg and steroids versus 59% in the group treated with IVIg only [$p = 0.01$]), no significant differences in mean neonatal PLT count or severe thrombocytopenia were observed. The remaining 5 studies reported comparable neonatal PLT counts in women treated with IVIg only and IVIg combined with corticosteroids as well.[20,22,24,36,37]

Of the 4 studies that compared different IVIg regimens, 2 found comparable neonatal PLT counts with doses of 0.5 g/kg/week, 1 g/kg/week and 2 g/kg/week.[19,30] Van Der Lugt et al[31] reported a non-significant, lower mean PLT count in five women treated with 1 g/kg/week (63×10^9/L) compared to 17 women treated with 0.5 g/kg/week (104×10^9/L).

Treatment-related complications

Of 24 studies in which FBS was performed with or without IUPT, 2 studies reported no procedure-related complications and 12 studies reported a total of 53 complications with a frequency ranging from 3% to 39% per treated pregnancy (Table 5.3). One study reported complications in more detail elsewhere.[20,43]

Overall, the proportion of treated cases with complications due to either FBS or IUPT was 11% (54 complications in 497 treated pregnancies). The most frequently described complication was the performance of an emergency cesarean section, mainly due to fetal distress (persisting bradycardia or fetal decelerations), of which approximately half resulted in a delivery before 34 weeks' gestation. Fourteen of the 54 complications resulted in a fetal or neonatal death (26%).

Of the 26 studies that used either IVIg or corticosteroids, 11 reported the side effects of the treatment. The most commonly reported side effect of dexamethasone treatment was the occurrence of oligohydramnios. Headache and rash were the most frequently reported side effects of IVIg treatment, leading to discontinuing of the treatment in only 1 patient.[37]

Discussion

Main findings

A non-invasive management approach in pregnancies complicated by FNAIT was found to be equally effective as compared with IUPT in preventing fetal and neonatal bleeding due to thrombocytopenia. Our analysis revealed a relatively high complication rate of antenatal management by FBS and IUPT of 11%, with 1 in 3 of these leading to fetal or neonatal loss. The most common non-invasive treatment administered to pregnant women was IVIg, primarily in a weekly dose of 1 g/kg. IVIg only had a 98.7% success rate for preventing ICH (4 ICHs occurred in 315 pregnancies).[16,17,19-24,26-32,34,35] This is consistent with the 97.3% found in the Cochrane analysis reported by Rayment et al[16], which included 37 pregnancies treated with IVIg only. However, none of the studies were powered to detect a significant difference in bleeding outcomes.

Strengths and limitations

Besides the obvious lack of randomized studies with an adequate control group (placebo or no treatment), the main limitation of our review is the heterogeneity of the extracted data from the primary studies. Although neonatal outcomes are generally well reported and appear quite homogenous, the crux of the heterogeneity is the diversity of study designs. First, there is an extensive variation in treatment strategies used, especially in different combinations. For example, Sainio et al[42] described 15 women treated with 6 different strategies (IVIG only, IVIg and steroids, IVIg and IUPT, IVIg and steroids and IUPT, as well as weekly IUPT or FBS only). Secondly, the dosage of specific treatments differed considerably (eg, prednisone was prescribed as 0.5 to 1 mg/kg/day as well as 10 mg, 20 mg, 30 mg and 60 mg per day). The interval and duration of therapeutic strategies also differed considerably between studies. For example, mean duration of IVIg treatment varied from 2 weeks[18,23] to 22 weeks.[19] Additionally, in 3 of the 4 RCTs, treatment intensification was applied to increase fetal PLT counts, which could have led to underestimation of the difference between treatment arms when comparing neonatal PLT counts.[20,35,37] Lastly, there was great variability in the risk of ICH when determined by the proportion of siblings with ICH not only between studies, but also between study arms.

The 2 most commonly used endpoints for studies are ICH and neonatal PLT counts. Whereas antenatal strategies target the prevention of bleeding complications in fetuses and neonates, preferably mortality and long-term neurodevelopmental impairment should be the gold standard outcomes. Because these outcomes are rare, most studies are not powered to detect significant differences between treatment strategies and must resort to using PLT counts as surrogate outcome measurements.

In this regard, there appears to be a correlation between PLT count and risk of bleeding, but this does not appear to be a linear relationship.[41] Although the neonatal PLT count appears to be a logical and best available surrogate outcome in evaluating antenatal treatment strategies, this parameter has limitations. Comparing treatment modalities based on mean or median PLT counts may therefore show some effect, but may not be meaningful clinically.[44] In addition, very low PLT counts were often found in fetuses or neonates without any bleeding. Although it is unclear to what extent animal studies can be used for understanding pathophysiology in humans, there is increasing evidence suggesting impairment of angiogenesis and endothelial integrity as a possible cause of increased bleeding tendency, leading to the assumption that thrombocytopenia is not the sole cause of bleeding complications in FNAIT.[3,45,46]

Our systematic review was designed to evaluate the effect of antenatal treatment options on neonatal outcome including neonatal PLT count, ICH and mortality, but it did not facilitate any conclusions on the need for centralized care, the optimal timing or mode of delivery; nor whether pre-delivery FBS should be performed to determine mode of delivery, neonatal brain imaging or the need for matched PLTs.

Ultimately, to our knowledge, this is the first systematically performed review that considers all available evidence, including randomized as well as nonrandomized studies. Despite the size and heterogeneity of the studies limiting the strength of this evidence, we used predefined outcome measures of all available evidence on antenatal management in pregnancies complicated by FNAIT.

Interpretation

This review suggests that non-invasive treatment strategies are safe and effective options for the antenatal management of pregnancies complicated by FNAIT, with a lower risk of severe complications compared with FBS and/or IUPT. The gestational age at which to start antenatal IVIg treatment in FNAIT has, however, not been well defined. It is reasonable to consider the severity of the disease in previous pregnancies when making treatment decisions. An earlier start of IVIg treatment will not necessarily result in a linear increase in the amount of IgG transported to the fetus.[47] The amount of IgG that traverses the placenta depends on gestational age (with the greatest placental transport taking place in the third trimester), the IgG subclass, maternal IgG levels, and placental integrity.[47]

In cohort analyses performed by Bussel et al[19] and Van der Lugt et al[31], pregnancies were divided into risk groups based on the only established risk factor for recurrent ICH, whether the sibling had (high risk) or did not have (standard risk) an ICH and when the ICH occurred in pregnancy (high risk, very high risk, and extremely high risk).[48,49] The time of initiation of IVIg treatment was based on this stratification, and the dosage used relied on the presumption that ICH recurred

in 79% of subsequent pregnancies.[7] An analysis of 43 cases of ICH performed by Tiller et al[44] suggested that in order to reduce the risk of recurrent ICHs in subsequent pregnancies, IVIg should be initiated before 20 weeks gestation.

Whether the commonly used dose of 1 g/kg/week is the best treatment for all FNAIT pregnancies, or whether this could be reduced or increased in certain subgroups remains unclear. Data from the previously described RCT and retrospective data provided by Van Der Lugt et al[31] showed that the lower dose of 0.5 g/kg/week appeared not to be inferior to the 1 g/kg/week IVIG in standard risk (ie, a previous sibling that did not have an ICH) populations. Given the dose-related side effects and costs, a dose of 0.5 g/kg/week could be regarded suitable for these women. A limited number of patients were treated with the lower dose and therefore more data are probably required to change practice. Conversely, higher doses (ie, 2 g/kg/week) have also been used but the studies analysed were limited by adequately comparable treatment arms.[19,37]

The use of IVIg in pregnancies at risk for FNAIT is still off-label and the possible immunostimulative or immunosuppressive effect of exposing the maturing fetal immune system to IVIg has not been adequately addressed. One cohort study by Radder et al,[18] attempted to address this by examining the neurodevelopmental outcome of 50 children, at a median age of 5 years, of which 37 were exposed to IVIg during fetal life. A higher incidence of otorhinolaryngological and hearing disability in the group that did not receive IVIG was found. IgG, IgG subclass, IgA and IgM levels were comparable between groups. A trend was found between high plasma IgE levels and in utero IVIg exposure; nonetheless, no difference in eczema or allergies was observed between the 2 groups. Although, based on this small cohort study, in utero exposure to IVIg seems to have no clinically apparent adverse effects in early childhood, further immunological research with a larger group of patients is needed to fully answer this question.

The benefit of adding corticosteroids to IVIg is unclear. One study found improvement in PLT counts (defined as a PLT > 25×10^9L at second sampling, an increase by > 10×10^9L compared to the first sampling, or PLT > 40×10^9L that was not decreased by > 10×10^9L).[16,35] The remaining 8 studies comparing treatment with IVIg to IVIg with steroids did not show significant differences in the PLT count, ICH or mortality.[19,20,22,24,32,35-37] More data from randomized studies comparing IVIg to IVIg with steroids that include an adequate control group are needed to reach any firm conclusions.

To achieve a major improvement in the treatment and prevention of FNAIT, physicians need to be able to prevent index cases, a strategy that was proven to be highly successful in hemolytic disease of the fetus and newborn, caused by the red cell counterpart of FNAIT. In order to do so, population-based screening programs are needed to identify first pregnancies at risk in time to start effective antenatal prophylaxis or treatment.

In conclusion, this article represents a systematic review on the effectiveness of different antenatal treatment strategies in pregnancies complicated by FNAIT, aiming to prevent ICH and bleeding-related fetal/neonatal losses. Our summary provides the best available evidence that suggests that the optimal approach is a non-invasive approach, involving weekly administration of IVIg, with or without the addition corticosteroids. Regarding the optimal dose and start of the treatment, there are insufficient data to recommend a specific gestational age or specific dose. However, the data support the treatment of high-risk pregnancies (ie, sibling suffered from an ICH) with 1 g/kg/week IVIg, started between 12 and 20 weeks gestation. For standard risk pregnancies (ie, no sibling suffered from an ICH) the data support starting treatment between 20 and 24 weeks gestation, and to use IVIg 1 g/kg/week with or without steroids. Additional data, especially a reliable biomarker of severity in a patient known to be affected, might allow the use of a lower dose IVIg (ie, 0.5 g/kg/week) or, alternatively, a higher dose IVIg (ie, 2 g/kg/week) with or without corticosteroids, depending upon severity.

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Supplemental material

Supplementary table S5.1 – Study characteristics

First author, year, country, center	*n*	ICH sibling (%)	Identification of cases	HPA-1a *n*/N	GA first FBS
Randomized Controlled Trials					
Padriaans, 2015[27] Netherlands, MC	23	0/23	Previous FNAIT without ICH and incompatible fetus	22/23	None
Berkowitz, 2007[34] USA/ Canada, MC	74	0/74	Previous FNAIT and incompatible fetus	NR	32
Berkowitz, 2006[33] USA, MC	79	7/79 (9)	Previous FNAIT and incompatible fetus	74/79	20
Bussel, 1996[17] USA, MC	54	10/54 (19)	Previous FNAIT with fetal PLT < 100 and incompatible fetus	52/54	26
Prospective Studies					
Kanhai, 2006[14] Netherlands, SC	7	7/7 (100)	Previous FNAIT with ICH and incompatible fetus	7/7	Pre-delivery
Bertrand, 2006[36] France, SC	19	2/19 (11)	Previous FNAIT and incompatible fetus	19/19	24
Radder, 2004[15] Netherlands, SC	50	8/42 (19)	Previous FNAIT with PLT<50 or with ICH and incompatible fetus	37/42	29
Silver, 2000[20] USA, SC	10	3/10 (30)	Previous FNAIT with signs of bleeding and incompatible fetus	10/10	22-28
Lynch, 1992[19] USA, MC	18	10/18 (56)	Previous FNAIT with PLT<40	17/18	26
Retrospective Studies					
Lugt, 2015[28] Netherlands, SC	22	2/22 (9)	Previous FNAIT and incompatible fetus	19/22	None
Bertrand, 2011[29] France, MC	92	9/66 (14)	Previous FNAIT and incompatible fetus	92/92	None
Mechoulan, 2011[21] France, MC	23	7/21 (33)	Previous FNAIT with bleeding and incompatible fetus	23/23	22
Bussel, 2010[16] USA, MC	37	37/37 (100)	Previous FNAIT with ICH and incompatible fetus	35/37	20-24

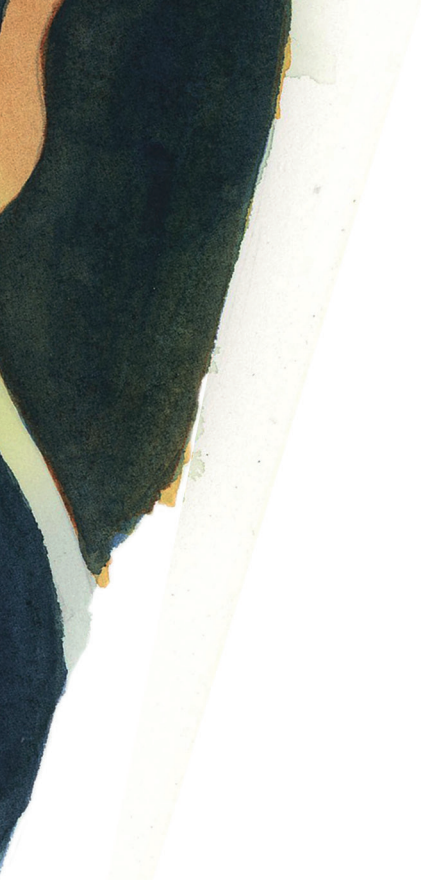
	Indication IUPT	GA start IVIg	Dose IVIg (cases treated)	Corticosteroid dose (cases treated)	Risk stratification for outcome (cases treated)
	None	28	0.5g/kg/wk (12) 1g/kg/wk (11)	None	None
	None	20	1g/kg/wk (37) 2g/kg/wk (37)	Prednisone 0.5mg/kg/day (37)	None
	None	24	1g/kg/wk (79)	Prednisone 1mg/kg/day (39)	Standard: fetal PLT>20 (39) High: fetal PLT<20 or sibling ICH (40)
	None	26	1g/kg/wk (54)	Dexamethasone 1.5mg/day (26), 5 changed to: Prednisone 60mg/day (4)	None
	PLT<100	16	1g/kg/wk (7)	None	None
	NR	23	NR	13/19, not otherwise specified	None
	PLT<100 or PLT<50 IVIG	32	1g/kg/wk (37)	Prednisone 60mg/day for 3wks (1)	None
	PLT<150	NR	1g/kg/wk (8) Fetal 1g/kg (2)	None	None
	PLT<100	26	1g/kg/wk (17) 0.5g/kg/wk (1)	Dexamethasone 5mg/day (3), 3mg/day (2), 1.5mg/day (2) Prednisone 10mg/day (2)	None
	None	16 or 28	0.5g/kg/wk (17) 1g/kg/wk (5)	None	Standard: sibling no ICH (20) High: sibling ICH (2)
	None	NR	1g/kg/wk (81) 1g/kg/2wk (1)	Prednisone 0.5mg/kg/day (65)	None
	None	24	1g/kg/wk (22) 1g/kg/2wk (1)	6/23, not otherwise specified	None
	PLT<50	13, 16 or 20	1g/kg/wk (24) 2g/kg/wk (13)	Prednisone 1mg/kg/day (5)	Based on GA of ICH sibling High: perinatal (12) Very high: 28-36 wks (17) Extremely high: <28 wks (8)

Supplementary table S5.1 – Continued

First author, year, country, center		ICH sibling	Identification of cases	HPA-1a	GA first FBS
	n	(%)		*n*/N	
Giers, 2010[18] Germany, SC	10	NR	Previous FNAIT with PLT<100 and skin bleeding or miscarriage with high anti-HPA-1a titre	9/10	20
te Pas, 2007[23] Netherlands, SC	13	5/13 (38)	Thrombocytopenic neonates due to FNAIT	12/13	NR
vd Akker, 2007[31] Netherlands, SC	99	16/99 (16)	Previous FNAIT and incompatible fetus	76/85	NR
Gheveart, 2007[38] UK, SC	55	NR	Previous FNAIT and incompatible fetus	49/55	NR
Yinon, 2006[35] Israel, SC	30	0/30	Previous FNAIT and incompatible fetus	21/30	Pre-delivery
Tilblad, 2003[24] Sweden, SC	18	1/18 (1)	Previous FNAIT	17/18	NR
Birchall, 2003[25] UK, MC	56	12/49 (24)	Previous FNAIT due to anti-HPA-1a	55/55	25
Sainio 1999[39] Finland, SC	15	2/15 (13)	Previous FNAIT or anti-HPA-1a and incompatible fetus	13/15	25
Kaplan, 1998[26] Europe, MC	37	4/27 (15)	Previous FNAIT	37/37	22-28
Kornfeld, 1996[30] Canada, SC	10	2/10 (20)	Previous FNAIT and incompatible fetus	9/10	16-24
Murphy, 1994[37] UK, SC	12	11/12 (92)	Previous FNAIT and incompatible fetus	12/12	26
Wenstrom, 1992[32] USA, SC	6	NR	Previous FNAIT and incompatible fetus	5/6	20-32
Kaplan, 1988[22] France, SC	9	1/9 (11)	Previous FNAIT or sister with FNAIT and incompatible fetus	9/9	20-22

FBS, fetal blood sampling; FNAIT, fetal/neonatal alloimmune thrombocytopenia; GA, gestational age (weeks); HPA, human platelet antigen; ICH, intracranial hemorrhage; IUFD, intrauterine fetal demise; IUPT, intrauterine platelet transfusion; IVIg, intravenous immunoglobulins; MC, multi center; NR, not reported; PLT, platelet count; SC, single center.

	Indication IUPT	GA start IVIg	Dose IVIg (cases treated)	Corticosteroid dose (cases treated)	Risk stratification for outcome (cases treated)
	Pre delivery	20	Fetal 1g/kg/wk (10)	None	None
	NR	NR	1g/kg/wk (19)	None	None
	PLT<100 or PLT<50 IVIG	18	1g/kg/wk (86)	None	Standard: sibling no ICH (83) High: sibling ICH (16)
	NR	NR	NR	16/51, not otherwise specified	None
	None	18-24	1g/kg/wk (24)	None	None
	NR	15-28	1g/kg/wk (9)	Prednisone, dose not specified (1)	None
	PLT<20 or PLT<40	26	0.8g/kg/wk (18) 1g/kg/wk (1)	Prednisone 0.5mg/kg/day (2)	None
	NR	NR	1g/kg/wk (11)	Prednisone 20-30 mg/day (2)	None
	NR	NR	1g/kg/wk (27)	0,5mg/kg/day, not further specified (10)	None
	NR	NR	1g/kg/wk (10)	None	None
	NR	20	1g/kg/wk (7) 0.4g/kg 5days (1)	Prednisone 20mg/day (5)	None
	None	NR	1g/kg/wk (6)	Dexamethasone 1,5mg/ day (3), one switch to Prednisone 60mg/day (1)	None
	NR	35	0,4g/kg/day for 5 days (1)	None	None



Chapter 6

Treatment and outcomes of fetal/neonatal alloimmune thrombocytopenia: a nationwide cohort study in newly detected cases

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Summary

Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is the most important cause of thrombocytopenia in term-born infants and can cause severe haemorrhages. Postnatal management strategies aim to reduce bleeding tendency by increasing platelet counts, but evidence for the optimal treatment is lacking. In a nationwide cohort, we reviewed postnatal management strategies and outcomes of all newly detected FNAIT, diagnosed and treated in the first week of life ($n = 102$). Postnatal strategies included no treatment ($n = 34$), platelet transfusion (PTx) with compatible ($n = 24$) or random-donor platelets ($n = 16$), or both ($n = 6$), and IVIg (with ($n = 9$) or without PTx ($n = 9$)). In all strategies, a median platelet count $> 50 \times 10^9$/L was reached within four days after birth without the occurrence of new haemorrhages. Highest and fastest increment in platelet count was observed after HPA-compatible PTx, median platelet count 151×10^9/L at five days of age. Treatment with IVIg was associated with the smallest increment in platelet counts, median platelet count 67×10^9/L at day 6. Random-donor PTx were not associated with a higher use of additional transfusions, which suggests that if HPA-compatible platelets are not directly available transfusion with random-donor platelets may be a more appropriate first line therapy in FNAIT.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is the leading cause of severe thrombocytopenia in term born new-borns.[1] FNAIT is a rare condition that occurs in approximately 1 in 1000 new-borns. Incompatibility in human platelet antigens (HPAs) between mother and fetus may lead to a maternal alloimmune response with formation of maternal IgG class alloantibodies. During pregnancy, there is an active placental IgG transport. Therefore, these alloantibodies will enter the fetal circulation, where they can destruct fetal platelets as well as damage endothelial cells, which can result in bleeding complications.[2,3] The severity of these bleedings can vary, from minor skin manifestations to major organ haemorrhages, of which the most feared complication is an intracranial haemorrhage (ICH) and subsequent neurological sequelae or even mortality.[4,5] The primary goal of treatment of patients with FNAIT is to prevent severe bleeding complications, antenatal as well as postnatal. During the antenatal period, the optimal strategy appears to be weekly maternal infusions with intravenous immunoglobulin (IVIg).[6] After birth, however, no clear consensus exists and currently applied strategies are highly inconsistent and primarily based on expert opinion and clinical experience.[7-9] The two largest cohorts published to date consist of our previous series of 22 cases with known immunisation (anticipated FNAIT) that received antenatal IVIg and an Australian registry based study including 44 cases of confirmed and newly detected FNAIT.[10,11] Thus far, these two studies did not provide comparisons of different treatment strategies for patients with FNAIT. Despite this shortage of evidence, transfusion with HPA-compatible platelets is generally considered the treatment of choice.[12] Small, heterogenic studies implicate that, compared to random-donor PTxs, transfusions with HPA-compatible platelets seem to give a larger increment of platelet count, with a longer sustained effect and therefore fewer transfusions.[11-14] However, in case of emergency, when HPA-compatible platelets are not available, treatment with random-donor platelets has been suggested to be a safe alternative.[15,16] In contrast to the great efficacy of IVIg in antenatal preventive treatment, the role of IVIg in the postnatal management of FNAIT remains unclear.[15,17,18]

Endeavouring to fill the gap in knowledge on postnatal management of FNAIT, we provide the largest cohort analysis reported thus far. While considering the guidelines and clinical features on which the choice for postnatal management is based, we analysed different postnatal management strategies. We set out to describe clinical and laboratory parameters and outcomes of patients with newly detected FNAIT according to whether or not their postnatal treatment comprised either HPA-compatible transfusions, transfusions with random-donor platelets or IVIg.

Methods

Study design and participants

We performed a nationwide cohort study on all neonates suffering from FNAIT born between 1-1-2006 and 1-1-2017 identified at the Leiden University Medical Centre (LUMC, national reference hospital) as well as at Sanquin Diagnostics, Amsterdam (national reference laboratory), the Netherlands. All cases were confirmed by the presence of specific HPA antibodies of the IgG-class in the maternal serum, directed against fetal/neonatal platelets as confirmed by crossmatch and HPA genotyping. HPA typing of the child was performed by genotyping. We excluded cases with insufficient clinical information, defined as no information on postnatal treatment strategy or course of platelet count. Solely unanticipated cases were included, defined as newly detected cases, diagnosed because of FNAIT related symptoms. To optimally assess the outcome of the postnatal treated we excluded cases that were detected and treated antenatally. We intended to describe the course in platelet counts and bleeding tendency in the first week of life. Ethical approval was provided by the Committee of Medical Ethics at the LUMC (G17·007).

Data Collection

Data were collected at both institutes (LUMC and Sanquin). Laboratory data included type of HPA-alloantibodies, HPA geno-/phenotype of the patients with FNAIT and their parents and the course of platelet count over time. Obstetric data included antenatal therapy (type, dose and duration), obstetric history, gestational age at birth, birth weight, mode of delivery, Apgar score after 5 minutes. Neonatal data comprised bleeding symptoms, postnatal treatment strategy, and the duration of admission. Bleeding symptoms were divided into minor or severe. Severe bleeding was classified as an ICH, an intraventricular haemorrhage (IVH) grade 3-4[19] or other major organ haemorrhage such as a severe pulmonary or gastrointestinal bleeding, requiring a red blood cell transfusion. All other uncomplicated haemorrhages (e.g. skin manifestations, hematomas or grade 1-2 IVH) were classified as minor bleedings. Indications for the measurement of neonatal platelet count were assessed. These indications can be one of the following factors that may be associated with thrombocytopenia as well: proven sepsis defined as a positive blood culture in a neonate with clinical signs of infection, small for gestational age (SGA, defined as a birth weight below the 10th centile), asphyxia, chromosomal abnormalities (trisomy 21) and prematurity. In case of insufficient data, referring hospitals were contacted for additional information. Thrombocytopenia was defined as a platelet count below 150×10^9/L and a severe thrombocytopenia was defined as a platelet count below 50×10^9/L. A platelet count below 20×10^9/L was classified as a very severe thrombocytopenia.

Treatment protocol

Postnatal treatment protocol comprised PTxs whenever the platelet count was below 30 $\times 10^9$/L for all cases treated before 2010 and whenever the platelet count was below 20 $\times 10^9$/L from 2010 onwards in non-bleeding infants. This threshold was 50 $\times 10^9$/L in case of manifest bleeding or need of procedure with a risk of bleeding, and for neonates with a birth weight <1500 g and gestational age < 32 weeks that were clinically ill. Standard dose was 10 – 20ml/kg or 10 – 20 $\times 10^9$/kg. First choice is a with HPA-compatible platelets. In cases of emergency one might resort to a transfusion with random-donor platelets, a product that is, in the Netherlands, composed of material from five donors. An HPA-compatible product is a single donor apheresis product. Both products are comparable in the concentration of platelets, 1.2 $\times 10^9$/mL for the HPA-compatible product and 1.1 $\times 10^9$/mL for the random-donor product. Postnatal IVIg was indicated in case of an insufficient rise in platelet count after two HPA-compatible PTxs (platelet count < 50 $\times 10^9$/L).

Statistical analysis

The incidence of minor or severe bleeding, first and lowest platelet count and clinical course of FNAIT (days to platelet count > 50 $\times 10^9$/L, platelet count > 100 $\times 10^9$/L, discharge) were compared between new-borns receiving different postnatal treatment strategies. Examination of platelet count course for different treatment groups, subgroup analysis of the group of cases caused by HPA-1a alloantibodies and of cases diagnosed the first two days of life were performed as well. These subgroup evaluations were performed in order to display the course of platelet count in a less heterogeneous group. Distributions in categorical variables between groups were compared with Chi-square test or Fisher's exact test, as applicable (Fisher's exact test for observed counts < 10). Comparisons for continuous variables were performed using student's t-test, one-way ANOVA or Mann Whitney U-test, as applicable. Tests were performed using IBM SPSS (version 23.0.0.2) and GraphPad Prism (version 7.02).

Results

During the 11-years study period, a total of 139 new-borns with newly detected FNAIT were identified, of which 31 cases had to be excluded, due to loss to follow-up, after transfer to an unknown hospital, or insufficient clinical information (Figure 6.1). Of the remaining 108 cases, six were excluded for analysis of postnatal treatment due to antenatal IVIg treatment for an ICH detected during pregnancy, which left 102 newly detected cases eligible for inclusion.

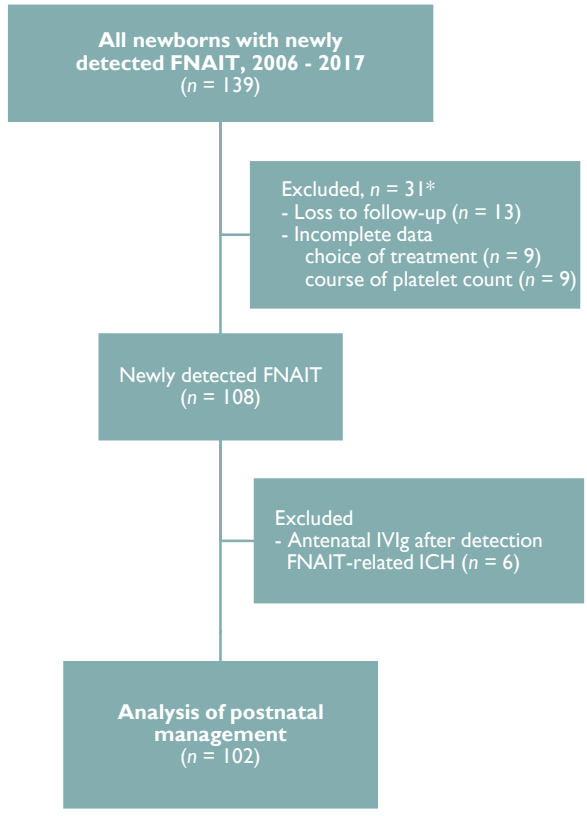

Figure 6.1 – Flowchart and description of study population

** Excluded cases did not differ from the included cases for HPA-type or, when known, the severity of the disease.*

FNAIT, fetal and neonatal alloimmune thrombocytopenia; ICH, intracranial haemorrhage; IVIg, intravenous immunoglobulin.

Demographic and clinical characteristics

Involved antibodies were directed against HPA-1a (*n* = 78), HPA-5b (*n* = 19), HPA-15a (*n* = 2), HPA-6 (*n* = 1) and in two cases alloantibodies directed against a newly detected private antigen, located on glycoprotein complex Ia/IIa (Table 6.I). Cases were detected because of bleeding symptoms (in 68/102 (67%) of all cases, or 64/98 (65%) of all living new-borns) or because of platelet count measurement as part of the work-up for prematurity (9/98, 9%), suspicion of an infection (9/98, 9%), asphyxia (2/98, 2%), small for gestational age (SGA, 6/98, 6%), trisomy 21 (1/98, 1%), chance finding due to hypoglycaemia or hyperbilirubinaemia (6/98, 6%), or because of suspicion of neonatal anaemia after large intrapartum haemorrhage of 3L (1/98, 1%). The proportion of new-borns that were SGA was higher than expected, 22% (22/98) versus 10% in general population. In six new-borns diagnostic work-up for FNAIT was performed because of the SGA and in the remaining 16 new-borns that were SGA, FNAIT was diagnosed because of bleeding symptoms.

Table 6.1 – Demographics and clinical characteristics

Antibodies directed against, *n* (%)	
HPA-1a	78 (77)
HPA-5b	19 (19)
Other*	5 (4)
Gravidity, median (range)	2 (1-5)
GA at birth†, in weeks, median (range)	38+3 (30+1-41+4)
Birthweight†, in grams, mean (SD)	2960 (733)
Small for gestational age†, *n* (%)	22 (22)
Apgar < 7 at 5 minutes†, *n* (%)	5 (5)
Infection†, n (%)	6 (6)
Minor bleeding, n (%)	65 (64)
Severe bleeding, n (%)	8 (8)
of which resulted in mortality	*4 (50)*
Platelet count†, $\times 10^9$/L, median (range)	
nadir platelet count	17 (3-91)
< 50	89 (91)
< 30	70 (82)
< 20	57 (58)
Postnatal treatment†, n (%)	64 (65)

* HPA-15a ($n = 2$); HPA-6 ($n = 1$) and private antigen ($n = 2$).

† 4 cases of intrauterine death excluded.

A total of 14 severe haemorrhages had occurred (Figure 6.1, Table 6.I). Six of these haemorrhages concerned antenatally detected ICHs that subsequently received antenatal IVIg and therefore had to be excluded from analysis of postnatal treatment. Of the eight included severe haemorrhages, four led to decease of the fetus; one infant died in utero because of a severe ICH detected at 29 weeks' gestation, a second infant died in utero due to a severe gastro-intestinal bleeding and in two cases with a severe ICH, the pregnancy was terminated at 19 and 22 weeks' gestation, respectively. In the four surviving children, the severe haemorrhages comprised three ICHs and one pulmonary bleeding, that were detected in the first days of life.

A total of 98 live new-borns with newly detected FNAIT remained. Of these, 34 new-borns received no treatment, 55 new-borns received one or more PTx and nine new-borns received only postnatal IVIg treatment (Table 6.2).

Course of platelet counts

As expected, the median of the first platelet count was higher in new-borns who were not treated compared to treated infants; 34×10^9/L versus 14×10^9/L (Table 6.2). Individual first and lowest platelet counts per treatment strategy are displayed in figure S1. Figure 6.2 depicts

the courses of platelet counts for the different treatment strategies. The corresponding values of the median platelet count, the interquartile range per treatment group and the number of cases contributing to the cohort per day are provided by supplemental table S6.I. In all children, irrespective of the postnatal treatment strategy, a median platelet counts above 50×10^9/L was reached within four days after birth (Figure 6.2A). Cases of FNAIT caused by HPA-1a or cases diagnosed in the first two days of life showed similar patterns of recovery of platelet count as compared to the whole group (Figure 6.2B and 6.2C versus figure 6.2A).

Table 6.2 – Distribution and effect of postnatal treatment

		No treatment	**Only compatible PTx**	**Only random PTx**	**Compatible after random PTx**	**PTx + IVIg**	**Only IVIg**
		n = 34	*n* = 24	*n* = 16	*n* = 6	*n* = 9	*n* = 9
First platelet count, $\times 10^9$/L median (range)		34 (14-128)	13 (4-61)	23 (3-56)	7 (3-12)	8 (4-15)	9 (6-39)
No clinical bleeding, *n* (%)		22 (65)	5 (21)	6 (38)	1 (17)	1 (11)	0
>1 PTx, *n* (%)		-	3 (13)	2 (13)	6 (100)	6 (67)	-
Platelet count above	***Days after diagnosis****						
20×10^9/L	1, *n* (%)	33 (97)	21 (88)	13 (81)	5 (83)	7 (78)	5 (56)
	2, *n* (%)	34 (100)	24 (100)	15 (94)	6 (100)	9 (100)	7 (78)
	3, *n* (%)	34 (100)	24 (100)	15 (94)	6 (100)	9 (100)	8 (89)
50×10^9/L	1, *n* (%)	14 (41)	16 (83)	10 (63)	2 (33)	2 (22)	3 (33)
	2, *n* (%)	21 (62)	20 (83)	14 (88)	5 (83)	4 (44)	3 (33)
	3, *n* (%)	23 (68)	21 (88)	14 (88)	6 (100)	5 (56)	4 (44)

PTx, platelet transfusion; IVIg, intravenous immunoglobulin.

*Day after diagnosis of thrombocytopenia, after which no drop below the given threshold of platelet count.

Transfusion with random-donor platelets, compared to HPA-compatible platelets, gave comparable amounts of new-borns that reached the transfusion threshold of 20×10^9/L or a platelet count of 50×10^9/L in the first days after diagnosis and treatment (Table 6.2). At three days after diagnosis of thrombocytopenia, treatment with PTx and IVIg or IVIg only had the lowest proportion of new-borns with a platelet count > 50×10^9/L (56% and 44%, respectively).

A total of 46 newborns were treated with (one or more) PTxs, solely. Of these, the first transfusion was with HPA-compatible platelets in 24 cases and with random-donor platelets in 22 cases (Table 6.3). After an HPA-compatible transfusion, three cases received one or more additional transfusions and eight cases received another PTx after a random transfusion (Supplemental S6.2). Of these 11 additional transfusions, four were adequately administered because of a drop

in platelet count below 30 × 10^9/L after the first transfusion. This included 2/24 initial HPA-compatible transfusions (8%) and 2/22 initial random-donor PTxs (9%). In the remaining seven cases the additional transfusion was administered almost simultaneously with the first PTx (*n* = 2, HPA-compatible directly after a random transfusion) or despite a platelet count above 30 × 10^9/L was already reached (*n* = 5).

Table 6.3 – Cases treated with one or more platelet transfusions

	First transfusion Compatible, *n* = 24		**First transfusion Random, *n* = 22**	
	Received 1 PTx *n* = 21	Received >1 PTx *n* = 3	Received 1 PTx *n* = 14	Received >1 PTx *n* = 8
First platelet count, × 10^9/L, median (range)	13 (4-61)	10 (8-58)	23 (3-56)	9 (3-24)
No clinical bleeding, *n* (%)	5 (24)	0	5 (36)	3 (38)
Day first PTx, median (range)	1 (1-4)	1	2 (1-52)	1 (1-4)
Dropped to platelet count < 30 × 10^9/L after first PTx, n (%)	-	2 (8)	-	2 (9)
Dropped to platelet count < 20 × 10^9/L after first PTx, *n* (%)	-	0	-	1 (5)

PTx, platelet transfusion.

Eight neonates with a nadir platelet count of 30 × 10^9/L or higher, above transfusion threshold in guidelines, received postnatal therapy (Figure 6.3). Four of these cases presented with minor bleeding (platelet counts 30, 35, 35 and 38 × 10^9/L, respectively). The remaining four cases were without clinical bleeding, though showed additional complications. One neonate was premature (gestational age 30^{+1}; platelet count 41 × 10^9/L), one was both premature (gestational age 32^{+6}) and SGA (platelet count 33 × 10^9/L), one other suffered from asphyxia (Apgar 2/2/5; platelet count 30 × 10^9/L) and the last was diagnosed with a transposition of the great arteries (platelet count 30 × 10^9/L). In seven of these eight cases, a random-donor PTx was administered and one case was treated with an additional HPA-compatible PTx.

Clinical presentation and choice of postnatal treatment

Neonates suffering from FNAIT with clinical bleeding were treated more frequent than asymptomatic new-borns (Figure 6.3). One case (HPA-5b) with an ICH received no treatment, because the nadir platelet count was 75 × 10^9/L. Despite very severe thrombocytopenia (platelet count < 20 × 10^9/L), seven new-borns did not receive any form of postnatal therapy (Figure 6.3). Of these cases, two had no signs of bleeding, and were detected because of a suspicion of infection one with FNAIT caused by HPA-1a and one by HPA-5b (both platelet count 17 × 10^9/L). Another two cases presented with skin bleeding; one with alloantibodies against HPA-1a and

one with anti-HPA-15a (both platelet count 14 × 10^9/L). In the last three cases, two caused by anti-HPA5b, the other by anti-HPA-1a, no signs of bleeding were found, but one child suffered from sepsis, one was diagnosed with trisomy 21 and one was small for gestational age (platelet count 15, 15 and 17 × 10^9/L, respectively). In six of these seven cases, a rapid increase of platelet count was seen, with a platelet count well above 20 × 10^9/L within one day.

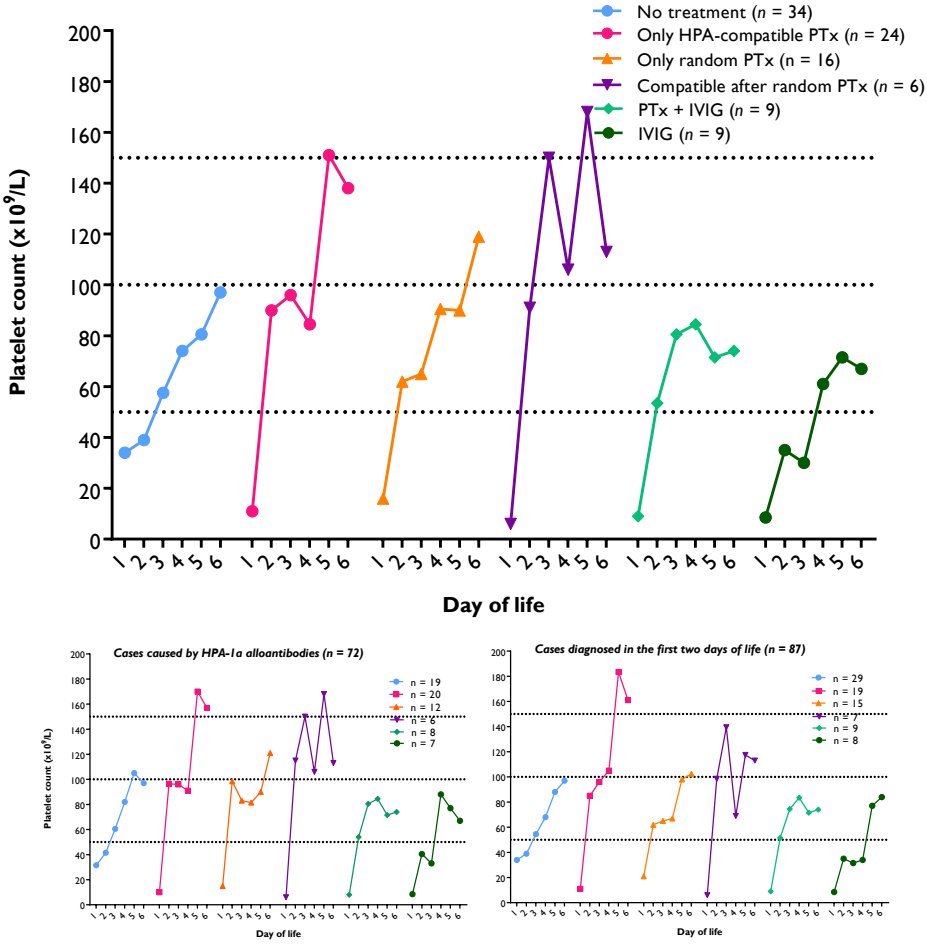

Figure 6.2 – Course of platelet count per postnatal treatment strategy

A. All live born neonates, *n* = 98. **B**. Only cases caused by HPA-1a, *n* = 71. **C**. Only cases diagnosed in the first two days of life.

In addition, nine new-borns with platelet counts between 20 × 10^9/L and 30 × 10^9/L did not receive postnatal treatment. Of these, eight were caused by HPA-1a and one by HPA-15a. Five children presented with skin bleeding, two children were premature and the other three were a chance finding of thrombocytopenia.

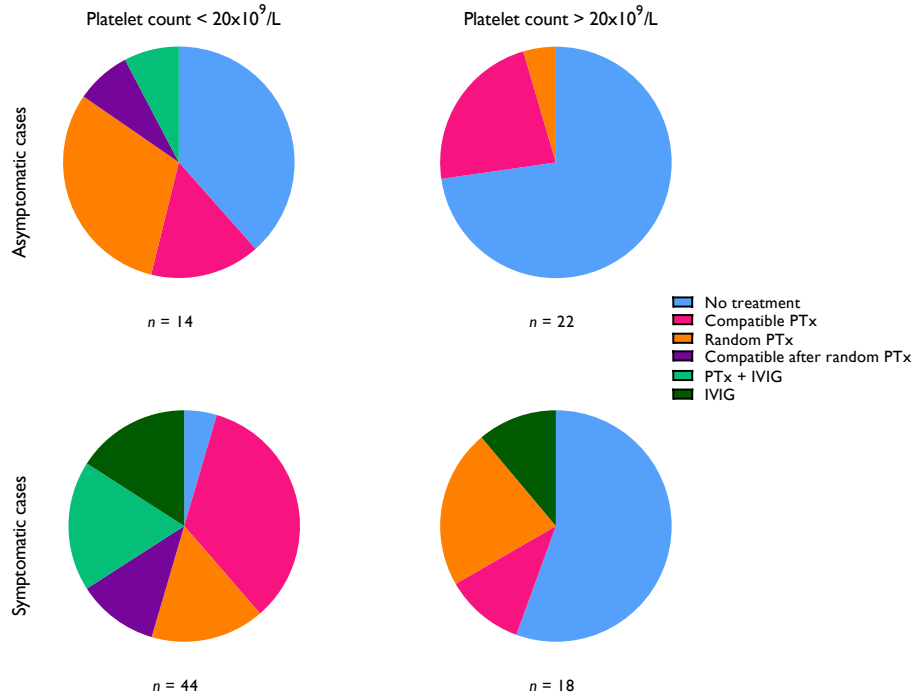

Figure 6.3 – Treatment choice based on platelet count and clinical outcome

A. Asymptomatic cases (without clinical bleeding). **B**. Symptomatic cases (with minor or severe bleeding).

Treatment with IVIg before 2010 in 7/30 (23%) and from 2010 in 2/68 (3%).

Discussion

This study represents the largest cohort of patients with FNAIT with recorded postnatal treatment strategies. Over the 11-years inclusion period, we collected data of 102 cases with newly detected FNAIT. These cases concerned children with newly detected FNAIT, in which a diagnostic FNAIT work-up was performed because of either an unexpected bleeding or severe thrombocytopenia. Despite national guidelines on the postnatal management of these children, a variety of strategies were applied in numerous combinations; transfusions with HPA-compatible platelets, random-donor platelets and IVIg administration. Overall, patients' outcomes were favourable, independent of postnatal treatment strategies. A median platelet count > 50×10^9/L was reached within four days after birth in every treatment group and none of the children showed new haemorrhages. This is in line with previous observations, that severe postnatal bleeding in FNAIT rarely occurs.[20] As expected first and nadir platelet count were important indicators of the administration of postnatal therapy.

Interpretation of these results should be done with care and caution, due to the observational nature of this study, the missing data, confounding by indication and selection bias. Next to the knowledge of existing guidelines and experience of the physician, the choice for a specific treatment is influenced by the overall clinical presentation and severity of the disease. This severity, however, is amongst others determined by our primary outcome measure, the (course of) platelet count. In order to assess and reduce the risk of bias towards less severe cases, the results were displayed for separate subpopulations as well, for example HPA-1a-mediated FNAIT or early diagnoses, presumably more severe cases, in the first two days of life. Also, it should be taken into consideration that the ultimate goal of treatment in FNAIT is to prevent (severe) bleeding, which in clinical practice is usually objectified by aiming for a platelet count above a certain level (20 or 30×10^9/L).

Consistent with international series, transfusion with HPA-compatible platelets was associated with the fastest and highest increment in platelet counts in our cohort.[11,12] Despite a possible shorter half-life and less pronounced increment, a median rise of platelet count over 100×10^9/L after six days was achieved in cases treated with only random-donor transfusions, as well. Between the group of cases treated with HPA-compatible or that treated with random-donor PTxs only, there was no significant difference in the amount of children reaching a platelet count of 20×10^9/L or 50×10^9/L within the first days of life. In our cohort, transfusion with random-donor platelets did not seem to lead to a higher need for additional transfusions, in terms of a drop in platelet count below 20×10^9/L or 30×10^9/L. Smaller observational studies have reported on the effect of random-donor platelets in FNAIT before. Kiefel and colleagues[16] reported a rise in platelet count of $> 80 \times 10^9$/L after treatment with one or two transfusions in ten newborns and Backchoul and colleagues[15], demonstrated a platelet count above 30×10^9/L for longer than 24 hours after one random-donor PTx in 5 out of 7 cases compared to 2 out of 4 cases treated with HPA-compatible platelets. It can be taken into account that our group of 16 treated cases with only random-donor PTxs included four children with anti-HPA-5b mediated FNAIT, for whom the five-donor product might have partially contained compatible platelets, since an estimated 81·3% of donors is HPA-5b negative.[21]

Postnatal treatment with IVIg seemed to be the least effective in our cohort. These results are in line with previously reported data.[22-24] Besides two case reports, Mueller-Eckhart and colleagues[22] demonstrated an increase of 30×10^9/L after 3-5 days in 10 out of 13 children treated with IVIg. Likewise, in the nine cases in our cohort treated with IVIg only, a platelet count above 50×10^9/L was reached after 5-6 days versus 2-3 days in new-borns treated with random-donor platelets or HPA-compatible platelets, thus leading to a longer admission. So, treatment with IVIg only might not a preferable postnatal treatment strategy.

We observed a large proportion of new-borns that were SGA in our cohort, 22/98 (22%). This may be partly due to a detection bias, whereas SGA can be the cause for measuring the neonatal platelet count. However, this was the case for only six of the 22 children that were SGA. Additionally, it has been suggested that interaction of anti-HPA-1a with trophoblast cells, which also express the HPA-1a epitope, affect normal placental development.[25,26]

All postnatal treatment strategies resulted in an increase in median platelet counts and no new haemorrhages occurred. Or, inversely, one could state that a safe platelet count will be reached regardless of the specific strategy applied and that it is all just natural course. This might especially be displayed by the gradual rise in platelet count in the group of new-borns that did not receive any postnatal treatment. The natural course of FNAIT, however, cannot be predicted and extrapolated from the untreated children in our cohort, due to their likely less severe disease. Also previous studies that identified the risk of FNAIT upon screening for HPA-1a negativity in pregnancy were not able to describe the natural course of disease, due to the taken interventions to minimize the risk of perinatal bleeding.[20,27]

In our cohort 16 new-borns with severe thrombocytopenia ($< 30 \times 10^9$/L, of which seven $< 20 \times 10^9$/L) did not receive a PTx or other treatment. In all cases, platelet counts increased spontaneously above the transfusion threshold within two days and no new haemorrhages were detected. In this regard, our data confirm that the transfusion threshold of 20×10^9/L was not associated with an increased risk of bleeding.

Overall, our data suggest that a transfusion with HPA-compatible platelets induces the fastest and highest increment in platelet count. In the Netherlands, HPA-1a and 5b-negative donor platelets are directly available from shelf in two blood bank distribution centers, which facilitates our quick administration of HPA-compatible transfusions. Further, our data illustrates that a transfusion with random-donor platelets may also lead to sufficient rise in platelet counts (well above transfusion triggers of 20 or 30×10^9/L), without an increase in the need for additional transfusions or the occurrence of new haemorrhages. A randomized study comparing bleeding tendency and rise of platelet counts upon transfusions with random-donor platelets or HPA-compatible platelets in homogenous treatment groups would provide more insight for optimal postnatal management of FNAIT.

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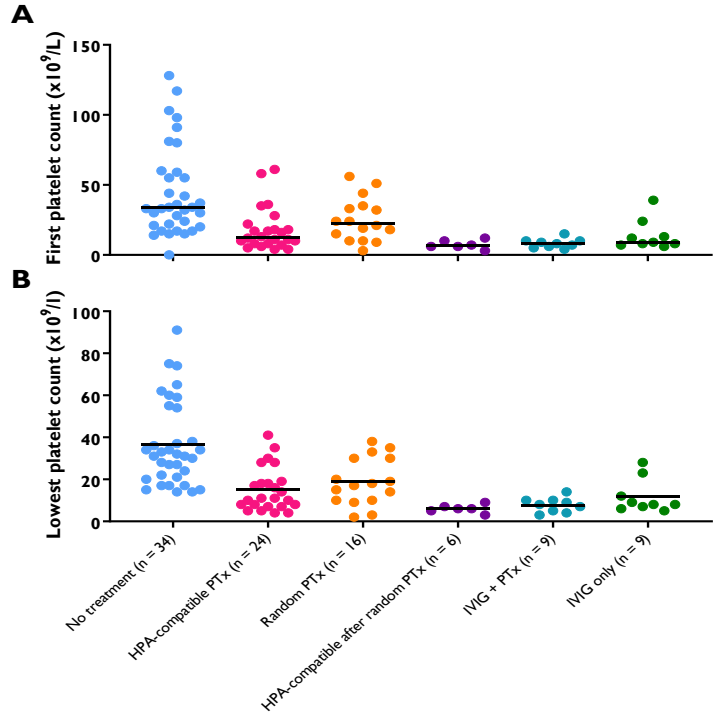
Supplemental material

Supplemental table S6.I – Course of median platelet count and interquartile range per postnatal treatment strategy

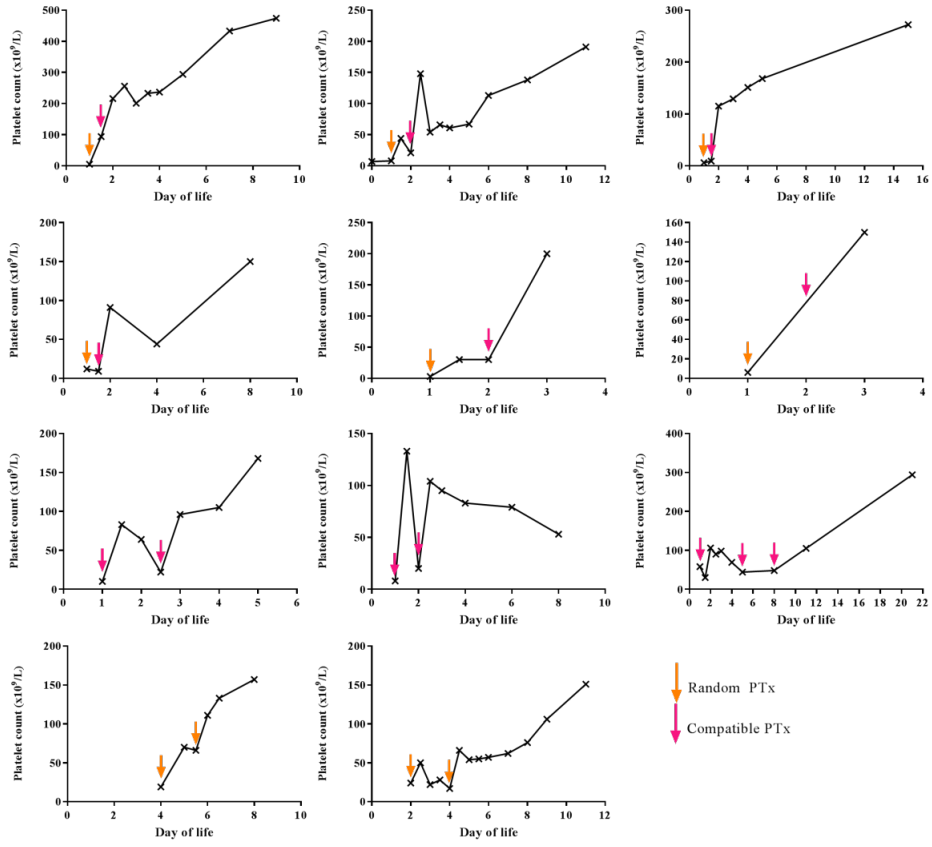
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
No treatment	34 (22 – 80)	39 (35-72)	58 (38-80)	74 (44-94)	81 (34-110)	97 (55-165)
Number of cases	*23*	*29*	*31*	*31*	*34*	*34*
Compatible PTx	11 (8-19)	90 (67-104)	96 (62-132)	85 (43-167)	151 (44-222)	138 (79-178)
Number of cases	*20*	*21*	*23*	*24*	*24*	*24*
Random PTx	16 (10-34)	62 (31-108)	65 (30-144)	91 (59-111)	90 (66-100)	119 (55-128)
Number of cases	*10*	*14*	*15*	*16*	*16*	*16*
Compatible after random PTx	6 (5-8)	91 (26-166)	150 (98-201)	106 (48-216)	168 (67-168)	113
Number of cases	*6*	*6*	*6*	*6*	*6*	*6*
PTx + IVIg	9 (6-12)	54 (43-83)	79 (35-91)	94 (66-108)	72 (53-119)	74 (63-178)
Number of cases	*9*	*9*	*9*	*9*	*9*	*9*
IVIg only	9 (7-21)	35 (15-63)	30 (13-48)	61 (27-117)	72 (52-211)	67 (37-67)
Number of cases	*8*	*8*	*9*	*9*	*9*	*9*

IVIg, intravenous immunoglobulins; PTx, platelet transfusion.



Supplemental figure S6.1 – Individual first and lowest platelet counts per postnatal treatment strategy

A. First platelet count. **B.** Lowest platelet count.



Supplemental figure S6.2 – Course of platelet count of cases receiving > 1 platelet transfusion

Chapter 7

Fast and low-cost direct ELISA for high-throughput serological HPA-1a typing

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Abstract

Background. Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal alloantibodies against fetal human platelet antigens (HPAs), mostly caused byanti-HPA-1a. Population-based screening for FNAIT is still a topic of debate. Logistically and financially, the major challenge for implementation is the typing of pregnant women to recognize the 2% HPA-1a-negative women. Therefore, there is need for a high-throughput and low-cost HPA-1a-typing assay.

Study design and methods. A sandwich ELISA was developed, using a monoclonal anti-GPIIIa as coating antibody and horseradish-peroxidase-conjugated recombinant anti-HPA-1a, as detecting antibody. The ELISA results were compared to an allelic discrimination PCR-assay. In phase I, samples from unselected consecutive pregnant women were tested with both assays. Phase II was part of a prospective screening study in pregnancy and genotyping was restricted to samples with an, arbitrary set, OD < 0.500.

Results. The ELISA was optimized to require no additional handling(swirling or spinning) of stored tubes. During phase I, 506 samples were tested. In phase II, another 62,171 consecutive samples were phenotyped, with supportive genotyping in 1,902. In total 1,585 HPA-1a negative and 823 HPA-1a positive women were genotyped. The assay reached 100% sensitivity with a cut-off OD from 0.160, corresponding with a 99.9% specificity and a false-HPA-1a negative rate of 0.03.

Conclusion. A high throughput, low-cost and reliable HPA-1a phenotyping assay was developed which can be used in population-based screening to select samples for testing of presence of anti-HPA-1a. Because plasma from tubes of 3 – 6 day-old samples can be used, this assay is applicable to settings with suboptimal conditions.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) can occur after maternal alloimmunization against foreign, paternally-derived human platelet antigens (HPAs) on fetal cells. Clinical consequences of FNAIT range from an asymptomatic thrombocytopenia to a severe internal organ hemorrhage, such as intracranial hemorrhages (ICHs) or even perinatal death. Antenatal treatment with weekly intravenous immunoglobulin (IVIg) infusions can effectively prevent these bleeding complications. However, in current practice without population-based screening, it is solely applicable in pregnancies with known alloimmunization, recognized in previous pregnancies.[1] Generally, FNAIT is considered the platelet counterpart for hemolytic disease of the fetus and newborn (HDFN) caused by red blood cell (RBC) alloimmunization. Whereas anti-RhD is the predominant cause of (severe) HDFN, alloimmunization against HPA-1a is the major cause of bleeding complications in FNAIT, accountable for approximately 80% of cases.[2,3] In the Caucasian pregnant population, 2.1% is HPA-1a negative, of which 10% will produce alloantibodies against HPA-1a (1 in 500 pregnancies), leading to a severe thrombocytopenia in approximately 1 in 1500 pregnancies.[4] The introduction of a screening and prevention program for HDFN caused by anti-RhD, has further decreased morbidity and fatal complications of anti-RhD-mediated HDFN hardly ever occur anymore in the Western world.[5,6] Its pathophysiologic similarities to HDFN, together with the availability of an effective and non-invasive preventive treatment, have stirred up the debate on implementing population-based screening for FNAIT caused by HPA-1a as well.[7] An important advantage is that only a small proportion of the pregnant population needs to be screened for alloimmunization, that is the approximately 2% HPA-1a negative women. In terms of cost-effectiveness and practical implications, the major component will be HPA-1 typing. Because currently used assays, applied in diagnostic settings, are complex and time consuming, there is need for a reliable, quick, simple and low-cost assay for high-throughput HPA-1 typing. We describe an enzyme-linked immune sorbent assay (ELISA) for HPA-1a phenotyping, modified from the assay described by Garner and colleagues.[8] The assay was extensively validated with supportive genotyping, allelic discrimination polymerase chain reaction (PCR), in exclusively pregnant women and in a high number of HPA-1a negative samples. Furthermore, the material used was plasma from three to six days-old stored tubes without handling (swirling or spinning), instead of whole blood, isolated platelets or platelet rich plasma (PRP) in order to eliminate a time-consuming and labor-intensive step and make the assay applicable to a setting for multiple testing from one tube of blood with material of suboptimal quality.

Materials and Methods

Patient samples

As part of the Dutch antenatal Screening Program for Infectious diseases and Erythrocyte immunization (PSIE), repeated red cell antibody screening and fetal *RHD* typing is offered free-of-charge at 27 weeks' gestation to all RhD and/or Rhc negative pregnant women. For this screening program, nine milliliter ethylenediamine tetra-acetic acid (EDTA) anticoagulated blood is drawn at certified, local laboratories all over the Netherlands ($n = \pm 90$) and transported to our laboratory by regular surface mail or Sanquin private courier service. Upon receipt at Sanquin, blood tubes receive a numerical code with barcode, that enables automated pipetting, testing and connecting the results to clinical laboratory information system (CLIS). After regular testing for prenatal screening program, 1 – 4 days after blood drawing (depending on the day of the week and time of drawing), the tubes were stored, upright in racks without cap, for two more days at 4°C, awaiting authorization of fetal *RHD* and red cell antibody screening. After this, the samples could be used anonymously for HPA-1a testing. To ensure the applicability for high-throughput testing in a potential screening setting, the tubes were loaded into the Hamilton STARlet workstation (Hamilton Robotics, Bonaduz, Switzerland) and 20 µL of the uppermost plasma of these 3 – 6 days-old, stored tubes was automatically pipetted into a plate, without first swirling or spinning them. Platelet counts of this plasma were determined using 20 µL of the same uppermost material from the tubes that would be used in the ELISA with a Coulter T-890 counter (Coulter Electronics Ltd, Luton, United Kingdom). All subjects gave passive consent for the study. No directly linked personal or medical information was used. The study was performed in accordance with the World Medical Association Declaration of Helsinki. Confidentiality was appropriately protected according to the Dutch Medical Treatment Agreement Act, expanded in the Codes for Good Behavior and Good Use.

The collection of samples was split into two phases. During the first phase, we included 506 consecutive samples of RhD and Rhc negative pregnant women. The second phase took place as part of a current prospective observational screening study (HIP-study, HPA-screening In Pregnancy), that is aimed at gathering missing knowledge on incidence, natural history and risk assessment of FNAIT. During this second phase, 62,171 consecutive samples of the same cohort of RhD and Rhc negative pregnant women were tested.

Antibodies

A recombinant, human monoclonal antibody against HPA-1a (B2G1) was used (kindly provided by Dr. W. Ouwehand and Dr. C. Ghevaert, University of Cambridge, NHS, Blood and Transplant, Cambridge, UK)[8,9] in a concentration between 1.1 and 1.8 µg/mL in PBS with 0.2% (v/v) BSA. B2G1 was conjugated with horseradish peroxidase (HRP), using Lightning-Link HRP Conjugation

Kit (Innova Biosciences, Cambridge, United Kingdom), according manufacturers' instructions. Murine monoclonal antibody CLBthromb/1 (C17, IgG1) was used as antibody directed against glycoprotein IIIa or CD61 (Sanquin Reagents).[10]

Enzyme-linked immunosorbent assay (ELISA) based HPA-1a typing

Flat-bottom Maxisorp 96-well plates (Nunc) were coated with C17 at a concentration of 3 µg/mL in 0.1M sodium carbonate buffer (Merck) and then incubated for a minimum of 12 hours to a maximum of five days at 4°C. Before use, the plates were washed with wash buffer (phosphate-buffered saline (PBS), 0.05% Tween-20) and blocked for one hour with NaCl 0.2% bovine serum albumin (BSA), to prevent non-specific binding. After proving the ability of using supernatant, from tubes stored in racks at 4°C for 3 – 6 days, the assay was optimized for optimal concentrations for type and dilution of monoclonal antibody used for coating of the plate (C17, 3 µg/mL), amount of plasma (20 µL), HRP-incubation time (45 minutes), washing method (3 washing steps with phosphate-buffered saline (PBS), 0.05% Tween-20), substrate incubation time (30 minutes) and optimally HRP-conjugated (1:2 ratio) B2G1 (supplemental table S7.1). A 6-step protocol for the HPA-1a ELISA was applied: 20 µL of plasma sample was automatically pipetted using a Hamilton STARlet workstation (Hamilton Robotics, Bonaduz, Switzerland) into each well. Subsequently, 20 µL HRP-conjugated B2G1 was added to each well and plates were centrifuged for 5 minutes at 550 g and incubated for a total of 45 minutes, including spinning time, in the dark at room temperature. Thereafter, plates were washed three times with the washing buffer and 50 µL of HRP-substrate solution (TMB/DMSO 10 mg/mL) was added and incubated for 15 minutes in the dark at room temperature. The reaction was stopped with 50 µL of sulfuric acid (H_2SO_4) and quantified using an ELISA reader (Biochrom Anthos, Cambridge, United Kingdom). An overview of this assay is depicted in supplemental figure S7.1. Single samples of HPA-1bb and HPA-1aa platelets isolated from healthy donors, stored at 4°C, were diluted to a concentration of 7.0×10^7/mL and used as positive and negative control in each plate.

HPA-1 Genotyping using allelic discrimination polymerase chain reaction (PCR)

A Xiril robotic workstation (Xiril) was used for automatic pipetting of the samples into the plate. Automated DNA extraction was performed with M-PVA magnetic beads (PerkinElmer Chemagen) from 200 µL EDTA anticoagulated blood. After normalizing DNA level to 5 ng/µL, an allelic discrimination PCR assay using FAM-labeled HPA-1a and VIC-labeled HPA-1b MGB-probe. Primer and probe sequences were as follow: forward primer, 5' – CTG ATT GCT GGA CTT CTC TTT GG – 3'; reverse primer, 5' – AGC AGA TTC TCC TTC AGG TCA CA – 3'; HPA-1a MGB probe, 5' – CTG CCT CTG GGC TC – 3' (5' FAM labelled); HPA-1b probe, 5' – CTG CCT CCG GGC TC – 3' (5' VIC labelled). We used Xiril robotic workstation for pipetting of 25 µL, using 12.5 µL Sensifast High ROX mastermix (Bioline), 5 µL extracted DNA, and primers and probes at final concentrations of 10 and 5 pmol/µL, respectively Cycling conditions were 30 seconds at 63°C and 5 seconds at

95°C, followed by 45 cycles of denaturation for 10 seconds at 95°C and annealing and elongation for 20 seconds at 63°C, completed by 30 seconds of 63°C using StepOne-Plus Real-Time PCR System (Applied Biosystems). Interpretation of results was performed with StepOne™ software (version 2.3).

Statistical analyses

Validation and standardization were performed by testing the intra-assay and the inter-assay variation and calculation of coefficients of variation (CV). Inter-assay was assessed twice, by ten-fold repeated measurement of 30 samples in the same assay by the same technician under the same conditions. Intra-assay variation was calculated in three ways. First, by measuring 30 samples on the same day by three different technicians (operator-to-operator variation). Second, by measuring the same 30 samples on three different days by the same technician (day-to-day-variation), this was performed twice. Third, by measuring the same 30 samples by both the pipetting robot and technicians in different rooms. Mean CVs were calculated based on variation in ELISA OD values. Diagnostic accuracy was evaluated using contingency tables, calculating sensitivity, specificity, predictive values, and cases falsely detected as HPA-1a positive or negative. The diagnostic value for different cut-off values was assessed by plotting sensitivity vs. specificity, and analyze the area under these curves (AUC). Statistical analyses were performed using SPSS (version 23.1) and GraphPad Prism (version 8.0) was used for producing plots and graphs.

Results

ELISA optimization

The CV of the intra-assay variation was between 2.9% and 4.7%. The mean CV in the inter-assay variation test, 30 samples tested by three different technicians on three (sequential) days, was 5.2% (range: 1.4% – 10.2%). The HPA-1a positive control platelets had a mean OD value of 1.720 (range 1.577 – 1.849) and were clearly discriminated from HPA-1a negative control platelets, which had OD values ranging from 0.021 to 0.042 with a mean OD of 0.031.

Effect of platelet counts

Platelet counts of 162 samples were determined. The mean platelet count in the supernatant, that was pipetted from the stored tubes, was 7.1×10^7/mL (range 1.5 – 34.8 10^7/mL; IQR 43 – 140 x 10^7/mL). Of 70 samples (HPA-1a positive, $n = 59$; HPA-1a negative, $n = 11$) the platelet counts are plotted against the corresponding ELISA OD (Figure 7.1). An ELISA OD below 1.000 in samples with HPA-1a positive platelets only occurred in samples with a platelet count below 8.0×10^7/mL. Regardless of platelet count (range 5.5×10^7/mL – 36.2×10^7/mL), all samples with HPA-1a negative platelets, all genotyped as HPA-1bb, tested in this series had an ELISA OD below 0.110 (range 0.021 – 0.104).

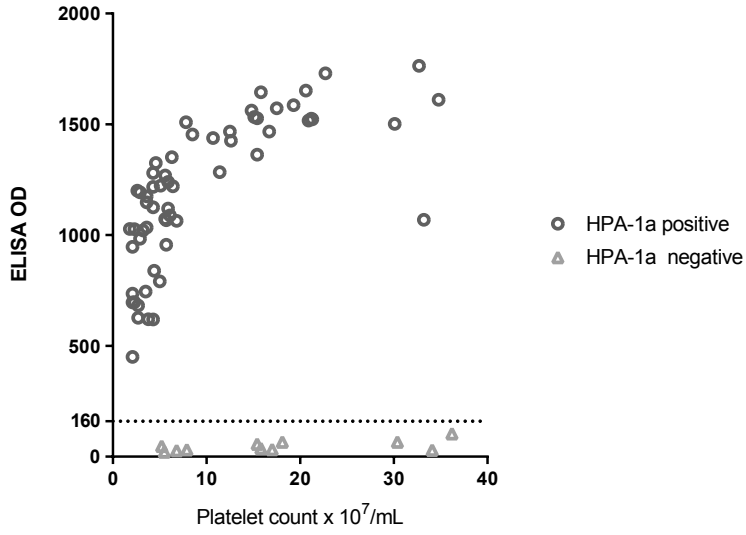

Figure 7.1 – Effect of platelet count on ELISA OD

ELISA, enzyme-linked immunosorbent assay; HPA, human platelet antigen; OD, optic density.

Samples and study population

Phase I. 506 consecutive samples of pregnant women were phenotyped by HPA-1a ELISA and genotyped by an HPA-1 allelic discrimination assay (Figure 7.2A). Of these, 13 women were genotyped as HPA-1bb (2.6%) and all had an OD < 0.100 (range: 0.028 – 0.058). All but two HPA-1a positive samples had an OD > 0.300. These two were both genotyped as HPA-1ab and had OD values of 0.072 and 0.149.

Phase II. During the second phase, 62,171 consecutive samples of pregnant women were tested (Figure 7.2B). To minimize the risk of missing HPA-1a negative pregnant women, the cut-off value for supportive genotyping of the HPA-1a ELISA was first set at 0.500 and later lowered to 0.300. A total of 1,902 samples underwent genotyping, 77 with OD < 0.500 but above 0.300 and 1,825 with OD < 0.300. The median OD of the 1,572 HPA-1bb samples was 0.035 (range 0.014 – 0.160). A total of 156 women ($n = 32 < 0.500$ and $n = 124 < 0.300$, respectively) were HPA-1ab and 174 ($n = 45$ and $n = 129$, respectively) were HPA-1aa. The mean OD positive controls was 1.591 (SD 0.084) and the mean OD of negative controls was 0.062 (SD 0.005).

Diagnostic test evaluation

Predictive values as well as diagnostic accuracy of the HPA-1a ELISA can only be optimally deduced from a tested population that reflects the prevalence of the tested condition in the total population, which is phase I of this study. With cut-off values of 0.075 – 0.200, sensitivity

remains 100% and diagnostic accuracy is 99.6 – 99.8% (Table 7.1, Figure 7.3A). HPA-1a positive predictive value is 100%, with HPA-1a negative predictive values varying from 87 – 93%, depending on different cut-offs.

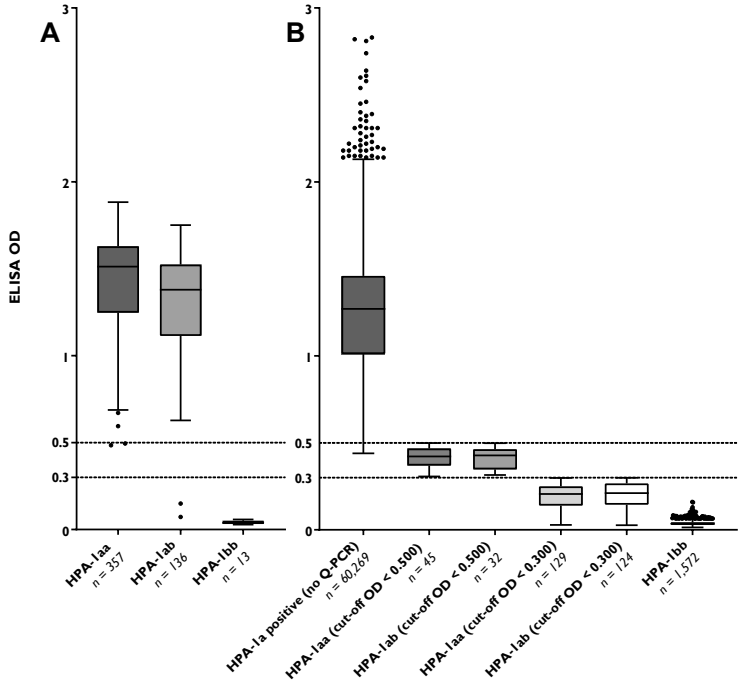

Figure 7.2 – Distribution of ELISA OD for different genotypes

A. Phase I, 506 consecutive samples. OD < 0.300 in 15 (HPA-1bb, *n* = 13; HPA-1ab *n* = 2; HPA-1aa, *n* = 0), OD > 0.300 in 491 (HPA-1bb, *n* = 0; HPA-1ab, n = 135; HPA-1aa, n = 357). **B.** Phase II, 62,171 consecutive samples. PCR in 1,902, because of OD from 0.300 - 0.500 (*n* = 77) or OD < 0.300 (*n* = 1,825).

Boxes represent 25th and 75th percentiles; lines in boxes represent median values; horizontal lines outside boxes represent 1.5 times IQR; dots represent outliers.ELISA, enzyme-linked immunosorbent assay; HPA, human platelet antigen; IQR, inter-quartile range; OD, optic density; PCR, polymerase chain reaction.

Extrapolation the diagnostic test evaluation towards phase II would imply considering that samples that did not undergo PCR (OD values: median 1.231, range 0.440 – 2.830) would have been genotyped as HPA-1a positive. Based on all genotyping data, phase I and II combined, threshold values above 0.160 would mean identifying all HPA-1a negative cases and resembles a sensitivity of 100%. This would correspond with a specificity of 99.9% (Figure 7.3B).

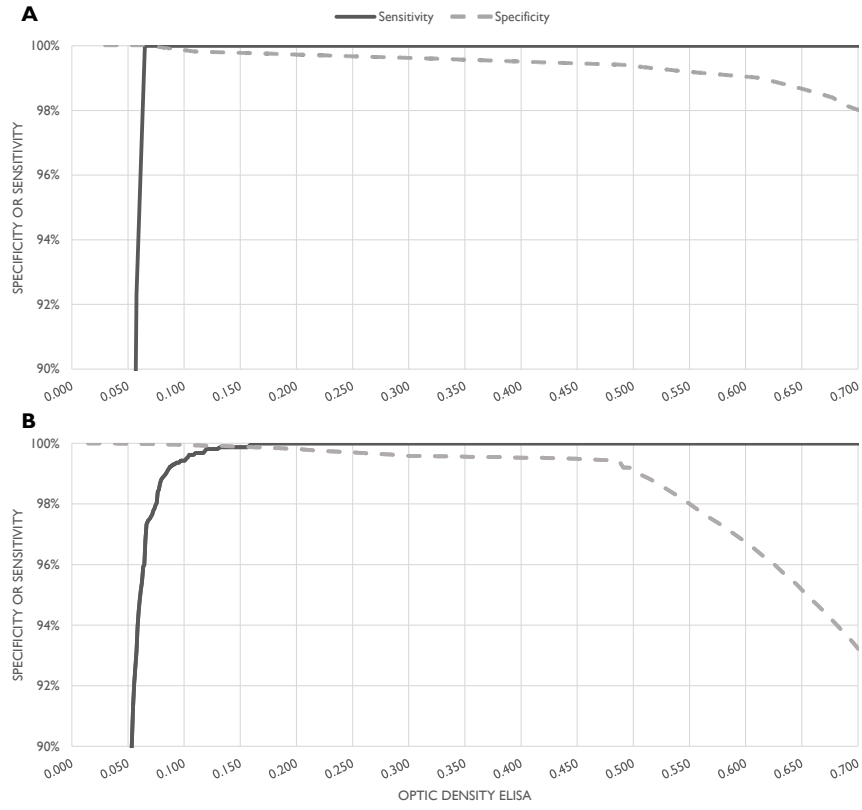

Figure 7.3 – Diagnostic evaluation of HPA-1a ELISA

A. Phase I, 506 consecutive samples. **B.** Phase I+II, 62,677 samples. During phase II all samples with an OD > 0.500 were marked as HPA-1a positive without genotyping.

ELISA, enzyme-linked immunosorbent assay; HPA, human platelet antigen; OD, optic density.

Costs

A potential screening setting for all pregnant women in the Netherlands would mean a total of 750 samples a day. By using in-house available MoAbs, equipment, reagents and disposables, costs are kept at a minimum. The total material costs per sample in a potential future screening setting would be €0,25 per sample, of these the biggest contributor is the amortization cost of a pipetting robot (€0.22 per sample). Another great part of costs for serological HPA-1a typing are labor costs. The hands-on time for this quick ELISA, testing three 96-well plates, is three hours for a single junior technician. Lastly, costs for follow-up testing, for example genotyping and antibody screening, need to be taken into account as well. These would mainly depend on the setting and design of the program, as well as on cut-off values used after validation with specific material sent in for testing in such programs.

Table 7.1 – Diagnostic evaluation of HPA-1a ELISA – Phase I (*n* = 506)

Cut-off OD value	Accuracy	Sensitivity	Specificity	AUC	HPA-1a negative predictive value	HPA-1a positive predictive value
<0.075	99.8%	100.0%	99.8%	99.9%	0.93	1.0
<0.100	99.8%	100.0%	99.8%	99.9%	0.93	1.0
<0.150	99.6%	100.0%	99.6%	99.8%	0.87	1.0
<0.200	99.6%	100.0%	99.6%	99.8%	0.87	1.0

AUC, area under the curve; ELISA, enzyme-linked immunosorbent assay; HPA, human platelet antigen; OD, optic density.

Discussion

Fetal and neonatal alloimmune thrombocytopenia is one of the most important causes of severe thrombocytopenia in term born neonates and a potentially life-threatening condition. Prevention of this disease by timely detection in potential antenatal routine HPA-screening has been debated for over decades. One of the unsolved complicating quests is the cost-effectiveness of such a screening program. Whereas just 2% of pregnant women is HPA-1a negative and will need follow-up testing, the major proportion of costs will be determined by HPA-1a typing of the complete pregnant population. Therefore, a reliable, quick, simple and low-cost ELISA assay, suitable for high-throughput serological HPA-1a typing was designed.

A couple of prospective HPA-1a screening studies that use an ELISA for HPA-1a typing have been performed thus far.[11-14] However, this study is the first to describe extensive validation with samples solely from pregnant women, to report in detail on (low) costs and to use moderate quality material, making it highly applicable for potential screening settings. Despite using these moderate quality samples the assay reaches a high diagnostic accuracy.

After phase I, setting the threshold to discriminate between HPA-1a positive and negative samples would lead to a cut-off value of 0.061 (mean + 2SD, 0.041 + 2*0.010). With cut-off values above 0.075 a 100% sensitivity is reached, which corresponds with a zero false-negative rate, so that no HPA-1a negative samples would be missed. In phase II the number of HPA-1a negative samples tested increased drastically, up to 1,585 in total. The cut-off value for discrimination between positive and negative would be 0.065 (mean + 2SD, 0.037 + 2*0.014). However, to achieve a 100% sensitivity, threshold for supportive genotyping or phenotyping should be increased to 0.160. Nonetheless, eliminating the possibility of missing HPA-1a negative samples, comes at the cost of falsely identifying some HPA-1a positive women as possible HPA-1a negative and therefore theoretically avoidable additional testing.

In the current prospective study HPA-1a negative cases are genotyped with allelic discrimination, which is a rather expensive step. One might argue that in a potential screening setting it is more cost-efficient to skip this step and either perform direct antibody screening in all women with ELISA results suggesting HPA-1a negativity or test maternal platelets by flow cytometry for the presence of the HPA-1a epitope with a monoclonal antibody before performing testing for the presence of anti-HPA-1a antibodies. For the tested cohort, a threshold of 0.160 resulted in 1,629 samples identified for additional testing, of which 57 would not have required this testing (false-HPA-1a negative rate 0.03). Also, in potential future screening setting, after identifying alloimmunized pregnancies, additional testing might to be performed to identify pregnancies at high risk of bleeding complications. For this, HLA-DRB3*0101 type, anti-HPA-1a antibody titer, Fc-glycosylation pattern or endothelial binding properties might be assessed.[15-20]

The strength of using moderate quality material in our assay is a limiting factor for not achieving an even higher accuracy and specificity as well. Also, the currently used volume is relatively small, 20 µL plasma. The amount of left-over material was carefully managed, because it was not only used in this study for the HPA-1a ELISA, but also for follow-up testing in the prospective HIP-study. Obviously, with increasing volume and quality of material in a potential screening setting, the amount of platelets added to the assay can be doubled or tripled. This will lead to higher OD values of the HPA-1a positive cases, but not the HPA-1a negative samples. Therefore, the line between HPA-1a positivity and negativity will be more discriminative, leading to even less falsely identified HPA-1a negative cases that need further follow-up testing and would undergo the above-mentioned, unnecessary antibody screening or repeated HPA-1a ELISA. Additionally, plasma platelet counts can be lowered by unnoticed thrombocytopenia in pregnant women as well. Although we did not specifically test our assay for this, this would not complicate the performance of the assay. No HPA-1a negative women will be missed, because these samples will still have low ELISA OD values. There is only a chance that samples of HPA-1a positive women have an ELISA OD below threshold and undergo avoidable follow-up testing.

By using a specific recombinant HPA-1a antibody that is directly labeled, there will be no cross-reactivity with specific or non-specific antibodies in maternal plasma.

This highly optimized assay minimizes the costs of serological HPA-1a typing to €0.25 per sample, excluding labor costs. Previously reported costs by Turner and colleagues were £3.01 per sample for ELISA and £16.19 for HPA-1 genotyping, without any further details or specification.[12] Reported costs from the largest prospective screening study, based on typing over 100,000 women, were €1.72 per sample when using flow-cytometry and €21.28 per sample when using HPA-1 genotyping by PCR, again without any further details or specification.[21]

Besides costs, prospective studies have reported on the use of serological HPA-1a typing in a screening setting as well. The first study screened a cohort 24,417 pregnant women, and besides notice of 20 cases that turned out to be HPA-1a positive after PIFT, no information on diagnostic accuracy or costs of the assay were provided.[11] A whole-blood ELISA kit for simultaneous HPA-1a typing and anti-HPA-1a detection, was described in two prospective studies in Ireland (4,090 women) and Scotland (26,206 women).[12,13] Supportive genotyping was performed in only 67 pregnant women, of which 54 turned out to be HPA-1bb. Again, no further information on diagnostic accuracy or performance of the ELISA was provided.[13] The largest prospective study screened 100,448 pregnant women in Norway.[14] Screening was performed by ELISA, flow-cytometry and PCR, with unknown distribution. Their ELISA assay used was previously described by Garner and colleagues, and used whole-blood samples.[8] The performance analysis of the assay was done in samples of 1,947 (random) donors and no pregnant women. Genotyping and PIFT were performed in samples that were considered HPA-1a negative and an equivalent of randomly selected HPA-1a positives. Overall both ELISA and supportive testing (genotyping and PIFT) were performed in 91 samples, of which 44 were HPA-1a negative. The flow-cytometry assay was previously described by Killie and colleagues and used a commercially available FITC-labeled mouse IgG1 anti-CD61 (clone SZ21) specific for HPA-1a and is a very quick assay with only a 10-minute incubation without washing.[22] They tested 45,960 samples and genotyped the 1,121 samples that were typed HPA-1a negative after flow-cytometry. Of these, 1,112 were typed HPA-1bb and 9 HPA-1ab. These samples tested were either pregnant women or blood donors, the exact distribution was not described.

Compared to the previously described assays in screening settings this study adds an extensive validation in a high number of pregnant women instead of healthy blood donors. Furthermore, since we have genotyped the 330 samples with the lowest OD values above the threshold, of which none were found to be HPA-1bb, we were able to demonstrate the excellent sensitivity of the assay. Further, the use of low-moderate quality material that was not spinned or swirled, like in the other studies that used blood drawn only for the purpose of HPA-1a typing, is important and reduces labor costs. When potentially adding a HPA-1a screening to an already existing serological prenatal screening it is highly preferable that the sample can be used from the same tube or same blood drawing moment. Also, without having to twist or spin for example 750 tubes a day (in case of a Dutch national screening program) the assay becomes more fit for high-throughput usage. Lastly, because the validation and confirmation with genotyping was also part of our national screening study, we were able to include a large amount of HPA-1a negative pregnant women.

Acknowledgements

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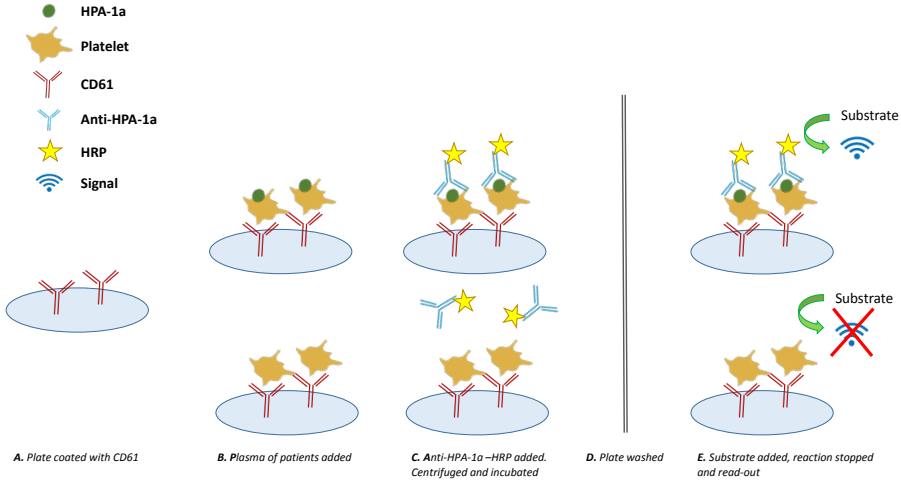
Supplemental material

Supplemental table S7.1 – Optimization of ELISA

Variable	Conditions tested
Coating plate	Y2. RFGP56, **C17** (1ug/mL and **3ug/mL**)
Amount of plasma	10ul, **20ul,** 30ul, 40ul
HRP-incubation time	30 minutes, **45 minutes** and 60 minutes
Washing method	**3** or 5 washing steps
Substrate incubation	10 minutes, 15 minutes and **30 minutes**
Concentration B2G1	1.8mg/ml: 1:500, **1:1000**, 1:5000
HRP conjugation B2G1	1:1, **1:2** and 1:4

ELISA, enzyme-linked immunosorbent assay; HRP, horse-radish peroxidase.



Supplemental figure S7.1 – HPA-1a ELISA

Chapter 8

Women's attitude towards routine HPA-screening in pregnancy

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Abstract

Introduction. Fetal and neonatal alloimmune thrombocytopenia is a potentially life-threatening disease with excellent preventative treatment available for subsequent pregnancies. To prevent index cases, the effectiveness of a population-based screening program has been suggested repeatedly. Therefore, we aimed to evaluate women's attitude towards possible future human platelet antigen-screening in pregnancy.

Material and Methods. We performed a cross-sectional questionnaire study among healthy pregnant women receiving prenatal care in one of seven participating midwifery practices. Attitude was assessed using a questionnaire based on the validated Multidimensional Measurement of Informed Choice model, containing questions assessing knowledge, attitude and intention to participate.

Results. A total of 143 of the 220 women (65%) completed and returned the questionnaire. A positive attitude towards human platelet antigen-screening was expressed by 91% of participants, of which 94% was based on sufficient knowledge. Attitude was more likely to be negatively influenced by the opinion that screening can be frightening. Informed choices were made in 87% and occurred significantly less in women from non-European origin, 89% in European women vs. 60% in non-European women ($p = 0.03$).

Conclusions. Pregnant women in the Netherlands expressed a positive attitude towards human platelet antigen-screening in pregnancy. We therefore expect a high rate of informed uptake when human platelet antigen-screening is implemented. In future counselling on human platelet antigen-screening, ethnicity and possible anxiety associated with screening test need to be specifically addressed.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is the most important cause of severe thrombocytopenia in term infants, affecting approximately 1 in 700 newborns.[1] As the platelet counterpart of hemolytic disease of fetus and newborn, it is triggered by maternal alloantibodies against incompatible, paternally derived, fetal human platelet antigens (HPA). FNAIT may induce bleeding complications, such as bruising or in severe cases an intracranial hemorrhage as well as massive internal organ bleedings.[2] Unlike for red cell alloimmunization in pregnancy, there is no government-organized population based screening program for FNAIT in the Netherlands. Therefore, FNAIT is usually diagnosed only after the occurrence of fetal or neonatal bleeding complications, or the chance finding of asymptomatic neonatal thrombocytopenia. Fortunately, for subsequent pregnancies there is a highly effective antenatal therapy available, using weekly infusions of immunoglobulins, preventing almost all bleeding complications in following incompatible gestations.[3]

The potential effectiveness of a screening program for FNAIT has been suggested repeatedly.[4,5] Ideally, in order to ascertain its usefulness and validity, population-based screening fulfills the Wilson and Jungner screening criteria.[6] Irreversible brain damage and perinatal death resulting from intracranial bleeding represent a serious health problem, a reliable and acceptable diagnostic test is available and there appears to be an adequate preventive treatment in latent stage. Whereas HPA-1a is the predominant cause of severe FNAIT, screening in studies is focused on identifying HPA-1a alloimmunization, occurring in approximately 1 in 600 pregnancies.[5] Several modeling studies, suggested cost-effectiveness of routine HPA-1a screening.[7,8] The Wilson and Jungner criteria were recently revised, with addition of recognition that a screening program should ensure informed choice and should respond to a recognized need.[9] In general, there is increasing interest in patients' attitudes towards health care, in particular, population-based screening programs.[10-12]

Although many have advocated HPA-screening in pregnancy to prevent FNAIT, no studies evaluating women's attitude towards such a screening program have been performed. Therefore, we performed a cross-sectional questionnaire study among healthy women in the first half of their pregnancy, assessing their attitude towards implementing a nationwide HPA-screening program in pregnancy and their ability to make an informed decision on participating.

Material and methods

From April 2016 through June 2016, women in the first half of their pregnancy, attending one of the seven participating midwifery practices in the Leiden region, were invited to participate. A questionnaire together with an information flyer was provided by their obstetric caregiver (Supplemental material). Women with a limited knowledge of the Dutch language were excluded from participation. After completion, the questionnaires were returned to the Leiden University Medical Center by using provided, pre-paid envelopes. The questionnaire as well as the information flyer were pilot-tested before the study onset.

To test women's attitude towards HPA-screening in pregnancy, the validated Multidimensional Measurement of Informed Choice (MMIC) model developed by Marteau and colleagues was used, which contains three dichotomized elements: knowledge, attitude and uptake.[13-15] Accordingly, an informed choice is based on sufficient knowledge and is value consistent. Value consistency is defined as a behavior that corresponds with the decision maker's attitude, i.e. negative attitude and declining the test or a positive attitude and uptake of the test. Conversely, uninformed choices are either value inconsistent and/or based on insufficient knowledge.

Attitude

The attitude towards HPA-screening was assessed by a 5-point Likert scale adapted from the MMIC model, which consisted of four items (Supplemental table S8.1). The scores ranged from 4 to 20, with a median of 12 to classify women's attitude. Scores higher than 12 indicated a positive attitude and scores equal to or lower than 12 indicated a negative attitude. The four items were sufficiently correlated with a Cronbach's alpha of 0.85.

Knowledge

Whereas no screening program and therefore no information booklets exist yet, an information flyer was established, containing information based on a list of domains of screening, suggested to be essential for informed decision making.[16,17] Knowledge was measured using 14 items, with response options 'correct' and 'incorrect' (supplemental table S8.2). These items were developed and evaluated by experts in obstetrics and gynecology, immunohematology and medical decision making. The items were based on the content covered by the information flyer, divided in three topics: characteristics of FNAIT, characteristics of the screening program, implications of the screening test. The outcomes were dichotomized into either sufficient or insufficient knowledge. Sufficient knowledge was defined as 11 or more questions answered correctly (79%).

Intention to participate

The actual uptake cannot be measured without an implemented screening program. Therefore, we assessed the intention to participate in HPA-screening, instead.

Questions regarding demographic (age, ethnicity, education level and marital status) and obstetric characteristics (parity, previous pregnancies, uptake of other prenatal screening, previous abnormal test results in pregnancy, and intended place of delivery) were included in the questionnaire. Educational level was divided into three levels: 'high' in case of higher vocational or academic degree, 'intermediate' in case of lower vocational or higher secondary school and 'low' in case of lower secondary or primary school. To estimate or define ethnicity, we focused on the geographical land of origin or ancestry, as described previously.[18] Women were defined as European if they themselves and their parents were born in Europe.

Data analysis was conducted in SPSS (version 22.0, SPSS Inc., Chicago, IL, USA). Group differences were tested using Pearson's chi-square test or Fisher's exact test for categorical variables and an unpaired t-test or analysis of variance were used for continuous variables. Potential association between quasi-interval variables (knowledge and attitude) was calculated using Pearson correlation test. To adjust for the influence of one or more continuous variables (educational level, ethnicity), partial correlation was used. Differences with $p < 0.05$ were considered to be statistically significant.

The study proposal was approved by the Ethical Committee of the Leiden University Medical Center in Leiden (reference: P15.351).

Table 8.1 – Participants' characteristics (*n* = 143)

	n	**%**
Age (years)[a]		
< 26	12	8.7
26 – 30	53	38.4
31 – 35	51	37.0
≥ 36	22	15.9
Parity		
Nulliparous	81	57.0
Multiparous	61	43.0
Ethnicity[a]		
European	127	92.0
Non-European	11	8.0
Education level[a]		
High	83	60.1
Intermediate	35	25.4
Low	20	14.5
Religion[a]		
Religious	39	28.3
Not religious	99	71.7

[a] Based on *n* = 138, due to missing data.

Results

During the study period, 220 women were invited to participate and received an information flyer on FNAIT together with a questionnaire form. A total of 143 women returned the questionnaire, a response rate of 65% (Supplemental figure S8.1). There were no characteristics of non-responders available. Participants' characteristics are displayed in Table 8.1. The mean age was 31 years (standard deviation 4.1) and mean gestational age 18.8 weeks (standard deviation 5.9). Of the 81 nulliparous women, 59 women reported to be pregnant for the first time (42%). The majority of the participants was highly educated (60%). The intended place of delivery was at home for 21%. Non-European origin included Aruba, Azerbaijan, Curacao, Ecuador, New Zealand and Russia once, Morocco twice and Turkey in three cases.

Attitude

The attitude towards future HPA-screening was positive in 124/137 cases (91%). This positive perspective was founded on sufficient knowledge in 116 out of 124 cases (one missing value). The item for measuring attitude that received the lowest scores was whether or not the screening was thought to be reassuring (Figure 8.1); for the other items, scores were comparable.

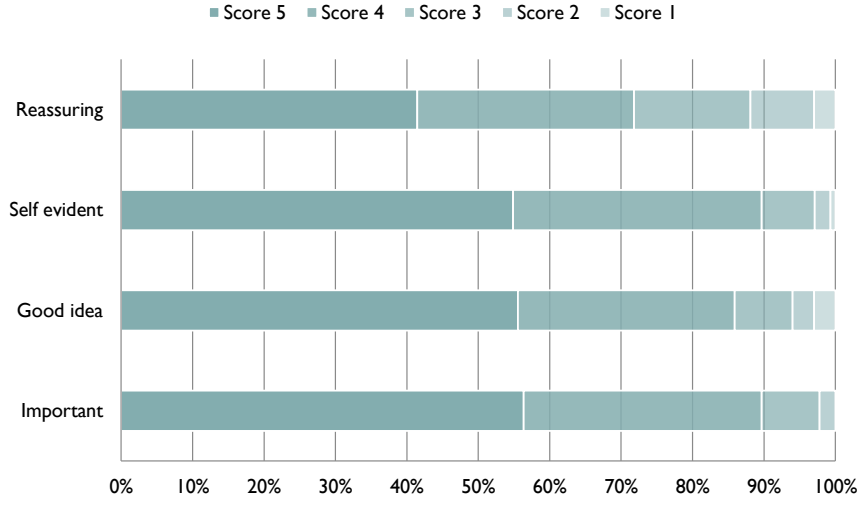

Figure 8.1 – Attitude scores towards human platelet antigen-screening in pregnancy

Thirteen women expressed a negative attitude towards future HPA-screening, in 11/13 cases based on sufficient knowledge, but all resulted in a willingness to participate in HPA-screening and thus representing value inconsistency. Characteristics of these women did not statistically differ from the whole study population, except for the intended location of birth. Women who intended to deliver at home were less frequently positive towards screening than women who planned to give birth in a hospital or birth hotel (81% vs 95%, $p = 0.02$). Of these negative attitudes ($n = 5$), one was based on insufficient knowledge, one was value consistent and declined screening, and the other three resulted in an intention to participate.

Knowledge

Overall, 93% of all participants were scored to have sufficient knowledge. None of the 10 women with more than three of the 14 questions answered incorrectly, would decline participating in future HPA-screening. Of these, six had a high educational level, four were nulliparous and six were religious. Rate of sufficient knowledge or mean knowledge scores did not differ significantly for ethnicity, educational level, parity or religion (Table 8.2).

Table 8.2 – Association between ethnicity/educational level and attitude and informed choice

	Sufficient knowledge		Positive attitude		Value consistent		Informed choice	
	n/N	%	*n*/N	%	*n*/N	%	*n*/N	%
Total	129/139	93	124/137	91	127/137	93	118/136	87
Educational level								
High	77/83	93	71/82	87	73/81	90	68/81	84
Intermediate	33/35	94	34/35	97	34/35	97	32/35	91
Low	17/19	90	18/19	95	18/19	95	16/18	89
Ethnicity								
European	119/127	94	114/125	91	116/124	94	110/124	89[a]
Non-European	8/10	80	9/11	82	9/11	82	6/10	60[a]

N, total number, [a]$p = 0.03$.

Intention to participate

Two women (2%) who returned the questionnaire had no intention to participate in possible HPA-screening in pregnancy, both were informed choices and both women were highly educated.

Informed choice

An informed choice was made by 118 of 136 participants (87%), of which 116 were informed choices with the intention to participate (Table 8.3). In the remaining 18 cases there was no informed choice because of insufficient knowledge ($n = 10$) or due to value inconsistency ($n = 8$). A significantly lower rate of informed choices was found in non-European women, with 60% making an informed choice vs. 89% ($p = 0.03$) in the European population (Table 8.2).

There was no correlation between knowledge scores and total attitude scores ($r = 0.10$, $p = 0.23$). Also, knowledge scores had no influence on the subscale score regardless of whether the test was perceived as reassuring ($r = 0.07$, p = 0.39). Correcting for educational level had very little influence on these correlations (attitude $r = 0.13$ $p = 0.12$; reassuring $r = 0.07$, $p = 0.40$).

Table 8.3 – Informed choice. Intention to participate in human platelet antigen-screening

	Yes	No	Total
Sufficient knowledge, positive attitude	116	0	116
Insufficient knowledge, positive attitude	8	0	8
Sufficient knowledge, negative attitude	8	2	10
Insufficient knowledge, negative attitude	2	0	2
Total	134	2	136

Discussion

In addition to the actual and ongoing debate on implementing HPA-screening in order to prevent the high morbidity and mortality caused by FNAIT, this is the first study assessing women's attitude towards such a screening program. Women's attitude towards HPA-screening in pregnancy was overall very positive, with 91% of all participants expressing a positive opinion. In 94% of these cases, positive attitude was based on sufficient knowledge, all resulting in the intention to participate in such a screening program. Less positive attitude scores were mainly obtained on the item 'reassuring', indicating that women are most concerned that HPA-screening could lead to anxiety during their pregnancy. The potential to cause anxiety need to be carefully considered in designing an HPA-screening program.

Almost all participants indicated they would participate in a HPA-screening program. However, not all of these choices were based on an informed choice. The choices for participation not based on informed choice were equally due to value-inconsistent choices as well as decisions based on insufficient knowledge. Compared to the composition of the whole study population, a higher proportion of uninformed decisions was made by women of non-European origin (22%

vs. 8%), again equally explained by value inconsistency and insufficient knowledge. Although the actual number of non-European pregnant women in the study was low, this was still a significant difference. In a future screening program, information needs to be adapted to the pregnant women's background and language.

The proportion of highly educated women in our study population (60%) was somewhat greater compared to the 48% in the general 25-45 year-old Dutch female population.[19] This could introduce bias in our results. However, since knowledge did not differ significantly between education groups and it was not correlated with attitude scores, we regard this overrepresentation of highly educated women as having no effect on our results and conclusions. In addition, due to the slightly less positive attitude towards screening in the highly educated women, if this overrepresentation of highly educated women would have had any influence on our results at all, it would be an underestimation of the positivity of women's attitude towards HPA-screening.

To estimate the third topic, 'uptake', of the validated MMIC model, 'intention to participate' was used as a surrogate measurement. Although this calculation is used by various studies to predict actual uptake of a test, we cannot as yet verify the strength of this prediction.[20,21]

Another limitation worth mentioning is the underrepresentation of non-Dutch or non-European participants due to the fact that only a Dutch information flyer was constructed and therefore women that were not able to read and understand the Dutch language had to be excluded from participation in our study. Possibly, this might contribute to an overestimation of the overall rate of informed choices made, which of course, in case of future screening, can be avoided by for example providing multilingual information flyers.

Strengths of our study were its careful design, using a validated model for assessing attitude and measuring informed choice. The response rate of our study was relatively higher than other questionnaire studies assessing patient's attitude towards population based screening.[10,12,20,22-24] The scale for measuring attitude had a high reliability and was sufficiently internal consistent, with a Cronbach's alpha of 0.85.

Pregnant women, informed using a carefully designed flyer, appeared to accept and welcome a general screening program for HPA-immunization. Higher knowledge scores did not have any effect on overall attitude scores or to the extent to which HPA-screening was regarded as reassuring. This is in line with previous studies reporting no association between a higher knowledge of a screening test and increased anxiety.[20,23,25] Knowledge questions that were more likely to be answered incorrectly were the questions regarding the implication of screening results. Basic clinical and procedural aspects of FNAIT screening were answered best.

This study confirms the importance of adapting counseling to women's ethnicity and educational level, especially as a non-European origin seemed to correlate with a decreased ability to make an informed decision.[24] Also, the main factor contributing negatively to the overall attitude is whether a future HPA-screening would be reassuring, which stipulates other aspects to be taken into consideration when informing and counseling pregnant women.[20] This is in line with the results from a review of 34 original studies on prenatal screening tests performed by Dahl *et al*[26], who concluded that the second most important reason for declining a screening test was its potential to cause anxiety and uncertainty.

Not every disease will be suitable for population-based screening, and before implementing such a program, predefined criteria have to be fulfilled.[6] In addition to Killie *et al*[7] speculating the cost-effectiveness, Tiller *et al*[27] as well as Kamphuis *et al*[5] advocating the importance of the health problem, the availability of a rapid and reliable screening test[28] and the current development of international treatment guidelines, our study shows fulfillment of yet another criterion of Wilson and Jungner – the acceptability of the screening to the population. Nonetheless, before proceeding to making a decision on implementing HPA-screening, additional research, focused on the natural history of FNAIT and the consequences of positive screening results, needs to be conducted. Prospective pilot screening studies should allow the identification of factors predicting high risk pregnancies that would benefit from treatment and would enable a more detailed economic analysis.

In summary, our study shows that with adequate provision of information, pregnant women are positive towards an HPA-screening program and are capable of making an informed choice on participating in such a program. Additionally, our study identifies important focus points to be taking into account when informing and counseling pregnant women. Implementation of HPA-screening in pregnancy in order to prevent FNAIT appears to be acceptable to the target screening population of pregnant women.

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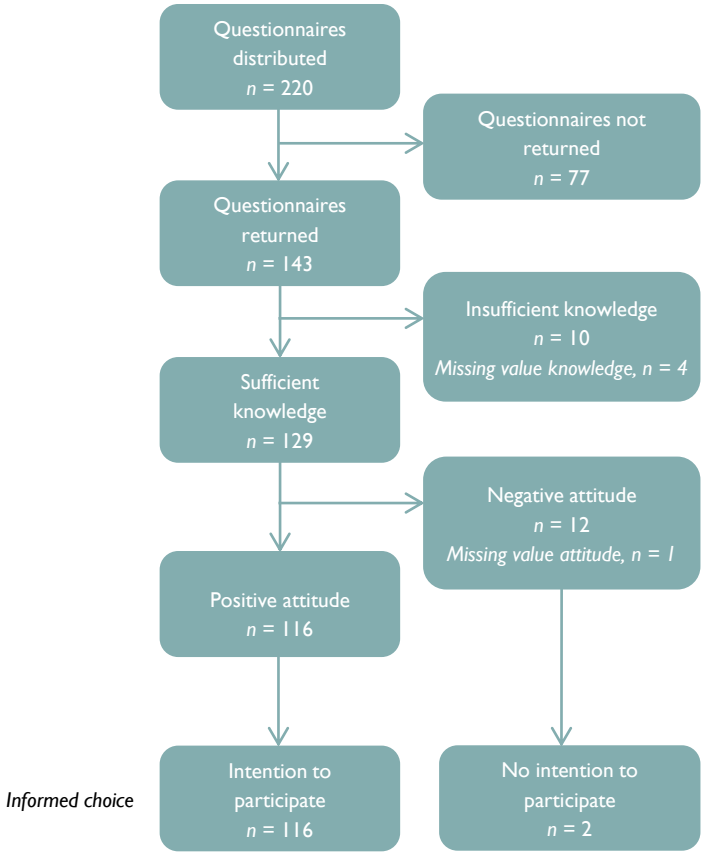
Supplemental material



Supplemental figure S8.1 – Flowchart of study response and informed decision making

Supplemental table S8.1 – Attitude measurement

For me, future HPA-screening would be …						
Unimportant	1	2	3	4	5	Important
Bad idea	1	2	3	4	5	Good idea
Useless	1	2	3	4	5	Beneficial
Frightening	1	2	3	4	5	Reassuring

HPA, human platelet antigen.

Supplemental table S8.2 – Knowledge measurement

Clinical aspects of FNAIT	**Correct**	**Incorrect**
Pathophysiology of FNAIT resembles that of Rhesus disease	O	O
In FNAIT the mother gets clinically ill	O	O
An intracranial hemorrhage is a severe symptom of FNAIT	O	O
Bleeding problems occur in every case of FNAIT	O	O
Diagnosis of FNAIT and HPA-screening	**Correct**	**Incorrect**
Diagnosis is often too late	O	O
Potential future HPA-screening will be applied postnatal	O	O
Screening can be performed by assessing mother's blood	O	O
Screening will detect all cases of FNAIT	O	O
A positive screening result indicates the baby will always suffer bleeding complications	O	O
One in 10 pregnant women will be screen-positive	O	O
Treatment of FNAIT	**Correct**	**Incorrect**
There is no preventative treatment available for FNAIT	O	O
Pregnant women need to be sedated before treatment	O	O
Treatment can be applied during pregnancy	O	O
With treatment, intracranial hemorrhages can be prevented	O	O

HPA, human platelet antigen; FNAIT, fetal and neonatal alloimmune thrombocytopenia.

Supplemental Questionnaire

Leids Universitair Medisch Centrum

Congratulations with your pregnancy!

We want to ask you to participate in a questionnaire study that focuses on screening for antibodies against platelets in pregnancy. Filling in the questionnaire will take about **5-8 minutes.**

We are very interested in **your opinion** about a potential new screening program during pregnancy. Attached you will find a flyer with information about antibodies against platelets in pregnancy. Please, read this flyer carefully.

Participation is completely voluntary. If the questionnaire is completed, it can be turned in at you midwife or send to us in the supplied envelope (free of costs). The questionnaire consists of a total of 25 questions. All results will be processed anonymously.

For questions or the need for more information, please contact: mailto:r.m.loeff@lumc.nl

Research team: Rosanne M. Loeff, LUMC
drs. D. Winkelhorst, medical doctor LUMC
prof. dr. D. Oepkes, gynecologist LUMC

GENERAL

1. What is your age?

……. years

2. What is your highest completed education?

o Higher vocational / Academic
o Lower vocational / Higher secondary school
o Lower secondary school / Primary school / none

3. What is your relationship to the father of your baby?

o Married / Living together
o Not living together, in relationship
o Not living together, no relationship

4. What is the land of birth of you, your parents, your potential partner and his/her parents?

Land of birth - you	……	Land of birth – your partner	……
Land of birth – your father	……	Land of birth – your partner's father	……
Land of birth – your mother	……	Land of birth – your partner's mother	……

5. Are you and your partner religious?
If so, please specify:

	You	**Your Partner**
None	o	o
Catholic	o	o
Protestant	o	o
Islamic	o	o
Hindoeïstic	o	o
Other, ……	o	o

ABOUT THIS PREGNANCY AND EARLIER PREGNANCIES

6. How many weeks are you pregnant?

…… weeks and ….. days

7. Who is your primary obstetric caregiver?

o midwife
o gynecologist
o general practitioner

8. Where do you intend to deliver?

o Home
o Hospital
o Birth clinic
o I don't know

9. Have you been pregnant or gave birth before?

This includes miscarriages.

o yes (go to question 10) o no (go to question 12)

10. Have you had a miscarriage before?

o yes (go to question 11) o no

…… miscarriage

11. How many times have you delivered before?

A delivery is called a delivery from 16 weeks gestational age onwards.
A delivery of twins counts as one delivery.

…… deliveries

12. Did you ever have an abnormal test result during pregnancy?

o yes o no

If so, please specify:

……
……
……
……

KNOWLEDGE - FETAL AND NEONATAL ALLOIMMUNE THROMBOCYTOPENIA (FNAIT)

We would like to ask you to read the flyer with background information. Below, you can find a couple of statements, please state if you agree or disagree with these statements.

13. Pathophysiology

	Agree	Disagree
FNAIT resembles Rhesus disease	O	O
FNAIT is caused by a difference in blood type between father and child	O	O
FNAIT causes illness in the mother	O	O
A intracranial hemorrhage is a severe symptom of FNAIT	O	O
All children with FNAIT will suffer from a hemorrhage	O	O

14. Diagnosis and screening

	Agree	Disagree
The diagnosis FNAIT is often made too late	O	O
Screening for FNAIT will take place after birth	O	O
Screening is possible with a blood test in mother	O	O
Screening will detect all cases of FNAIT	O	O
A positive screenings result always results in disease in the child	O	O
1 in 10 women has a positive screenings result	O	O

15. Treatment

	Agree	Disagree
There is no preventive treatment for FNAIT	O	
For preventive treatment mother needs to be sedated	O	O
Preventive treatment can take place during pregnancy	O	O
With preventive treatment during pregnancy intracranial hemorrhages can be prevented	O	O

YOUR OPINION ON THE FLYER

We would very much like to know what your opinion on our flyer is.
For example: If you think the flyer is very clear, please encircle '5'
If you think the flyer is somewhat unclear, please encircle '2'.

16. The flyer supplies on screening for antibodies against platelets in pregnancy and FNAIT was:

Unclear	1	2	3	4	5	**Clear**

17. Would you have wanted more information on screening to timely detect FNAIT?

- o No
- o Yes, I would have likes some more information:
- o more information on paper
- o more spoken information from my obstetric caregiver
- o through a special telephone number
- o other .....

18. Please specify below the kind of information that your missed in our flyer or the information that was unclear

.....
.....
.....
.....

ATTITUDE

Please let us know what your opinion is on a potential screening program to timely detect FNAIT. Please encircle the number that best fits your opinion.

For example: *If you think screening is very important, please encircle '5'.*
If you think screening is absolutely not important, please encircle '1'.

19. In my opinion, screening for FNAIT during pregnancy is:

Unimportant	1	2	3	4	5	**Important**
Bad idea	1	2	3	4	5	**Good idea**
Useless	1	2	3	4	5	**Beneficial**
Frightening	1	2	3	4	5	**Reassuring**

20. Would you participate in a potential screening program for FNAIT:

	Yes	**No**
I would participate in a screening program for FNAIT	O	O

21. If you want to elaborate on questions 19 and 20, please do so below:

.....
.....
.....
.....

PARTICIPATION IN CURRENT SCREENING PROGRAMS

With the following questions we would like to know your opinion and participation in current national screening programs in the Netherlands.

22. Did you know participation in screening programs is voluntary?

o Yes, I knew
o No, I did not know

23. Did you know that the blood testing for antibodies against red blood cells and infectious diseases in the first trimester of your pregnancy is part of a screening program?

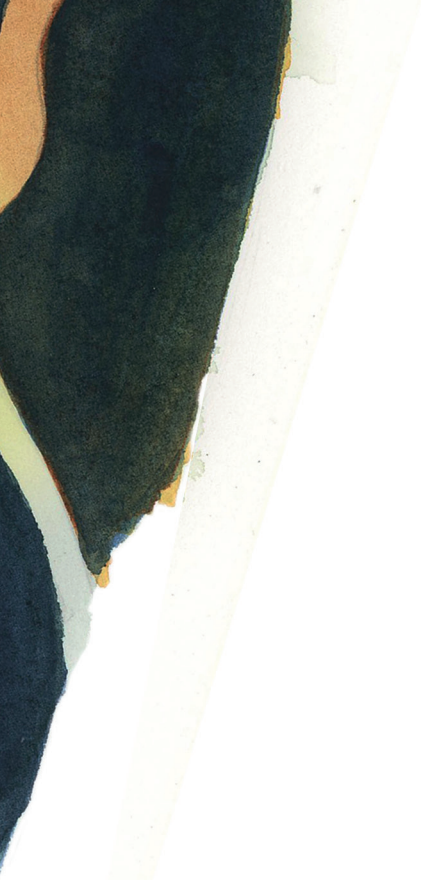
o Yes, I knew
o No, I did not know

24. Did you participate in this screening program?

o Yes
o No
o Not applicable: no blood test is performed yet

25. Did you participate during previous pregnancies?

o Yes
o No
o Not applicable: this is my first pregnancy



Chapter 9

HIP-study (HPA-screening In Pregnancy): interim-analysis

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Manuscript in preparation

Abstract

Introduction. Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a potentially life-threatening disease. In subsequent pregnancies, when immunization is known, devastating bleeding complications can be effectively prevented with weekly maternal intravenous immunoglobulins (IVIg) infusions. Population-based screening to identify alloimmunized pregnancies that would benefit from treatment is a highly debated topic. Missing knowledge on natural history and lack of diagnostic tools to select immunized pregnancies at high-risk is currently complicating implementation of such screening.

Materials and Methods. We performed a nation-wide, prospective, non-interventional cohort study. At 27 weeks' gestation serological HPA-1a typing was performed with enzyme-linked immunosorbent assay (ELISA) and antibody screening using a bead-based PAKLx assay. All women gave consent for antibody testing and clinical data collection.

Results. A total of 40,945 pregnant women were typed for HPA-1a, of which 986 (2.4%) were HPA-1a negative. Within 262 HPA-1a negative cases that gave consent for further testing, 24 immunizations were detected (9.2%). These resulted in 4 cases of clinical relevant FNAIT, 3 with minor hemorrhage and 1 with a severe ICH leading to late pregnancy termination at 34 weeks' gestation. Compared to a HPA-1a positive control group, HPA-1a immunized cases had a higher risk at premature delivery before 34 and 37 weeks' gestation (risk ratios, 8.1; 95% CI, 1.8 to 36.2 and 3.9; 95% CI, 1.7 to 8.9), a higher risk at hemorrhage (risk ratio, 13.0; 95% CI, 4.4 to 38.5), a higher risk at hypertensive disorders (risk ratio, 3.1; 95% CI, 1.4 to 7.0) and significantly more miscarriages in there obstetric history (risk ratio, 1.6; 95% CI, 1.1 to 2.4).

Conclusions. Incidence numbers of HPA-1a negativity and anti-HPA-1a alloimmunization are in line with numbers reported in literature. For careful extrapolation of incidence of clinical relevant FNAIT to the general pregnant population, data from the completed study have to be awaited.

Introduction

Bleeding problems and severe thrombocytopenia in otherwise healthy term-born infants are most likely to be caused by fetal and neonatal alloimmune thrombocytopenia (FNAIT).[1,2] Incompatibility between fetus and mother, for human platelet antigens (HPAs), might result in the formation of specific alloantibodies during pregnancy. These maternal alloantibodies can enter the fetal circulation by active transport across the placenta and lead to platelet destruction and possibly also endothelial damage.[3,4] Clinical consequences of FNAIT can vary from asymptomatic thrombocytopenia to minor skin hemorrhage, such as hematomas or petechiae, or ultimately result in severe internal organ hemorrhages and perinatal death.[5,6] FNAIT is considered to be the platelet counterpart of hemolytic disease of the fetus and newborn (HDFN), because of the similar pathophysiologic fundaments. In this comparison, HPA-1a, that causes the vast majority of the (severe) cases of FNAIT, is the equivalent of RhD in HDFN.[7] Accordingly, a lot of interest has been gone into the prevention of FNAIT by population-based screening, comparable to the RhD prophylaxis and screening program.[8-10] However, besides their resemblance, important differences complicate the design and implementation of such population-based screening, in order to timely detect, prevent and treat FNAIT.

First, despite a couple of large prospective cohort studies, the natural history of the disease is still undetermined. Most of these screening studies, were not completely observational and performed some kind of intervention, thereby prevent drawing any firm conclusion on the natural history of FNAIT.[11-15] Additionally, to implement a screening program in the Netherlands, more accurate estimates of incidence and prevalence of the disease in the Dutch population need to be known. Second, one of the most important differences from HDFN, making it even less achievable to implement population-based screening for prevention of FNAIT, is the lack of diagnostic tools to identify pregnancies at high risk for bleeding complications. In HDFN, alloimmunization is monitored by both laboratory and clinical assessment. High risk cases are identified by laboratory assessment (antibody titer and antibody-dependent cellular cytotoxicity), followed by ultrasound assessment of pre-selected cases (estimation of fetal anemia by Doppler-based assessment of flow velocity in the middle cerebral artery). These cases most likely benefit from fetal blood sampling (FBS), followed by an intrauterine transfusion (IUT).[16] In FNAIT, without the clinical applicability of such (non-invasive) diagnostic parameters, all known alloimmunizations are treated with non-invasive intravenous immunoglobulin (IVIg) infusions.[17,18] The vast majority of these alloimmunizations are currently detected because of the disease they've already caused. However, in a potential screening scenario, far more alloimmunized pregnancies will be detected, among which pregnancies that would have never resulted in fetal or neonatal bleeding problems. Treating all these alloimmunizations in a potential screening setting would result in considerable overtreatment.

To obtain insight in these two important features of the disease (natural history and risk assessment severe course of disease) and to facilitate assessment of the feasibility of screening, we designed the HIP-study (HPA-screening In Pregnancy). The study is completely observational. This way, besides the incidence of HPA-1a alloimmunizations and clinical manifestations, we will also be able to conclude on the natural history of FNAIT. Ultimately, by collecting both blood samples and testing for antibody characteristics, genetic markers and data on bleeding symptoms in children from alloimmunized and non-alloimmunized pregnancies, we aim to develop a diagnostic tool. This tool will allow the identification of immunized pregnancies at high risk for bleeding complications, that would benefit from treatment. Which would not only be beneficial in current management of FNAIT, but most of all in a potential future screening setting.

Materials and methods

Study population

From March 2017, all RhD or Rhc negative pregnant women in the Netherlands were eligible for enrolment in the study. As part the Dutch nationwide Prenatal Screening for Infectious diseases and Erythrocyte immunization (PSIE), these women are offered a free of charge red blood cell (RBC) antibody screening and fetal *RHD* typing at 27 weeks' gestation. Blood for this screening is drawn at certified, local laboratories all over the Netherlands ($n = \pm 90$) and transported to our laboratory.[19] Left-over material of the ethylenediamine tetra-acetic acid (EDTA) anticoagulated blood tubes was used. Pregnant women with known HPA alloimmunization, and that received IVIg treatment, were excluded. All pregnant women gave informed consent for inclusion in the HIP-study. The study was approved by the Committee of Medical Ethics at the Leiden University Medical Center (P16.002).

Laboratory analysis

After regular testing and reporting of these results, left-over material of the blood samples, mainly 3-6 days-old, was used. Plasma containing platelets was used for serological HPA-1a typing, using an enzyme-linked immunosorbent assay (ELISA), as described previously.[20] In short, 20µl of plasma was automatically pipetted onto 96-well microtiter plates coated with anti-CD61 (C17). Then, recombinant HRP-labeled anti-HPA-1a (B2G1) was added, plates were centrifuged and incubated for 45 minutes. After 15 minutes of incubation with anti-HRP, reaction was stopped with sulfuric acid (H_2SO^4) and results were obtained with an Anthos reader. HPA-1a typing was performed in all samples that did not decline consent. In all samples with an ELISA OD below 0.160, supportive genotyping using allelic discrimination polymerase chain reaction (PCR) assay was performed. Plasma and buffy-coat of samples that were typed HPA-1a negative were stored at -20°C. Additionally, for each HPA-1a negative case, one HPA-1a positive

control was stored as well. For further testing informed consent was needed. Well after the estimate due date, the incidence of alloimmunization was evaluated. With the stored, left-over plasma of all HPA-1a negative women a Pak Lx assay was a performed, a bead-based GP-specific HPA-antibody detection method (LIFECODES Pak Lx Assay, Immucor GTI Diagnostics, Norcross, United States of America) to screen for HPA-1a alloantibodies. Possible laboratory parameters that will be tested to assess risk at bleeding complications are: HLA-DRB3*0101 status, antibody level, Fc-core glycosylation and FcgRIII-binding index, endothelial cell binding and endothelial cell function.[4,21-26] Combining the clinical information with the laboratory results will enable the identification of two groups; immunized cases with disease and immunized cases without disease. The latter group will form a unique and optimal control group for identification of possible parameters to predict the development of (severe) bleeding complications.

Clinical data collection

Of all stored samples, obstetric care givers were contacted to obtain clinical information, through a digital case report form (CRF). This form contained data on maternal medical history, obstetric history, current pregnancy, delivery and neonatal period (first week of life). Primary outcome was clinically relevant HPA-1a mediated FNAIT, which was further classified into severe FNAIT (with severe bleeding, ICH or internal organ hemorrhage) or mild FNAIT (all other bleeding, petechiae, bruising, hematoma or mucosal bleeding, and/or treatment or clinical observation for thrombocytopenia).

Statistical analysis

Clinical data will be entered into a validated data capture system, called ProMISe, provided and designed by the LUMC. The system is protected by password and contains internal quality checks to identify inaccurate or incomplete data. Laboratory data will be entered in a separate password protected database by independent technicians, inaccessible to the researchers. Both clinical and laboratory data will be combined and further data management and analysis will be performed using SPSS and Graphpad. Included cases were divided into three groups: HPA-1a negative cases with anti-HPA-1a (referred to as immunized cases), HPA-1a negative cases without anti-HPA-1a (referred to as non-immunized cases) and HPA-1a positive controls. Comparisons between groups for categorical data were performed using chi-square or Fisher exact test. Risk ratios (relative risks) for immunized cases versus the HPA-1a positive control group were calculated for categorical data. For continuous variables groups were compared using student t-test or Mann-Whitney U, as applicable. A *p*-value smaller than 0.05 was considered statistically significant.

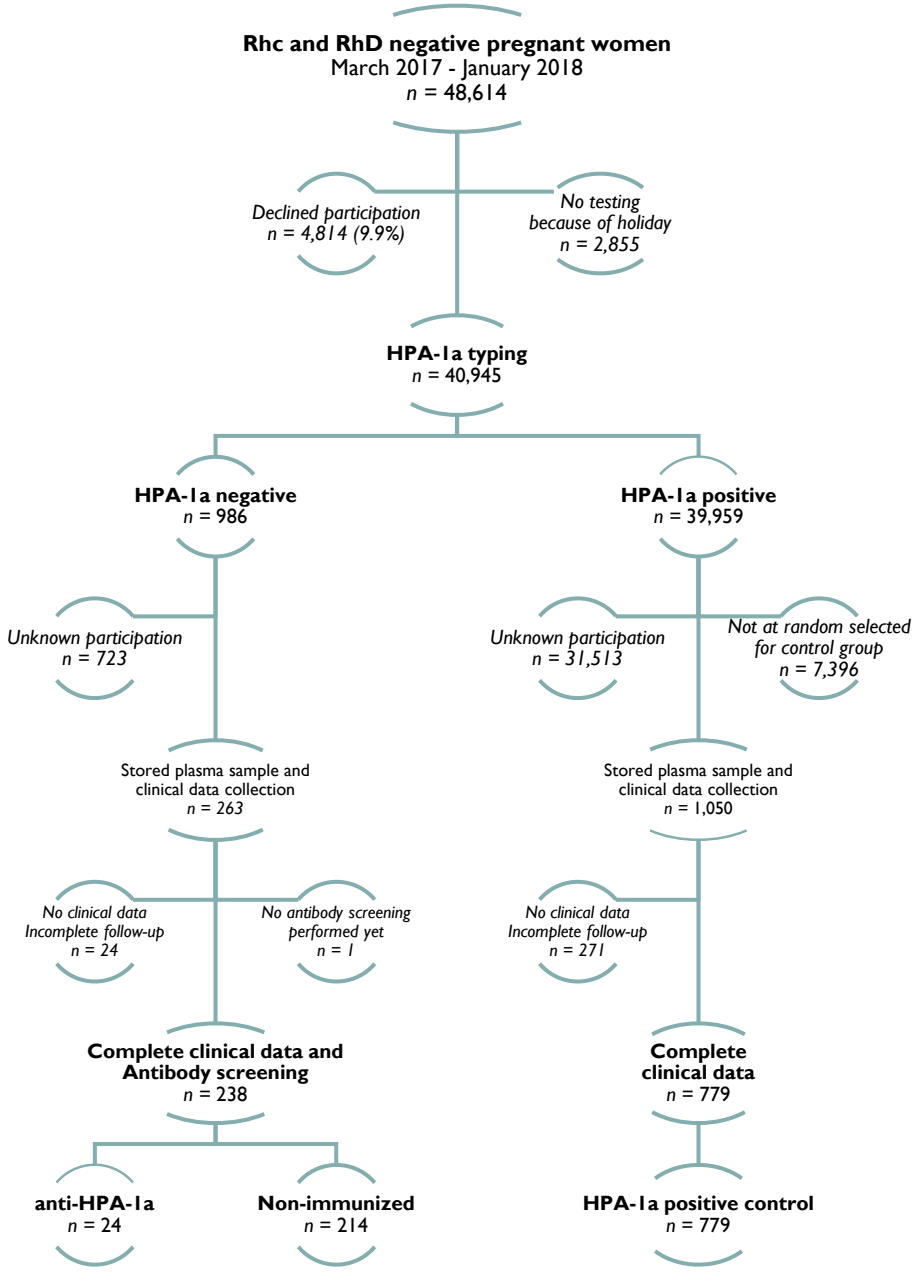

Figure 9.1 – Description of study population

Results

Participants

In a period of 10 months, from March 2017 to January 2018, 48,614 pregnant women were tested as part of the population-based screening program at 27 weeks' gestation for RBC antibodies or fetal *RHD* type because of their RhD-negative or Rhc-negative blood type (Figure 9.1). Of these, 4,814 (9.9%) declined participation to the HIP-study and in 2,855 serological HPA-1a typing was not performed due to holidays and/or understaffing. This leaves a total of 40,945 women that were serologically typed for HPA-1a. Of these, 986 (2.4%) were HPA-1a negative. For every HPA-1a negative sample a HPA-1a positive sample with consent was stored, in total we included 1,050 HPA-1a positive controls. Informed consent was registered for 27%, so a total of 263 HPA-1a negative samples were available for further testing. In all but one case antibody screening was performed, leading to the detection of 24 anti-HPA-1a immunizations (24/262, 9.2%).

Table 9.1 – Baseline Characteristics

	HPA-1a negative (*n* = 238)	**HPA-1a positive (*n* = 779)**	**Total (*n* = 1,017)**
Gravidity	2 (1-3)	2 (1-3)	2 (1-3)
Primigravidae	77 (32)	271 (35)	348 (34)
Parity	1 (0-1)	1 (0-1)	1 (0-1)
Nulliparae	95 (40)	342 (44)	437 (43)
Medical history ITP	0	0	0
Miscarriage in history	57 (24)	196 (25)	253 (25)
IUFD in history	2 (1)	6 (1)	8 (1)
Multiple gestation	4 (2)	13 (2)	17 (2)

Data presented as n (%) or median (interquartile range); HPA, human platelet antigen; ITP, immune thrombocytopenia; IUFD, intrauterine fetal demise.

Descriptive data

The clinical data collection was complete for 1,018 of 1,249 included cases (82%). We were able to analyze a total of 238 HPA-1a negative women and 779 HPA-1a positive controls. Baseline characteristics (gravidity, parity, medical history with ITP, obstetric history and multiple gestations) were comparable between both groups, no statistical significant differences were observed (Table 9.1).

Table 9.2 – Clinical Outcome

		HPA-1a negative (n = 238)	
		Immunized (n = 24)	Non-immunized (n = 214)
Perinatal	Gestational age at delivery	39^{+6} (37^{+3} – 40^{+5})*	39^{+6} (39^{+0} – 40^{+5})
	Premature delivery (< 37 weeks)	4 (17)*	6 (3)
	Premature delivery (< 34 weeks)	2 (9)*	2 (1)
Neonatal	Male sex	14 (58)	104 (49)
	Birth weight (g)	3195 (2950 – 3435)*	3480 (3194 – 3870)
	Small for gestational age (< p10)	2 (8)*	18 (9)
	Apgar score <7 at 5 minutes	1 (4)*	2 (1)
	Hemorrhage	4 (17)	2 (1)
	Of which severe hemorrhage	1 (4)	0
	Of which leading to perinatal death	1 (4)	0
	Perinatal death without bleeding	0	0

Data presented as n (%) or median (interquartile range); CI, confidence interval; HPA, human platelet antigen.

* one case of termination of pregnancy at 34 weeks' gestation excluded.

** risk ratio of immunized cases versus HPA-1a positive cases.

Clinical outcome

The clinical outcome of all three groups is presented in table 9.2. Median gestational age at delivery was similar between the three groups. A total of four pregnancies (17%) ended in premature delivery before 37 weeks' gestation in the immunized group and 42 (5%) in the HPA-1a positive control group (risk ratio, 3.9; 95% CI, 1.7 to 8.9). The proportion of premature deliveries before 34 weeks' gestation was 9% in the immunized cases and 1% in the HPA-1a positive controls (risk ratio, 8.1; 95% CI, 1.8 to 36.2). Median birth weight was lower in the immunized group (3195 gram) than in the non-immunized and HPA-1a positive control group (3480 gram and 3510 gram, respectively). There was no significant difference in the proportion of infants that were SGA (8% versus 9% and 11%, respectively; *p* 0.45). Four infants (17%) in the immunized group suffered from increased bleeding tendency and 10 infants in the HPA-1a positive control (1%) had any form of bleeding (risk ratio, 13.0; 95% CI, 4.4 to 38.5). One of the four infants with bleeding symptoms in the immunized group suffered a severe hemorrhage, an ICH. No severe hemorrhages were observed in the non-immunized group and the HPA-1a positive control group. Five cases (1%) of perinatal mortality were observed in the HPA-1a positive control group. One was a case of severe perinatal asphyxia, with no other abnormalities after MRI and pathologic examination. The other four were IUFDs of which one had fetal anemia due to a massive fetomaternal hemorrhage, one declined post-mortem examination and in two the cause was unknown after examination.

HPA-1a positive (n = 779)	*p*-value	Risk ratio (95% CI)**
39^{+6} (38^{+5} – 40^{+4})	0.407	-
42 (5)	0.017	3.9 (1.7 – 8.9)
8 (1)	0.038	8.1 (1.8 – 36.2)
396 (51)	0.750	1.1 (0.8 – 1.6)
3510 (3130 – 3845)	0.006	-
88 (11)	0.451	1.0 (0.9 – 1.2)
11 (1)	0.290	3.1 (0.4 – 22.7)
10 (1)	0.001	13.0 (4.4 – 38.5)
0	0.025	-
0	0.025	-
5 (1)	0.637	-

Immunized cases, non-immunized HPA-1a negative cases and HPA-1a positive cases had similar proportions of first pregnancies and first-born children (Table 9.3). Hypertensive disorders, either pregnancy-induced hypertension (PIH) or pre-eclampsia, were present in five (21%) immunized pregnancies and 53 (7%) pregnancies from HPA-1a positive controls (risk ratio, 3.1; 95% CI, 1.4 to 7.0). Obstetric history, if applicable, revealed a miscarriage in ten (63%) immunized cases and 196 (39%) HPA-1a positive controls (risk ratio, 1.6; 95% CI, 1.1 to 2.4).

Immunized cases

Of the 24 anti-HPA-1a immunized pregnancies, four resulted in clinically relevant FNAIT, one severe FNAIT and three cases with mild FNAIT (Table 9.4). The case of severe FNAIT was a severe bilateral intracranial hemorrhage detected at ultrasound that was performed due to reduced fetal movements at 29 weeks' gestation. In follow-up MRI assessment in the following weeks, extensive damage to the fetal brain tissue with large cysts was observed and after extensive counseling a late termination of pregnancy at 34 weeks' gestation was performed. The three cases of mild FNAIT consisted of petechiae in a male infant born after an emergency cesarean section due to fetal distress, a girl with a cephalic hematoma after an uncomplicated pregnancy and delivery and a case of hematomas in a female infant that was admitted to the neonatal intensive care unit (NICU) because of prematurity.

Table 9.3 – Obstetric characteristics

	HPA-1a negative (*n* = 238)		**HPA-1a positive**	***p*-value**	**Risk ratio**
	Immunized (*n* = 24)	Non-immunized (*n* = 214)	**(*n* = 779)**		**(95% CI)**
Primigravidae	8 (33)	69 (32)	271 (35)	0.764	0.9 (0.4 – 2.2)
Nulliparae	10 (42)	85 (40)	342 (44)	0.525	0.9 (0.4 – 2.1)
Hypertensive disorder this pregnancy	5 (21)	18 (9)	53 (7)	0.039	3.1 (1.4 – 7.0)
Miscarriage in obstetric history*	10 (63)	47 (32)	196 (39)	0.028	1.6 (1.1 – 2.4)
IUFD in obstetric history**	1 (7)	1 (1)	6 (1)	0.258	5.2 (0.7 – 40.2)

Data presented as n (%) or median (interquartile range); CI, confidence interval; HPA, human platelet antigen; IUFD, intrauterine fetal demise.

* risk ratio of immunized cases versus HPA-1a positive cases.

** primigravidae excluded; immunized *n* = 16, non-immunized *n* = 145, HPA-1a positive *n* = 508.

*** nulliparae excluded; immunized *n* = 14, non-immunized *n* = 128, HPA-1a positive *n* = 435.

Discussion

Implementing population-based screening in order to timely detect and prevent disease burden caused by HPA-1a mediated FNAIT is a long debated topic. Lacking knowledge on natural history and incidence of the disease and, more importantly, the absence of reliable diagnostic tools to select alloimmunized pregnancies at high risk for bleeding, complicate implementation. We present the preliminary results of a large, nationwide, non-interventional, prospective screening study.

The incidence of HPA-1a negativity and subsequent alloimmunization in our study can be best compared to the three largest prospective antenatal screening studies performed in Norway, Scotland and England (screening 100,448, 26,506 and 24,417 pregnant women, respectively).[15,27,28] In our cohort 2.4% of the pregnant women were HPA-1a negative, similar to the reported 2.1% – 2.5%. Although the material used for HPA-1a typing varied (platelet-rich plasma or whole blood in the previous studies and plasma in ours), all three studies performed serological HPA-1a typing (either ELISA or flow-cytometry), followed by supportive genotyping. Further, the studies describe an antenatal anti-HPA-1a detection of 7.2% – 9.6%, comparable to the 9.2% alloimmunizations detected in our HPA-1a negative samples. In contrast to our single measurement at 27 weeks' gestation, previously mentioned studied screened for antibodies 2 to 5 times during pregnancy, between 8 and 36 weeks' gestation. For anti-HPA-1a detection none of the studies used PAKLx bead-based assay, like was performed in our cohort. Both Kjeldsen-Kragh *et al.*[15] and Williamson *et al.*[27] used the monoclonal antibody immobilization of platelet antigens (MAIPA) assay and Turner *et al.*[28] used a somewhat comparable ELISA technique. A study that compared MAIPA to PAKLx in 100 cases with suspected FNAIT, detected 26 anti-HPA-1a immunizations were by both MAIPA and

PAKLx and one case with anti-HPA-1a that was only detected by PAKLx.[29] Despite we performed antibody screening only once during pregnancy, the use of a probably more sensitive technique might explain the relatively high immunization rate of 9.2%.

Within the 24 immunizations that we detected, three cases with only minor bleeding and one case with severe bleeding were identified. The comparison to reported incidences in literature is troubled by the absence of completely observational studies. The Norwegian screening study reported two cases of severe bleeding in 144 antenatally detected HPA-1a immunizations, the occurrence of minor bleeding complications was not described.[15] The Scottish study detected three minor and no severe bleeding complications in 25 immunizations and the English study found seven minor hemorrhages and one case of severe bleeding within 37 antenatally identified alloimmunizations.[27,28]

Obviously, the small numbers from our study make it difficult to adequately extrapolate. With a birth rate of approximately 170,000, this would result into the identification of 4,080 (2.4%) HPA-1a negative pregnant women and 375 anti-HPA-1a immunizations (9.2%) each year in the Netherlands.[30] Further extrapolation would mean that these immunizations lead to 63 cases of clinically relevant FNAIT (375/24*4), of which 47 with only minor hemorrhage and 16 with severe bleeding complications.

It should be noted that, because antibody screening was performed with the sample collected at 27 weeks' gestation, the detected alloimmunizations in our study might still be an underestimation. Besides potentially missing immunizations that occur in third trimester and after delivery, immunizations that resulted in severe bleeding and pregnancy termination or IUFD before 27 weeks' gestation will also not be included. Further underestimation might occur due to the absence of routine neonatal brain ultrasound. Intracranial hemorrhages that will not lead to clinical problems during pregnancy or in the first week of life can be missed. To estimate the extent of this problem, a prospective study with routine ultrasound examination and structured long-term follow-up will be necessary. The Norwegian screening study did perform routine cranial ultrasound of all newborn's brains.[15] They detected two ICHs. One was already detected on ultrasound at 34 weeks' gestation and both had petechiae at birth. The other was a small grade I bleed, also with petechiae but no neurological symptoms. Within our study design, the first and large ICH would have been detected, but we would not have identified the latter. There were two other prospective screening studies that detected an ICHs. The first, performed by Williamson *et al.*[27], performed routine cerebral ultrasound in all HPA-1a alloimmunizations, and detected a severe and symptomatic ICH, leading to the formation of porencephalic cyst and hydrocephalus. Blanchette *et al.*[11] detected the second, a symptomatic ICH amongst three anti-HPA-1a immunizations.

Table 9.4 – HPA-1a immunizations

Classification	G/P	Obstetric history	Pregnancy	Delivery	Sex
Severe FNAIT	G3P1	1 miscarriage	Bilateral ICH on US at 29 weeks	Late pregnancy termination at 34+0	Male
Mild FNAIT	G3P2		Gestational diabetes	Primary CS at 33+5	Female
	G1P0	NA		Spontaneous delivery at 40+6	Female
	G3P1	1 miscarriage	PIH	Secondary CS at 39+6 due to fetal distress	Male
No clinical bleeding problems	G2P0	1 miscarriage	PIH	Induction of labor at 34+1	Female
	G2P0	1 miscarriage		Spontaneous delivery at 39+6	Male
	G4P2	1 miscarriage		Spontaneous delivery at 39+3	Female
	G1P0	NA		Spontaneous delivery at 40+1	Female
	G1P0	NA	PPROM at 31 weeks	Forcipal extraction at 31+4, after PPROM	Male
	G3P2			Spontaneous delivery at 40+2	Female
	G1P0	NA		Spontaneous delivery at 38+5	Female
	G1P0	NA	Oligohydramnios	Induction of labor due to oligohyramnios at 41+2	Male
	G7P4	2 miscarriages, 1mola pregnancy		Induction of labor at 37+0	Male
	G1P0	NA		Spontaneous delivery at 39+1	Male
	G3P2		Anemia	Primary CS at 37+3	Male
	G1P0	NA		Spontaneous delivery at 39+6	Male
	G4P2	1 miscarriage		Spontaneous delivery at 41+0	Male
	G2P1			Spontaneous delivery at 40+5	Male
	G1P0	NA	PIH	Vacuum extraction at 40+5	Female
	G2P1		PIH	Induction and spontaneous delivery at 37+6	Female
	G2P1			Spontaneous delivery at 40+2	Male
	G3P1			Spontaneous delivery at 36+3	Male

Birth weight (g)	Signs of bleeding	Other	Other antibody	MFI anti-HPA-1a
2482	Massive ICH, hematomas (PLT 12)	Severe bilateral ICH, severe damage to brain tissue, large cysts	None	13485
2016	Hematomas (PLT 102)	Admission to NICU for prematurity, respiratory distress, hyperglycemia	None	11068
3140	Cephalic hematoma		None	6420
2960	Petechiae, conjunctival bleeding (PLT 80)	SGA (<p10)	None	9117
1715	None	SGA (<p2.3)	HLA, HPA-5b	1385
3655	None		None	3285
2970	None		HLA	2933
3410	None		None	163
1750	None	Admission to NICU because of prematurity	None	598
3245	None		None	1006
2790	None	Bilateral clubfeet	None	595
3405	None	Hypospadias	None	390
2950	None		HLA	18526
3045	None	Admission to NICU due to hyperbilirubinemia	None	1899
2975	None		None	1163
3320	None		None	5649
4410	None		None	443
4360	None		None	5694
3215	None	Pediatric consultation due to vacuum	None	365
3195	None	Apgar score 5/6	None	341
3715	None		None	145
2700	None	Admission to NICU and hypothermic treatment due to asphyxia	None	487

Table 9.4 – Continued

Classification	G/P	Obstetric history	Pregnancy	Delivery	Sex
	G7P1	4 miscarriages, 1 abortus provocatus		Spontaneous delivery at 39+2	Female
	G4P2	1 miscarriage		Spontaneous delivery at 40+6	Male

AS, Apgar score; CS, cesarean section; HLA, human leukocyte antigen; G/P, gravidity/parity; HPA, human platelet antigen; ICH, intracranial hemorrhage; IUFD, intrauterine fetal demise; NA, not applicable; NICU, neonatal intensive care unit; PIH, pregnancy-induced hypertension; PLT, platelet count; PPROM, premature prelabor rupture of membranes; SGA, small for gestational age; US, ultrasound.

The lack of routinely collected neonatal platelet counts in our study might be considered a limitation as well. Besides hampering comparison with the previously performed prospective studies, this complicates estimation of the consequences of our detected anti-HPA-1a alloimmunizations. Previous studies suggest that there will be more cases of severe thrombocytopenia than cases with minor hemorrhage.[12,14,27,28] However, we do feel that the complete neonatal follow-up data, including the occurrence of minor bleeding, compensates this lack of information. After all, within health care and screening the goal is to prevent clinical disease instead of laboratory values.

At the moment of performing and writing this interim-analysis, the collection of clinical data is still ongoing. The rate of cases with currently incomplete clinical data is larger in the control group compared to the immunized group (26% versus 10%, respectively). After completion of the collection we expect these proportions to be similar. Also, whereas data is collected blinded for outcome of serological HPA-1a typing or antibody screening and data is collected per health care institution (either hospital or midwifery practice), we do not expect this to introduce a possible selection bias. Further, because we used left-over material from the Dutch population-based screening program for RBC immunization, we only included RhD negative and Rhc negative pregnant women. Additionally, we missed pregnant women with anti-RhD or anti-Rhc alloantibodies in the first trimester of their pregnancy, because no additional RBC antibody screening at 27 weeks' gestation will be performed in these women. However, RhD negative or Rhc negative blood type has never been associated with HPA-type, HPA-alloimmunization or FNAIT disease severity. Neither has an association RBC alloimmunization and HPA-1a immunization or disease severity been described before. Therefore, we do not expect the use of this specific study population to introduce a selection bias.

	Birth weight (g)	Signs of bleeding	Other	Other antibody	MFI anti-HPA-1a
	3435	None		None	18726
	3460	None	Admission to NICU because of hyperbilirubinemia	None	18676

In conclusion, our prospective and more importantly non-interventional study reports incidence numbers of HPA-1a negative and subsequent HPA-1a alloimmunization in the Netherlands, that are comparable to the literature. Further, estimates on incidence of clinical relevant FNAIT are in line with expected numbers as well and do not seem to possibly hamper implementation of a population-based screening program. For exact extrapolation and firm conclusions, completion of the HIP-study needs to be awaited.

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Chapter 10

General discussion

Parts of this discussion have been submitted for publication as:

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General discussion

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) can be a highly unexpected and dramatic complication in pregnancies. This form of alloimmunization can lead to devastating bleeding problems that might have been prevented if the alloimmunization would have been known. In current practice, all known alloimmunized pregnancies are treated with highly effective, weekly, intravenous immunoglobulin infusions (IVIg). Without population-based screening, these pregnancies are virtually solely in focus after a previously affected pregnancy or newborn. Affected cases that might have been prevented if the alloimmunization would have been detected prior to the occurrence of bleeding. Identifying these pregnancies before they lead to clinical damage would mean a major improvement in the management of FNAIT, but will only be achievable by implementing population-based screening. Implementing screening for detection of several diseases, such as erythrocyte immunization, congenital infections, breast and cervical cancer, and a number of diseases identified by neonatal heel-prick screening, has drastically decreased associated morbidity and mortality.[1-4] New and promising population-based screening programs cannot be implemented without carefully considering the pros and cons. There should be an acceptable balance between offering treatment to those in need with undetected disease, while avoiding harm and overtreatment to those not in need. To guide this careful consideration and weighing of benefits, burdens and costs, the World Health Organization published ten screening criteria, posed by Wilson & Jungner (W&J, figure 10.I).[5]

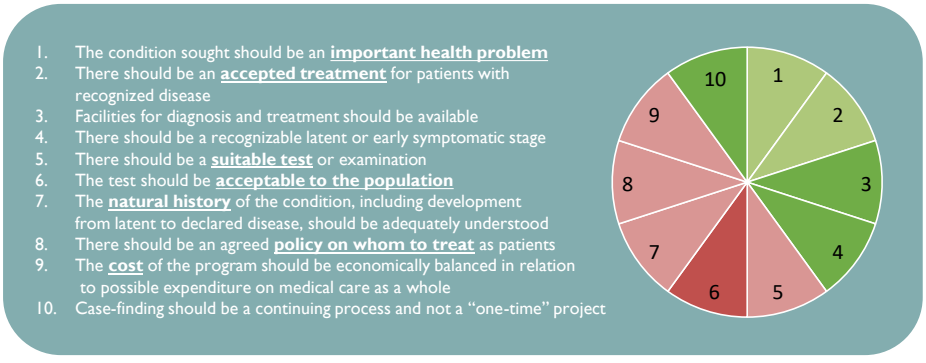

Figure 10.1 – Evidence for screening - Wilson & Jungner criteria[5]

Dark green, criterion fulfilled; light green, some extra information needed for fulfillment; light red, little information available but insufficient for fulfillment; dark red, no information available.

In this thesis, we aimed to gather and evaluate the evidence that is necessary to answer the highly-debated question whether it is feasible and sensible to implement prenatal screening to prevent the devastating consequences of FNAIT.

Important health problem

The importance of a health problem can be defined by both the severity of the disease and its incidence. A rare disease with major individual and social impact can be as important as diseases with milder outcome but higher incidence.[6] FNAIT fits the first category, a rare disease with major consequences. One of the most-feared consequences is a fetal or neonatal intracranial hemorrhage (ICH), due to its high mortality rates and possibly even worse, a high risk of lifelong handicaps and neurological sequelae in survivors.[7] This complication is generally well-known and described in most guidelines and literature. And whereas most case series and articles do report on highly unfavorable short-term consequences, no detailed and structured long-term follow-up data of children suffering ICH caused by FNAIT have been reported. Information that is indispensable in the screening debate. Indispensable, because the information is necessary for assessment of consequences and costs to be prevented, as well as for the counseling of parents. In *chapter 3* we describe ICHs that were managed at our center, which is the national referral center for neonatal alloimmune disease. The identification of 31 cases in 21 year is probably not an adequate representation of the true prevalence of ICH in our country, which has a birth rate of 170,000 per year. Current underdiagnosing of FNAIT in absence of screening might be a strong contributor to this small number of 1-2 cases per year. This was previously suggested by Davoren *et al.*[8], who reported a discrepancy between retrospectively collected cases and expected cases based on prospective screening studies.

Neurodevelopmental outcome of the surviving children in our cohort was unfavorable. More than half were severely neurodevelopmental impaired (NDI). This is in line with previously described case-series. Within a series of nine ICHs due to FNAIT, three infants died, and severe impairment was reported in all surviving children.[9] Another series reported three ICHs caused by FNAIT, of which one died and the two surviving children suffered severe NDI.[10] Both series are limited by the small numbers, absence of long-term follow-up and lack of standardized testing. Also, both might have been strongly biased by the selection of only severe cases. This selection bias might have occurred in our cohort as well. The fact that our study was single center at a referral hospital and data were retrospectively collected, together with considerable missing clinical data, might have led to an overrepresentation of the more severe cases. This is supported by the relatively high rate of perinatal mortality. Compared with the largest cohort of retrospectively collected ICHs, described by Tiller *et al.*[11], our mortality rate is somewhat higher (48% versus 35%). However, the proportion of first-born children affect by FNAIT is similar. Over half of the described severe cases occur in first-born children.

Prospectively collected data from non-intervention studies might tackle the risk of selection bias to more severe ICHs, but these numbers are too small to extract useful data from (Table 10.1).[17,21] Further, a prospective study that identifies all clinically overt cases of ICH that are symptomatic in the first day of life, might still miss cases that are initially asymptomatic. Intracranial bleeding,

causing discrete brain damage that is not big enough to be symptomatic (e.g. leading to epilepsy, reduced consciousness or other clinical problems), will remain undetected but might still lead to behavioral or cognitive problems in later life. Whether this milder, initially subclinical, phenotype of ICH exists as well, is something to be assessed in future prospective research with long-term follow-up.

Table 10.1 – Prospective cohort studies assessing incidence and natural history

Author, year	HPA-1a negative	Antenatal anti-HPA-1a	PLT <50 x 10^9/L	Mild bleeding	Severe bleeding	Intervention
Mueller-Eckhardt, 1985[12]	26/1,211 (2.1)	2/26 (7.7)	0	0	0	None
Reznikoff-Etievant, 1988[13]	27/860 (3.1)	0/27 (0)	0	0	0	None
Blanchette 1990[14]	81/5,000 (1.6)	3/50 (6.0)	1	0	1	NTCS, PP
Doughty, 1995[15]	74/3,473 (3.2)	1/71 (1.4)	0*	0*	0	FBS/IUPT, IVIg, PP
Durand-Zaleski, 1996[16]	52/2,066 (2.5)	4/45 (8.9)	1	0	0	FBS, IVIg, CST
Williamson, 1998[17]	618/24,417 (2.5)	37/385 (9.6)**	8	7	1	PP
Davoren, 2003[18]	54/4,090 (1.3)	2/34 (5.9)	1	1	0	FBS, IUPT, PP
Maslanka, 2002[19]	144/8,013 (1.8)	12/122 (9.8)	3	1	0	IUPT, IVIg
Turner, 2005[20]	546/26,506 (2.1)	25/318 (7.9)	5	3	0	PP
Kjeldsen-Kragh, 2007[21]	2,111/100,448 (2.1)	144/1,990 (7.2)	48	NR	2	NTCS, PP
Debska, 2018[22]	373/15,204 (2.5)	22/373 (5.9)	3	NR	NR	IUPT, IVIg
Winkelhorst, (HIP-study) *unpublished results*	986/40,945 (2.4)	24/262 (9.2)	NT	3	1	None

Numbers are *n*/N (%). CST, antenatal corticosteroids; FBS, fetal blood sampling; FNAIT, fetal and neonatal alloimmune thrombocytopenia; HPA, human platelet antigen; IUPT, intrauterine platelet transfusion; IVIg, antenatal intravenous immunoglobulins; NR, not reported; NT, not tested; NTCS, near-term cesarean section; PLT, platelet count; PP, postnatal platelets available for transfusion. Severe FNAIT is defined as neonatal platelet count at birth <50 x 109/L. Mild bleeding is defined as only skin or mucosal bleeding. Severe bleeding is defined as internal organ hemorrhage or ICH.

* One HPA-1a negative women delivered two severely affected twin children, anti-HPA-1a antibodies detected after birth, not detected by prenatal screening.

** Two pregnancies ended in loss of the baby, one at 15 weeks, another as neonatal death form immaturity after CS at 25 weeks for severe pre-eclampsia.

A fetal or neonatal ICH in an otherwise healthy, but thrombocytopenic infant would in most cases be strongly associated with FNAIT, especially when other causes for thrombocytopenia are excluded. Though seldom described in (international) guidelines or reports on FNAIT, the site of bleeding can conceptually be in every organ in the body. These other hemorrhages are therefore likely to be underdiagnosed, which might not only lead to inadequate diagnoses and treatment in the affected infants, but also lead to inadequate follow-up and management in potential subsequent pregnancies. In *chapter 4,* we described a case series and literature review

of these under-reported outcomes of FNAIT that were detected and diagnosed at our tertiary center. Two of the three described hemorrhages were fatal. Besides these gastrointestinal and pulmonary bleedings at our center, in literature we found cases of ocular, spinal cord, renal, subgaleal and genitourinary bleeding as well.[8,23-37] In absence of population based screening, to avert denying these infants and women of adequate care, a wider scope when dealing with bleeding problems in full-term newborns is necessary. Awareness is key!

Broadening the clinical spectrum of FNAIT

Classical features of FNAIT are a thrombocytopenia with or without mild to severe bleeding complications. These features have long been the only described clinical consequences. However, more and more indicative evidence implies that this might just be the tip of the iceberg. The cornerstone of this suggested broadening of the clinical spectrum of FNAIT is the placenta. The HPA-1a epitope, which is targeted in the vast majority of (severe) FNAIT cases, is expressed on the β3 integrin. Interestingly, this β3 integrin, besides being present on platelet membranes, complexed to α2b (α2bβ3 or glycoprotein IIbIIIa) as the fibrinogen receptor, is present on syncytiotrophoblast cells as well. Here, β3 is complexed to αV (αVβ3) as the vitronectin receptor.[38,39] Syncytiotrophoblast is the outer layer of the trophoblast that is predominantly involved in invasion of the endometrium and implementation in early pregnancy. After penetration of the endometrium, the syncytiotrophoblast cells eventually reach the maternal circulation and form chorionic villi, which is the start of placental development. Fetal β3 is expressed on these cells in blastocysts and early, first-trimester placentas.[38]

This early fetal β3 exposure to the maternal circulation might explain the increased occurrence of FNAIT in first pregnancies or first-born children, when compared to HDFN. Further, once alloimmunized, the possible binding and interaction of maternal anti-HPA-1a to syncytiotrophoblast might interfere with placental function as well. Generally, placental insufficiency can lead to various clinical problems, such as oligohydramnios, (asymmetric) intrauterine growth restriction (IUGR), premature birth, pre-eclampsia, miscarriage, fetal distress and intrauterine fetal demise (IUFD).[40-42] A recent study with a murine FNAIT model showed poor placental perfusion and abnormal placental vascularization in placentae of immune mice.[43] Further, they describe a decrease in fetal weight and increase in miscarriages and fetal death. A reduced birth weight has been reported in human studies as well, Tiller *et al* reported a reduced birth weight only in boys in their prospective study and a rate of 23% small for gestational age infants in their retrospective cohort of ICHs.[11,44] We have found similar results. For *chapter 6,* we analyzed neonatal treatment of all newly detected cases of FNAIT, born between 2006 and 2017, diagnosed at the national reference laboratory and therefore a complete presentation of all cases diagnosed in the Netherlands. Within this cohort, an increased number of small for gestational age (SGA) children, boys and girls, could be identified as well (Table 10.2).

Table 10.2 – Retrospective cohort (unpublished data)

	Newly detected FNAIT (n = 102)	General population* (n = 779)
GA at birth	38^{+2} (36^{+6} – 40^{+0})	39^{+6} (38^{+5} – 40^{+4})
Premature delivery (< 37wk)	22 (22)	42 (5)
Birth weight	3020 (2491 - 3414)	3510 (3130 – 3845)
Male sex	68 (67)	396 (51)
SGA	22 (22)	88 (11)
Obstetric history		
Miscarriage**	29 (41)	196 (39)
IUFD***	4 (7)	6 (1)

Numbers are n (%) or median (IQR). CS, cesarean section; GA, gestational age; FNAIT, fetal and neonatal alloimmune thrombocytopenia; IUFD, intrauterine fetal demise; SGA, small for gestational age.

* General population, numbers from HIP-study control group.

** primigravidae excluded ($n = 70$ in newly detected FNAIT, $n = 508$ in control group).

*** nulliparae excluded ($n = 57$ in newly detected FNAIT; $n = 435$ in control group).

Also, compared to numbers reported for the general pregnant population in the Netherland, an increased number of IUFDs and miscarriages in obstetric history and cases of pre-eclampsia were detected. An increased number of premature deliveries was also reported. Naturally, besides an interesting new insight, no conclusions regarding causal relationship between anti-HPA-1a alloantibodies and these outcomes can be drawn from these data. Besides an important bias of selection in this retrospective cohort, we should also take into account that confounding might play a role. First, the reason for assessing platelet counts in these infants might be the prematurity or birthweight itself, which introduces a considerable selection bias. Second, the thrombocytopenia that is detected can be caused by the prematurity or SGA as well. Although platelet counts in these cases are not usually below 50×10^9/L.[45] In our prospective cohort, described in *chapter 9*, this increase in the number of infants that were SGA was not observed. So this difference displayed in table 10.2 might very well be due to confounding and bias in selection. However, an increased number of miscarriages and IUFD in obstetric history in both cohorts as well as a higher proportion of premature deliveries was also observed in our prospective cohort. Whether the increased number of miscarriages and IUFD was a result or the cause of the immunization remains undetermined.

To investigate the interaction and role between anti-HPA-1a and placenta, we collected placental tissue of cases of newly detected FNAIT, placentae of FNAIT cases that were treated with IVIg and control placentae of uncomplicated pregnancies. Cases were identified retrospectively through our cohort described in *chapter 6*. At the moment of writing this thesis, our group is working on examining macroscopic and histopathologic characteristics of these placentae. Also, immunohistochemical staining assays will be performed to assess IgG deposition and possible placental damage and dysfunction through complement activation via the classical pathway.

Natural history

Without knowledge on natural history of the disease it is merely impossible to determine the potential health gain from screening programs. Assessing natural history should start with a complete and representative population and then monitoring subgroups at risk.[6] For FNAIT, this would mean starting with a large number of unselected pregnant women, then determining which women are HPA-1a negative and subsequently evaluating the alloimmunization rate.

Furthermore, conclusions on natural history of FNAIT can only be adequately drawn from prospective observational (thus non-interventional) studies. Aiming to obtain knowledge on natural history, there have been several large prospective screening studies performed (Table 10.1). Non-interventional studies, however, are ethically challenging. Testing for alloimmunization and subsequently observing its natural course would mean withdrawing antenatal treatment from pregnancies that would have been treated in current practice. Therefore, merely all studies applied some kind of intervention. Either fetal blood sampling (FBS), intrauterine platelet transfusion (IUPT), near-term cesarean section (NTCS), or a directly postnatal platelet transfusion. This prevents drawing conclusions on natural history of FNAIT. In contrast, alloimmunized pregnancies detected in current practice because of a (previous) affected child, which are now antenatally treated with IVIg, are arguably different from alloimmunized pregnancies detected through screening.

The HIP-study was designed as a prospective and observational cohort study to collect these missing data on incidence and natural history (*chapter 2*). To overcome the ethical dilemma even further, we decided to postpone screening maternal sera for anti-HPA-1a antibodies until after birth. Although the study, at the time of writing this thesis, is not yet completed, we performed an interim-analysis after the first 10 months (*chapter 9*) to carefully estimate preliminary natural history of FNAIT in the Netherlands. Within the 24 detected immunized cases, one severe case of FNAIT and three cases with only minor bleeding problems were identified. These numbers are comparable to those extracted from prospective studies without antenatal intervention and only postnatal platelets available for transfusion. Williamson *et al*. detected one severe bleeding in 37 immunizations and Turner *et al.* did not identify any severe bleeding complications in 25 immunisations.[17,20] The immunization rate of 9.2% and a HPA-1a negativity rate of 2.4%, in the HIP-study, the detected 24 immunizations would represent the screening of 9,958 unselected pregnant women. Assuming a total number of pregnancies of 170,000 per year in the Netherlands, this is approximately 1/17 of the total pregnant population in the Netherlands.[46] Because of the small numbers, extrapolation should be interpreted with caution. Extrapolation would mean identifying 4,080 HPA-1a negative pregnant women each year and 375 HPA-1a immunizations. Further deducing the findings from the HIP-study would indicate 17 severe bleeding complications and 51 minor cases per year.

To reflect on whether this incidence of FNAIT might justify prenatal screening, we can take a look at implemented perinatal screening programs that focus on identifying pregnancies and newborns at risk and initiating prenatal or postnatal treatment to preventing severe complications for newborns. In the Netherlands, five of such perinatal screening programs have been instated: 1) Prenatal screening of infectious diseases and red blood cell immunization, 2) Prenatal screening for Down's, Edwards' and Patau's syndrome, 3) Structural Ultrasound Scan; and 4) Neonatal blood spot screening and 5) Neonatal hearing screening (Table 10.3). With those numbers varying from 1 – 200 identified or prevented cases each year, the incidence of FNAIT is definitely comparable to other implemented perinatal screening programs.

Table 10.3 – Incidences of identified or prevented cases per year in the Netherlands

Prenatal screening for infectious diseases and erythrocyte immunization (PSIE)	
Severe RhD mediated HDFN*	320**
Severe Rhc-mediated HDFN*	6-7[1]
Congenital HIV	5
Active syphilis during pregnancy	100
Prenatal screening for Down's, Edwards' and Patau's syndrome	
Down syndrome	195
Edwards' and Patau's syndrome	17
Structural ultrasound scan	
Spina bifida or anencephaly	55
Neonatal blood spot screening[3]	
CAH, PKU	10-20
Galactosemia, metabolic diseases (e.g. MSUD, MCD, LCAD, OCTN2, 3-MCC)	<1
Alpha/beta-thalassemia, metabolic diseases (e.g. TYR-1 VLCAD)	1-5
Sickle cell disease	17
Cystic Fibrosis	23
Neonatal hearing screening	
Double sided hearing loss	119

3-MCC, 3-Methylcrotonyl-CoA carboxylase deficiency; CAH, congenital adrenal hyperplasia; HDFN, hemolytic disease of the fetus and newborn; HIV, human immunodeficiency virus; LCAD, Long-chain hydroxyacyl-CoA dehydrogenase deficiency; MCD, multiple CoA carboxylase deficiency; MSUD, maple syrup urine disease; OCTN2, Carnitine transporter deficiency; PKU, phenylketonuria; PSIE, prenatal screening infectious diseases and erythrocyte immunization; TYR-1, Tyrosinemia type 1; VLCAD, very-long-chain acyl-CoA dehydrogenase deficiency.
*Severe HDFN defined as perinatal death, the need for intrauterine transfusion (IUT) or exchange transfusion in the first week of life.
**Severe HDFN before 1969 $n = 350$[47] – severe HDFN 2008 $n = 30$[48].

A part from the difficulty of extrapolating due to small numbers, the numbers extracted from the HIP-study are likely still an underestimation. First, the antibody screening was performed only once in every pregnancy, at 27 weeks' gestation. Alloimmunization might occur throughout the whole pregnancy and especially during delivery. Previous studies have shown that in pregnancies with negative antibody screening at 28-34 weeks' gestation, anti-HPA-1a antibodies can be detected after delivery.[17,21] Although this might lead to a possible underestimation, alloantibodies formed only during and detected after delivery could not have resulted into severe FNAIT in that pregnancy, a case that would have been missed by screening. Second, because of our timing of screening, at 27 weeks' gestation, we miss alloimmunizations that have resulted in IUFD earlier in pregnancy. Third, one of the limitations of our study is the lack of routine neonatal platelet count measurements. This way we are unable to detect cases with an asymptomatic thrombocytopenia. Nonetheless, the goal of screening is not to prevent specific laboratory values but identifying cases at risk of symptomatic disease that can be prevented. Fourth, by obtaining clinical information from obstetric care givers, we will miss cases that were asymptomatic in the first eight days of life but developed complications thereafter. Lastly, besides absence of routine laboratory evaluation our study does not include routine ultrasound examination of the neonatal brain. In conclusion, taken all these limitations into account, the results and numbers that will be extracted from the HIP-study will not be a perfect reflection of the natural history of FNAIT. Yet, they will likely be an underestimation rather than an overestimation, which is preferable in evaluating feasibility and efficacy of implementing screening.

Accepted treatment

At present, the diagnosis of FNAIT is most often made postnatally, in case of an unexplained chance finding of an isolated neonatal thrombocytopenia or in case of a newborn with an unexpected bleeding and corresponding thrombocytopenia in the first days of life. In these cases, postnatal treatment that is aimed at reducing the (risk of) bleeding by increasing platelet counts, can be administered. Commonly, treatment is applied when platelet counts drop below a certain threshold or in case of major bleeding, mostly 20×10^9/L or 30×10^9/L.[49-51] In clinical guidelines for the treatment of neonatal thrombocytopenia in FNAIT, various treatment strategies exist. In *chapter 6* we analyzed different neonatal treatment strategies applied in over one hundred cases of newly detected FNAIT. Strategies were observation/no treatment, transfusion with HPA-compatible and/or random platelets, IVIg or a combination. A favorable outcome of all included cases, regardless of applied treatment strategy, was observed. All infants reached a platelet count > 50×10^9/L within four days after birth without the occurrence of new hemorrhages.

Obviously, the choice of treatment is strongly determined by the outcome that measures the effect of the treatment as well, namely platelet count. Therefore, we should be careful with posing strong statements on effectiveness. However, with comparable platelet counts at diagnosis and clinical disease, newborns that are treated with transfusions with random-donor

platelets do seem to have similar increases in platelet counts over time, compared to those treated with compatible platelet transfusions. Additionally, receiving a first transfusion did not seem to increase the need for another transfusion in these cases, when considering a threshold of a platelet count below 20 – 30 × 10^9/L for transfusion. Therefore, one might argue that these random-donor platelet transfusions can be safely used as first line therapy when encountering an unexpected case of neonatal thrombocytopenia, suspicious for FNAIT.

In a potential future screening program, however, these cases of neonatal thrombocytopenia will not be unexpected, but anticipated. Compatible platelets for transfusion is then logistically less challenging and preferable. In line, a recently published systematic review that included 14 studies with 754 infants, concluded that although compatible transfusions are more effective in increasing platelet counts, random-donor platelets are often effective enough to achieve clinical goals.[52] An appropriate threshold for transfusion should be a platelet count of 30 – 35 × 10^9/L, according to available evidence.[21,53] Although studies on transfusion thresholds in FNAIT are lacking, we would advise to lower this threshold to 25 × 10^9/L in newly detected as well as anticipated cases of FNAIT without major bleeding, as is currently used in our national transfusion protocol.[49] First, it seems that no new hemorrhages occur after the diagnosis of FNAIT, regardless of platelet count or initiation of therapy. Second, a recent study on thrombocytopenia in premature infants shows that a lower transfusion threshold (25 × 10^9/L instead of 50 × 10^9/L) actually leads to better outcome.[54]

In contrast to the treatment of unanticipated cases, which is administered postnatally, pregnancies with known alloimmunization can be treated antenatally in order to prevent the occurrence of bleeding complications. In the vast majority of these cases the immunization is known because of a previous affected sibling. In rare cases, antenatal treatment is applied because of antenatal diagnosis of FNAIT, either due to the detection of fetal bleeding on ultrasound or due to diagnostic work-up because of a family member (a sister) with FNAIT. Antenatal treatment is aimed at prevention of bleeding complications. Due to the similarity to HDFN, which for decades is treated with intrauterine blood transfusions, the first available antenatal treatment for this indication was serial intrauterine intravascular platelet transfusions. An invasive treatment that obviously introduces an additional and substantial bleeding risk. Puncturing the umbilical cord for fetal blood sampling, determining fetal platelet count and if necessary transfusion of platelets is much more risky in fetuses with low platelet counts then in 'just' anemic fetuses.[55] Quite remarkably, now that a safe and effective non-invasive antenatal treatment using IVIg is available, there are still specialized centers performing this procedure, either as antenatal treatment itself or as pre-delivery diagnostics to determine fetal platelet counts and decide on the mode of delivery.[22,56,57] In *chapter 5* we evaluated all available evidence on antenatal management strategies. Next to three published, randomized controlled trials we also critically appraised and analyzed all published cohort studies. This systematic review

did clearly show that non-invasive treatment with weekly IVIg infusions was as effective in preventing severe bleeding complication, as the invasive treatment using fetal blood sampling (FBS) with or without intrauterine platelet transfusion (IUPT). In 11% of pregnancies that were treated with FBS/IUPT complications occurred, of which a quarter resulted in fetal or neonatal death. More than half of the reported perinatal mortalities were associated, and likely caused by the invasive treatment with FBS/IUPT.

Non-invasive treatment consists of different dosages of IVIg with or without the addition of corticosteroids. No clear benefit of adding corticosteroids has been shown. The only study that did report a significant improvement in platelet counts when adding steroids used a somewhat questionable, not predefined, outcome measure for this effect.[58,59] With regards to both start and dose of IVIg therapy, pregnancies can be stratified into a high-risk and a standard-risk group, based on whether or not a sibling had an ICH.[60,61] The most commonly used dose is 1 g/kg maternal weight/week, and is adapted from treatment in immune thrombocytopenic purpura (ITP). However, our group has shown that for standard-risk pregnancies a lower dose of 0.5 g/kg/week, was not inferior to 1g/kg/week. Therefore, we advise to treat standard-risk pregnancies with 0.5 g/kg/week and high-risk pregnancies with 1g/kg/week. Because many ICHs occur before 30 weeks of pregnancy, therapy should start before.[11,62] Given the 79% recurrence rate of ICH we would advise in high-risk pregnancies to start treatment earlier in pregnancy, at 16 weeks' gestation, and in standard-risk pregnancies at 20-24 weeks' gestation.[7]

Although antenatal weekly IVIg treatment is considered to be the safest and most effective management for pregnancies at risk for bleeding complications, this has only been investigated in subsequent pregnancies. No data are available about the treatment of pregnancies at risk identified through screening. Therefore, whether weekly IVIg treatment is effective in a screening setting and can prevent the occurrence of bleeding complications in first cases as will remain unanswered until screening will actually be implemented.

A novel drug to be used in the treatment of fetal and neonatal alloimmune disease is M281, an anti-FcRn antibody. Blockade of FcRn and therefore preventing the interaction of IgG with FcRn, might inhibit fetal exposure to pathogenic IgG by preventing or minimizing transplacental transfer of pathogenic IgG as well as inhibiting IgG recycling.[63] The phase I human clinical study illustrated that M281 was safe and well tolerated in healthy human volunteers.[64] A clinical phase II studies in early onset severe HDFN is currently being set up (NCT03842189). If that study is successful, a study in FNAIT would be one of the logical next steps to consider.

Suitable test

In outlining population-based screening for FNAIT, one of the most challenging factors, financial as well as logistical, is identifying the 2% HPA-1a negative pregnant women. Because the number of women in need of follow-up testing decreases 50-fold after this, the first line of screening is strongly contributing to the total costs, and is therefore very important for the cost-effectiveness of a potential screening program.[65,66] In terms of costs, serological testing has benefits over genotyping. Different serological HPA-1a typing assays have been used in performed prospective screening studies, flow-cytometry and enzyme-linked immunosorbent assay (ELISA).[17,20,21] For the applicability in population-based screening the assay needs to be quick and suitable for high-throughput testing. Both serological assays, depending on the material used, are quick. They were both previously validated, but unfortunately only in a small number samples and merely in random donors instead of pregnant women. The ELISA used in Scottish screening study was reported to cost £3.01 per sample.[20] The largest screening study to date, performed in Norway, used both ELISA and flow-cytometry and reported €1.72 per sample for flow cytometry and €21.28 for genotyping.[21,65] Both assays are quick, but flow-cytometry needs considerable more analyzing time at a rather expensive flow-cytometer, which increases costs, therefore we used an ELISA assay for HPA-1a typing in our prospective study. Aiming to further reduce costs and time, to increase validation in pregnant samples and to increase applicability in future screening, the previously described ELISA was optimized and described in *chapter 7*. We used material of moderate quality, 3-6 days-old tubes without spinning or swirling them, to increase the applicability for high-throughput screening. Assay costs, excluding labor, were only €0.25 per sample. The goal of screening, obviously, is to identify all HPA-1a negative women, so the test needs to reach a 100% sensitivity (i.e. a zero false-HPA-1a positive rate). Achieving this comes at the cost of an increased the rate cases falsely identified as HPA-1a negative to 0.03. For future population-based screening, one might consider skipping the costly step of genotyping and perform follow-up antibody screening of all serologically HPA-1a negative cases.

Acceptability of screening for FNAIT

The success and effectiveness of any screening program will be determined by the uptake and willingness to participate to the program. Increasing consumerism in our current health care system leads to an important role of patient's attitude towards the screening in determining this participation rate. Thus, when evaluating the sixth W&J criterion, the acceptance of pregnant women to potential future prenatal screening for FNAIT, we need to assess women's attitude towards such a screening program. Obviously, we prefer women to take the decision to participate in a screening program to be based on knowledge and understanding of the disease, the test and the consequences of (a positive) outcome of testing. Informed decisions are determined by adequate knowledge and should be in concordance with their attitude towards the screening program. These three factors (uptake, knowledge and attitude), leading to informed choice, can be measured by using the validated Multidimensional Measurement

of Informed Choice (MMIC) model that is developed and described by Marteau and colleagues (Figure 10.2).[68] In *chapter 8* we report the first study that assessed women's attitude towards potential population-based screening for the prevention of morbidity and mortality caused by FNAIT. Overall, the attitude of women towards potential future HPA-screening in order to prevent FNAIT was very positive and the willingness to participate in a future screening was almost 99%. The (small) groups that were less likely to express a positive attitude were highly educated women and women that intended to deliver at home. Without any difference in knowledge or uptake, this led to a lower rate of value-consistent decisions in these groups. Within our participants, we had an underrepresentation of non-European women and a slight overrepresentation of highly educated women (60% compared to 48% reported for the general population of 25-45-year-old Dutch women). The first group had a higher rate of uninformed decisions. The latter had no significant differences in knowledge or attitudes scores. Based on these findings we would expect a high rate of informed uptake when implementing HPA-screening in pregnancy. Further, our data confirm that it is important to adapt the provision of information to women's ethnicity and to pay attention to its potential to cause anxiety and uncertainty.

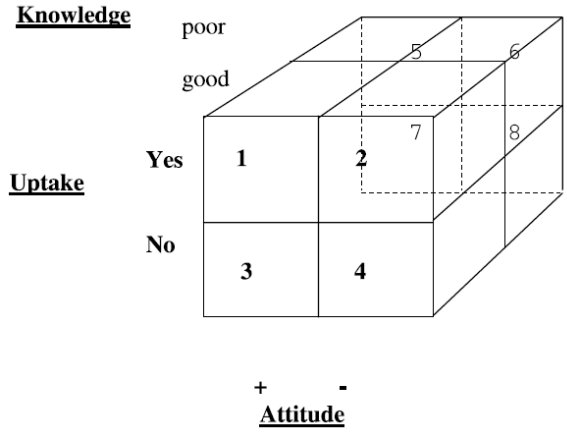

Figure 10.2 – Multidimensional Measurement of Informed Choice[67]

Box 1 and 4 represent informed choices. Box 3 and 2 represent value inconsistent choices, despite sufficient knowledge.
Box 5, 6, 7 and 8 represent uninformed choices based on insufficient knowledge.

Risk assessment

The greatest challenge still ahead is the identification of those alloimmunized pregnancies that are truly at high risk of developing fetal bleeding complications. The eighth W&J criterion demands an agreed policy who to treat as patients. In current practice, all known platelet alloimmunized pregnancies are antenatally treated. If this policy would be applied to a screening

program, there would be a considerable overtreatment whereas it is estimated that only around 5% of alloimmunized pregnant women will have a fetus or neonate with a significant bleeding complication (Table 10.2, *chapter 9*).[14,17,21] Therefore, there is a need of non-invasive tests to better predict fetal risk and outcome. Ideally, we would like to have maternal serum markers that reliably determine the biological effect of the alloantibodies and thereby predict disease severity. For this purpose, a couple of markers and assays have been suggested.

Antibody level - In some centers, antibody levels are monitored by titration and quantification. While high titers are significantly correlated with lower platelet counts and more severe disease, this does not seem to be a consistent relationship.[69] There are cases of severe hemorrhages with barely detectable antibody levels.[70-72] When trying to predict disease severity, antibody titer might be a useful contributor but not a reliable measurement to solely guide follow-up testing and management.

*HLA-DRB3*0101* – The majority of HPA-1a incompatible pregnancies, in which alloimmunization will occur, HLA-DRB3*0101 positive.[21,73-75] Only 0.5% of the HLA-DRB3*0101 negative incompatible pregnancies will result in the formation of anti-HPA-1a antibodies.[76] Recently, the presence of 1 or 2 HLA-DRB*0101 alleles was suggested to be significantly correlated to anti-HPA-1a levels in alloimmunized samples from a prospective study.[77] These authors reported this dose of the HLA-DRB*0101 allele to be significantly correlated to neonatal platelet counts as well. Whether this effect was through anti-HPA-1a levels and whether this has clinical implications independently of anti-HPA-1a levels, is unclear.

Fc-glycosylation of anti-HPA-1a antibodies – Immunoglobulin G (IgG) antibodies can vary in glycosylation pattern of the Fc-part, which influences the affinity to Fc-receptors and thus antibody effector activity.[78] This pattern is determined by the composition of a glycan attached to the Asn297 residue on the Fc-part and can vary in amount of, for example, galactose and core fucose. A lower level of this core fucose has been demonstrated to result in stronger binding to the Fc-receptors (FcgRIIIa and FcgRIIIb),[79] In archived samples of 48 cases of anti-HPA-1a mediated FNAIT, low levels of fucosylation on anti-HPA-1a specific antibodies led to an enhanced phagocytosis of platelets.[80] The same significant decrease in fucosylation was observed by Sonneveld *et al*.[81] in a cohort of 80 anti-HPA-1a immunized women, showing a significant correlation with bleeding severity. Unfortunately, despite the large numbers of samples in both studies, the lack of prospectively collected samples and the lack of an optimal control group of immunized cases without disease, hamper the current use of these markers for disease prediction.

Endothelial damage - As discussed previously in *chapter 1*, binding and interaction with endothelial cells have been proposed to be correlated to the occurrence of ICH. *In vitro* studies have illuminated the direct interaction between anti-HPA-1a and human umbilical vein endothelial cells (HUVECs),

demonstrated by a decreased HUVEC spreading as well as a decreased integrity of their monolayer in electric cell-substrate impedance sensing (ECIS) assays.[82] In addition, *in vivo* murine studies showed that mice without circulating platelets and or fibrinogen do not show any bleeding problems in utero. This supports the hypothesis that, instead of just the thrombocytopenia, another mechanism might play a key role in causing bleeding complications. Recently, a large study with both active and passive murine models of anti-HPA-1a mediated FNAIT and showed that ICHs in these mice occurred regardless of platelet count.[43] Also, HPA-1a antibodies inhibited angiogenic signaling, induced endothelial cell apoptosis and decreased vessel density in affected brains as well as retinas. The first analysis with a small number of human sera containing HPA-1a antibodies suggested that three subtypes of anti-HPA-1a alloantibodies exist.[83] The first type, that only interacts with HPA-1a on β3 when in complex with α2b, predominantly on platelets. A second type, interacting with HPA-1a on β3 regardless of the complex, so with platelets and endothelial cells as well. And a third type, that binds specific to HPA-1a on β3, when in complex with αV. This type of anti-HPA-1A alloantibodies might be αVβ3-specific, might therefore be responsible for and possibly predict the occurrence of ICH.[83]

To evaluate this interaction and its clinical consequence even further we selected a series of 55 serum samples of anti-HPA-1a mediated FNAIT cases, 5 samples of anti-HPA-5b mediated FNAIT and 13 negative controls of HPA-1a positive pregnant women without HLA antibodies. Our aim was to demonstrate binding of the anti-HPA-1a from the maternal sera to HUVECs as well as a functional effect on endothelial cells, decreased angiogenesis. For both assays we used purified IgG fractions from the sera.

Flow-cytometry showed increased binding to HUVECs in the ICH cases (Figure 10.3). Binding of anti-HPA-1a cases without ICH, anti-HPA-5b mediated cases and negative controls were comparable. Although the differences in median between the groups were significant, the ranges in delta MFI within the groups were quite large. Still, cases with ICH did not show any binding in this assay, and vice versa. Also, there is a clear increase in binding when comparing the cases with and without anti-HLA Class-I alloantibodies. To eliminate this effect of anti-HLA on HUVEC and therefore overestimation of the binding of anti-HPA-1a in the sera to HUVEC, we plan to repeat these experiments with an immortalized HUVECs that does not express HLA Class I, through a HLA-ABC heavy chain knock-out. Alternatives might be the use of beads that express either αVβ3 or α2bβ3 or to use HEK-cells that express either αVβ3 or α2bβ3. This is currently work in progress.

Further, we used the same IgG fractions from our retrospectively collected FNAIT sera to evaluate functional effect of this binding of anti-HPA-1a to endothelial cells. Sprouting angiogenesis is fundamental for the expansion of blood vessels during embryonic development.[84] To mimic sprouting angiogenesis *in vitro* we performed a 3D bead-based sprouting assay (in collaboration with Coert Margadant) based on previous protocols with some modifications.[85,86] At the time of writing this thesis, the first ten samples have been imaged, but not yet analyzed (Figure 10.4).

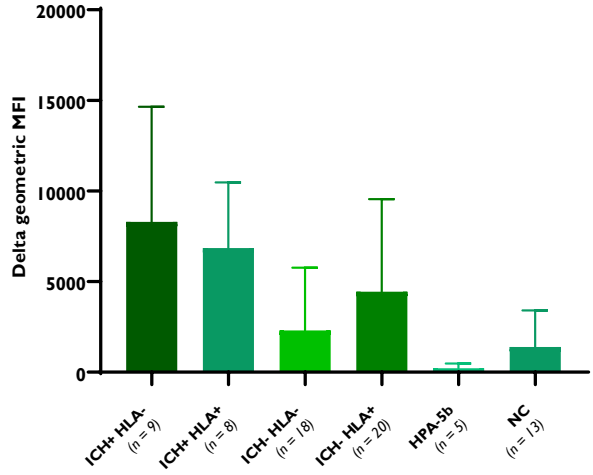

Figure 10.3 – Flow-cytometry assay: purified IgG from FNAIT sera and binding to HUVECs

HLA, human leukocyte antibody; HPA, human platelet antigen; HUVEC, human umbilical vein endothelial cell; ICH, intracranial hemorrhage; MFI, mean fluorescence index; NC, negative control.

Although it would be highly preferable, the chance of detecting that one marker that is perfectly correlated with disease severity and can be used for selecting pregnancies at high risk for adverse perinatal outcome is probably small. More likely, a set of the above-mentioned markers and/or assays is able to select the high-risk population that benefits from antenatal preventive treatment without the hazard of enormous overtreatment. An important pitfall with these identified markers is that they have never been tested on large series of samples and, even more important, they have never been compared to an adequate control group. For optimal identification of high-risk pregnancies, it is very important to compare immunized pregnancies with disease to immunized pregnancies without disease. The absence of this group in all previously described studies assessing potential predictors for disease severity hampered the identification of reliable and clinically applicable predictors. The HIP-study is collecting a large number of these cases, to be used for an adequate and specific risk assessment model. At this time it is uncertain if we can include the desired number of cases. Substituting this group with severe cases of ICH that were retrospectively identified, is complicated by the different time points at sampling (after birth in the retrospective cases and at 27 weeks' gestation in the prospective cases). Overall, whilst collecting a great and unique control group for the development of a specific risk-assessment model, still the hurdle of determining and optimizing sensitivity of such a model needs to be overcome.

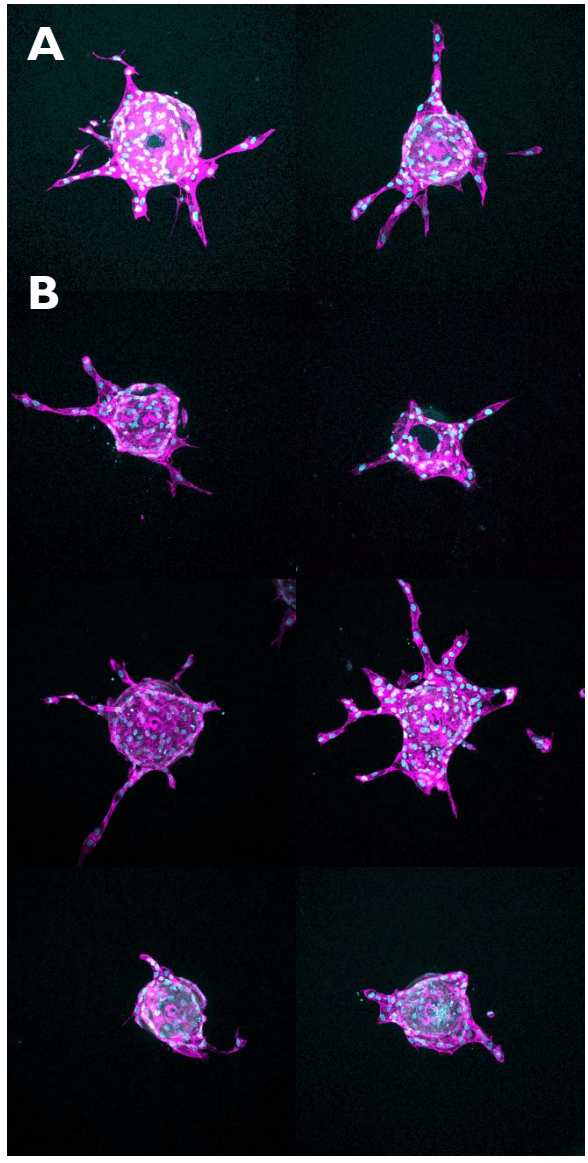

Figure 10.4 – Confocal images of sprouting assay

A. Two examples of beads without the added IgG fractions from FNAIT sera, positive control. **B.** Six examples of beads with the added IgG fractions from FNAIT sera.

Screening scenario

Timing of screening. The first decision to be made is whether screening should take place during pregnancy or postnatally. Killie and colleagues analyzed anti-HPA-1a immunizations and antibody levels from the Norwegian screening study and concluded that primary immunization occurs mainly during or after delivery and that FNAIT is very unlikely to occur in a first-time pregnancy.[87,88] In contrast, retrospective data show a high proportion of primigravidae or first-born children suffering from severe FNAIT. So, there might be a difference between the moment of immunization in prospectively identified cases, of which the majority does not lead to disease, and the immunizations that do result in severe FNAIT. The goal of screening would be to prevent the latter. So, when designing a screening program, it would make sense to do so during pregnancy. Additionally, since severe cases of ICH can already occur in second trimester, we would suggest starting screening early in pregnancy. To optimize logistics, it could be easily added to the already implemented screening program for red blood cell immunization in the 12th week of gestation (Figure 10.5). First, from the complete pregnant population the group of HPA-1a negative women will need to be identified. In case of suggested HPA-1a negativity, it might be cost-saving to skip supportive genotyping, as performed in the HIP-study, and perform anti-HPA-1a antibody screening in all cases. The optimal timing of antibody screening needs to be determined. Logistically easiest, and similar to the red blood cell immunization screening, this could be done at 12 weeks' gestation, possibly within the same sample. This could be useful in all pregnancies but the first one: in a first pregnancy, antibodies may not yet be detectable. A (second) antibody screening between 18 and 20 weeks may be more useful. Not later, because bleeding may have already occurred.[11] An option for easy logistics would be to combine this with the 20 week-anomaly scan, which has an uptake of around 99%.[49]

Non-immunized HPA-1a negative women. In case of negative antibody screening, adequate follow-up of HPA-1a negative women would be advised, at least a repeated antibody screening around 28-30 weeks and 6 weeks postpartum. These women might be the target population that would qualify for potential prophylaxis (NAITgam, developed by PROFNAIT project, described in *chapter 1*).[89]

Immunized HPA-1a negative women. When anti-HPA-1a is detected, ideally a combination of risk-assessment markers should separate a low-risk from a high-risk group. IVIg treatment can then be given only to the remaining small high-risk group (estimated 50-100 women per year). Whereas the low-risk group still consists of HPA-1a alloimmunized pregnancies and no prospective screening and follow-up data on this groups exists yet, careful monitoring, at least for the first period after implementation of population-based screening, seems justified.

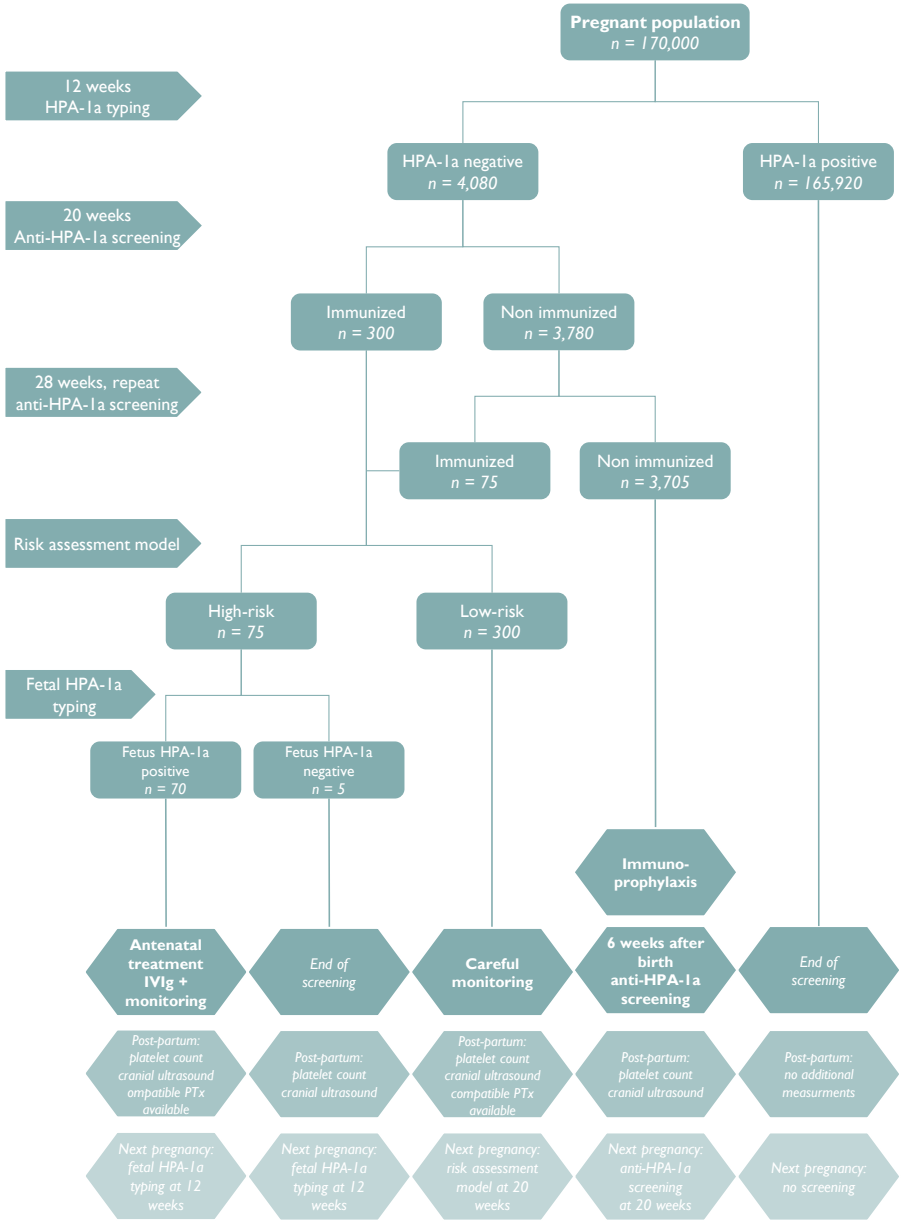

Figure 10.5 – Flow-chart of hypothetical screening scenario

With number of estimated cases per year in the Netherlands, based on a birth rate of 170,000[46].

Further, it might be considered to perform non-invasive fetal HPA-1a typing in either all identified HPA-1a negative pregnant women, to refrain from follow-up testing, or in (high-risk) immunized HPA-1a negative to refrain from unnecessary treatment if the fetus turns out to be HPA-1a negative as well.

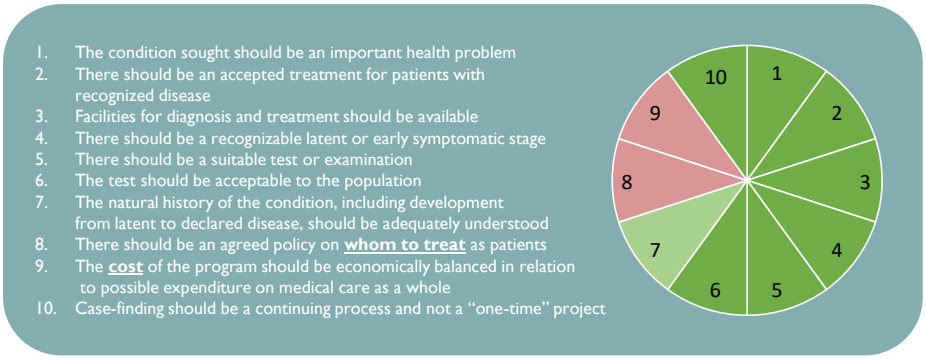

Figure 10.6 – Principles of early disease detection, Wilson & Jungner criteria[5]

Dark green, criterion fulfilled; light green, some extra information needed for fulfillment; light red, little information available but insufficient for fulfillment; dark red, no information available.

In conclusion - Evidence based screening

With this thesis, we have gathered a large amount of information for fulfillment of the W&J criteria (Figure 10.6). Furthermore, we describe the design and interim results of a promising prospective non-interventional study that is the base for collecting the remaining missing knowledge and for enabling a verdict on the feasibility and efficacy of potential population-based screening to prevent FNAIT. Thus far, we have established that FNAIT is an important health problem, for which acceptable and affordable diagnostic and treatment options are available. Further, we have developed a suitable test, we have reported on the acceptability of a potential screening program and presented the first preliminary results on natural history of the disease. These can be further confirmed when our prospective study (HIP-study) is completed in 2020. Still important knowledge, regarding the 8th and 9th criteria of W&J is missing (Figure 10.6). For this, we will need the prospectively collected samples of the HIP-study. Our unique, prospectively collected control group of immunized pregnancies without clinical disease will enable the development of a risk-assessment model to determine whom to treat as patients. Consequently, when we have more details of this policy, overall costs of the program can be calculated. We do expect that a national screening rand prevention program for FNAIT will be feasible and cost-effective, and that implementation in the coming years will lead to a significant reduction in fetal and neonatal bleeding complications due to this disease.

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Chapter 11

Summary
Nederlandse samenvatting

Summary

Fetal and neonatal alloimmune thrombocytopenia – evidence based screening

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is the most common cause of thrombocytopenia in otherwise healthy newborns. During pregnancy, fetal blood cells enter the maternal circulation and the mother will be exposed to unknown, paternally derived, antigens. This exposure might result in alloimmunization, the maternal production of antigen-specific alloantibodies. Through active transport across the placenta, these alloantibodies enter the fetal circulation, where they can cause damage. In FNAIT, these alloantibodies are targeted against human platelet antigens (HPAs) that are present on fetal platelets. Alloantibodies against HPA-1a are the most commonly involved in (severe cases of) FNAIT. Clinical presentation can vary from an asymptomatic thrombocytopenia or relatively harmless bruises and petechiae to severe life-threatening and invalidating intracranial hemorrhages (ICHs). Once alloimmunization is detected and diagnosed, subsequent pregnancies can be treated to prevent the recurrence of bleeding complications. Unfortunately, in absence of population-based screening, alloimmunization is virtually only known after an affected fetus or newborn. Affected infants that might have been prevented if only the alloimmunization was known and treated prior to the occurrence of bleeding complications.

1. **The condition sought should be an important health problem**
2. **There should be an accepted treatment for patients with recognized disease**
3. **Facilities for diagnosis and treatment should be available**
4. **There should be a recognizable latent or early symptomatic stage**
5. **There should be a suitable test or examination**
6. **The test should be acceptable to the population**
7. **The natural history of the condition, including development from latent to declared disease, should be adequately understood**
8. **There should be an agreed policy on whom to treat as patients**
9. **The cost of the case-finding program (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole**
10. **Case-finding should be a continuing process and not a "one-time" project**

Figure 11.1 – Principles of early disease detection – Wilson and Jungner screening criteria

Implementation of population-based screening in order to prevent FNAIT needs to be a carefully weighed decision. The benefits of screening need to outweigh the potential harm. To guide careful consideration and decision-making, Wilson and Jungner (W&J) proposed and published ten screening criteria that were adopted by the World Health Organization (Figure 11.1). For FNAIT, some of these criteria are already clearly fulfilled. For example, general

consensus exists on the fact that in current health care facilities for diagnosis and treatment are available and that these facilities are able to detect alloimmunization in pregnancy, before the occurrence of bleeding symptoms, a latent stage of FNAIT. Further, in present health care, a potential population-based screening program would definitely be implemented as a continuing process, until new insights prove otherwise. The remaining seven criteria are in need of additional evidence for fulfillment. This thesis aimed to contribute to this fulfillment and can hopefully enable future decision-making regarding implementation of population-based screening.

Within this consideration of implementation, knowledge on incidence and natural history of FNAIT is indispensable. Whereas anti-HPA-1a is the most commonly involved antibody in FNAIT, screening studies and literature focus primarily on detecting HPA-1a-mediated FNAIT. From prospective screening studies, we know that approximately 2% of the Caucasian population is HPA-1a negative and therefore at risk for alloimmunization. Further, we know that circa 10% of HPA-1a negative pregnant women will produce anti-HPA-1a alloantibodies. Because most of these studies applied a kind of intervention, adequate estimation of the incidence of clinically relevant FNAIT is nearly impossible. It is clear, however, that this only a small proportion of the alloimmunizations. This means that offering preventive treatment for all alloimmunizations will be considerable overtreatment and stipulates that there is need for (a set of) predictive marker(s) that can identify which alloimmunizations will lead to disease. To gather information on both 'natural history' and a 'policy whom to treat', we initiated a nation-wide, prospective, non-interventional cohort study. The design and set-up of this study, called HPA-screening In Pregnancy (HIP-study) are described in *chapter 2*.

In short, for the HIP-study a large group of pregnant women will be serologically screened for HPA-1a on their platelets. Plasma of HPA-1a negative women will be stored, and for every HPA-1a negative case, an HPA-1a positive control will be stored as well. Clinical data on medical history, obstetric history, pregnancy, delivery and neonatal health in the first week of life will be collected of all women of whom samples were stored. An HPA-1a antibody screening will be performed in stored plasma samples from HPA-1a negative women. The complete non-interventional set-up of the study enables an adequate estimation on natural history and incidence of the disease. Further, this approach facilitates the collection of an unique control group, namely alloimmunized pregnancies without clinical disease. Comparing these alloimmunized cases without disease to alloimmunized cases with disease will enable the detection of diagnostic markers that could predict which alloimmunized pregnancies are at high risk of bleeding and would benefit from treatment. At the same time, this would identify alloimmunized pregnancies with a low risk of bleeding, for which we can safely refrain from antenatal treatment.

The disease that screening is implemented for, needs to be an important health problem. This can be either due to its high incidence, or a result of its great clinical impact. In *chapter 3 and 4* we studied the disease burden of FNAIT. Clinically relevant FNAIT is characterized by an increased bleeding tendency, of which the most feared bleeding complication is an ICH. Short-term outcome of these bleedings is highly unfavorable, due to its high risk of perinatal mortality. Long-term outcome is suggested to be unfavorable as well, although no detailed and structured follow-up studies of children suffering from ICH solely due to FNAIT have been described. This is information that is indispensable in the counseling of parents. In *chapter 3*, we have performed an observational cohort study, assessing both short- and long-term outcome of 21 children that suffered an ICH due to FNAIT. We report a high mortality rate (48%) and the suggested poor neurodevelopmental outcome was confirmed as well. Six out of ten surviving children suffered severe neurodevelopmental impairment (NDI), and one child was moderately impaired. All impaired children had a cerebral palsy, a severe cognitive and severe motor delay. In four children there was visual impairment and/or epilepsy as well.

Besides ICH, a hemorrhage caused by FNAIT can occur in every kind of organ. In *chapter 4* the results of a retrospective chart analysis and literature review of cases of FNAIT that presented with severe bleeding other than ICH, are presented. In addition to the pulmonary hemorrhages and gastrointestinal bleeding treated at our center, we found reports of renal, ocular, spinal cord, subgaleal and genitourinary bleedings caused by FNAIT. With this chapter we indicate that although these bleedings are less likely to be associated with FNAIT, these hemorrhages can have severe consequences if not adequately treated. Every thrombocytopenic, otherwise healthy, newborn with an unexpected severe bleeding should be suspected for FNAIT and diagnostic work-up should be easily accessible and performed. That way, the affected infant can be treated adequately, but even more importantly, it enables appropriate follow-up for future pregnancies.

Of the W&J criteria, the WHO stated that perhaps the most important criterion to fulfill is that there needs to be accepted treatment for the disease. We have analyzed both the optimal antenatal and postnatal treatment in *chapters 5 and 6*. In *Chapter 5*, we present a systematic review that analyzed four randomized studies and 22 cohort studies. Antenatal treatment for FNAIT can be divided into invasive and non-invasive treatment. Invasive treatment consists of fetal blood sampling (FBS) with or without an additional intrauterine platelet transfusion (IUPT). Our review confirms that this is a risky procedure; in 11% of pregnancies treated with one or more FBS/IUPTs a complication occurred, of which one in four resulted in perinatal death. Strikingly, of all described fatalities, more than half were related to the procedure itself. Non-invasive treatment consisted of IVIg (mainly at a dose of 1.0 mg / kg maternal body weight / week) with or without corticosteroids. We found no data to support adding corticosteroids to IVIg treatment or the reducing or increasing of IVIg dose (0.5 or 2.0mg/kg/wk). We therefore

advise that antenatal treatment should consist of weekly maternal IVIg infusions at 1.0 mg/kg/wk. Treatment should be started between 20 and 24 weeks' gestation. Except for pregnancies that are at high risk because a sibling suffered an ICH. Here, treatment should start between 12 and 20 weeks' gestation.

In the cohort study, described in *chapter 6*, we analyzed postnatal management strategies and outcomes. We first concluded that, despite national guidelines, many different treatment strategies were applied; no treatment, platelet transfusion (PTx) with compatible or random-donor platelets or both, and IVIg or without PTx. Second, we concluded that in all strategies, a safe platelet count was reached within four days after birth without the occurrence of new hemorrhages. The highest and fastest increment was observed after HPA-compatible PTx and the smallest with IVIg. Treatment with random-donor PTx was not associated with a higher use of additional transfusions, which suggests that if HPA-compatible platelets are not directly available, transfusion with random-donor platelets may be a more appropriate first line therapy in FNAIT.

In *chapter 7*, we describe an enzyme-linked immunosorbent assay (ELISA) that can be used as a suitable test for screening. Whereas, in terms of screening, we focus on FNAIT that is caused by HPA-1a, the first goal is to identify all HPA-1a negative women. Considering that this is approximately 2% of the pregnant population, follow-up testing will only be necessary for 1 in 50 women. Therefore, the HPA-1a typing assay will be a major contributor to the logistical feasibility and cost-effectiveness of the program. In this chapter, we describe the low-cost ELISA that we have designed. The assay was optimized to require no additional handling (swirling or spinning) of stored tubes, which makes it applicable for high-throughput and reduces labor costs. The goal of screening is to identify all HPA-1a negative cases. Our assay reached this 100% sensitivity with still a very high specificity of 99.9% and only a false-HPA-1a negative rate of 0.03 (e.g. 3/100 samples identified as phenotypically HPA-1a negative will have a HPA-1a positive genotype).

Increasingly important in current health care, that is characterized by increased consumerism and individualism, is the acceptability of the screening to the population. Therefore, we performed a cross-sectional questionnaire study among healthy pregnant women that was aimed at assessing women's attitude towards potential future HPA-screening. For this purpose the validated Multidimensional measurement of informed choice model was used, that besides attitude, also measures knowledge and intention to participate. In *chapter 8* we demonstrate that this attitude was very positive, as expressed by 91% of participants, which was based on sufficient knowledge in 94%. Overall, 87% of the choices to intend to participate in the study were informed choices. We have shown that less informed choices were made in non-European women, stipulating the fact that it is important to adapt future counseling to women's ethnicity.

Results of the completed HIP-study, of which the set-up and protocol are described in *chapter 2*, are to be awaited. However, we did perform an interim-analysis after 10-months, which is described in *chapter 9*. During this period 40,945 pregnant women were serologically typed for HPA-1a, of which 986 women (2.4%) were HPA-1a negative. Of the HPA-1a negative cases, 263 (27%) gave informed consent for further testing and storage of material. Within these samples, 24 anti-HPA-1a immunizations (9.2%) were detected, leading to 4 cases of clinically relevant FNAIT. One case of severe FNAIT with a large ICH detected on ultrasound at 29 weeks' gestation that eventually led to a late termination of pregnancy at 34 weeks' gestation. The other three cases expressed minor bleeding symptoms; one cephalic hematoma and two cases with widespread hematomas or petechiae. So far, these numbers are in line with expectations and do not seem to be a potential hitch for the implementation of population-based screening.

With this thesis, important evidence is presented that can be used for the fulfillment of the W&J criteria. A discussion and interpretation of these results is provided in *chapter 10*. Additionally, the potential interaction of anti-HPA-1a antibodies with HPA-1a on integrin αVb3, expressed on placental tissue and endothelial cells, is discussed in this chapter. This interaction might lead to a broadening of the clinical spectrum of FNAIT and might lead to the identification of a new predictor for alloimmunized cases at high risk for bleeding. Further, we propose a potential scenario and address the remaining unanswered questions in the debate towards implementing routine HPA-screening in pregnancy in order to prevent clinically relevant FNAIT.

Nederlandse samenvatting

Foetale en neonatale alloimmuun trombocytopenie – evidence based screening

Foetale en neonatale alloimmuun trombocytopenie (FNAIT) is de belangrijkste oorzaak van neonatale trombocytopenie in verder gezonde, a-term geboren kinderen. Tijdens de zwangerschap komen foetale bloedcellen terecht in de maternale circulatie en wordt de moeder blootgesteld aan onbekende, van de vader afkomstige, antigenen. Deze blootstelling kan leiden tot een immuunreactie bij de moeder en de productie van alloantistoffen tegen deze foetale antigenen. Alloantistoffen worden tijdens de zwangerschap actief getransporteerd over de placenta en eenmaal in de foetale circulatie kunnen ze leiden tot schade. Bij FNAIT zijn deze antistoffen gericht tegen antigenen op de bloedplaatjes, ook wel humaan plaatjes antigen (HPA) genoemd. Hiervan zijn antistoffen gericht tegen HPA-1a het vaakst betrokken bij (ernstige) FNAIT. Het klinisch beeld bij FNAIT wordt daarom gekenmerkt door een tekort aan bloedplaatjes, een trombocytopenie. Deze trombocytopenie kan de enige uiting van FNAIT zijn en noemen we dan asymptomatisch, maar kan ook gepaard gaan met bloedingsproblemen, variërend van relatief onschuldige blauwe plekken en petechiën tot zeer ernstige en levensbedreigende hersenbloedingen. Wanneer bekend is dat een vrouw geïmmuniseerd is en HPA-alloantistoffen heeft gemaakt, kan zij in de volgende zwangerschap behandeld worden om ervoor te zorgen dat deze antistoffen niet meer tot bloedingsproblemen bij het kind leiden. Omdat er geen prenatale screening naar deze antistoffen bestaat, weten we doorgaans pas dat er sprake is van alloimmunisatie wanneer een vrouw al eerder een keer zwanger is geweest en bevallen is van een kindje met bloedingsproblemen. Bloedingsproblemen die theoretisch gezien voorkomen hadden kunnen worden, als we eerder hadden geweten dat de vrouw antistoffen had gemaakt en dus hadden kunnen starten met een preventieve behandeling.

1. **De ziekte moet een belangrijk gezondheidsprobleem representeren**
2. **Er moet een geaccepteerde behandeling voor geïdentificeerde ziekte zijn**
3. **Er moet beschikbare faciliteiten voor diagnose en behandeling zijn**
4. **Er moet een herkenbaar latent of vroeg-symptomatisch stadium zijn**
5. **Er moet een geschikte screeningstest zijn**
6. **De screening moet acceptabel zijn voor de populatie**
7. **Het natuurlijk beloop moet bekend zijn**
8. **Er moet een beleid zijn over wie te behandelen als patiënten**
9. **De kosten van de screening (inclusief behandeling) moeten in balans zijn met de mogelijke kosten die het bespaard**
10. **De screening moet een continue proces zijn en geen eenmalig project**

Figuur 11.2 – Principes voor ziekte opsporing – Wilson en Jungner criteria

Voordat een eventueel nieuw screeningsprogramma geïmplementeerd kan worden, moet deze aan belangrijke voorwaarden voldoen en moet er beoordeeld worden of de voordelen van een dergelijk programma opwegen tegen de nadelen. Om deze beoordeling te vergemakkelijken heeft de wereld gezondheidsorganisatie tien criteria hiervoor gepubliceerd, beschreven door James Wilson en Gunner Jungner (W&J criteria, Figuur 11.1I). Aan een aantal van deze criteria wordt al duidelijk voldaan. Zo zijn er adequate faciliteiten beschikbaar voor diagnose en behandeling van FNAIT en ook voor het detecteren van alloimmunisatie, een latent stadium voordat er symptomen optreden. Verder zal er geen twijfel zijn over het doel van implementatie, dit zal uiteraard zijn als continue proces een niet als eenmalige actie (criterium 10, Figuur 11.11). Voor de overige zeven criteria is voor een deel nog aanvullende informatie nodig. Het doel van dit proefschrift is het verzamelen van de informatie om de beoordeling over het nut en de effectiviteit van HPA-screening ter preventie van FNAIT mogelijk te maken.

Erg belangrijk en onmisbaar bij deze beoordeling is kennis over de incidentie en het natuurlijk beloop van de ziekte. Omdat HPA-1a verreweg het vaakste leidt tot FNAIT wordt in studies en literatuur over potentiele screening gefocust op FNAIT die wordt veroorzaakt door antistoffen tegen HPA-1a. Uit een aantal van deze prospectieve studies weten we dat circa 2% van de Kaukasische populatie HPA-1a negatief is, en dus anti-HPA-1a antistoffen zou kunnen produceren. Uit dezelfde studies weten we dat ongeveer 10% dit ook daadwerkelijk doet. Omdat deze studies vervolgens enige vorm van interventie hebben uitgevoerd in de geïmmuniseerde zwangerschappen is het nagenoeg onmogelijk hieruit te concluderen hoeveel geïmmuniseerde zwangerschappen vervolgens tot klinische ziekte leiden; met andere woorden, het natuurlijk beloop van FNAIT. Wel is duidelijk dat dit slechts een klein deel is. Dit betekent dat behandeling van alle geïmmuniseerde zwangerschappen leidt tot een aanzienlijke overbehandeling en dat het belangrijk is om een risico-inschatting te kunnen maken met betrekking tot welke geïmmuniseerde zwangerschappen tot ziekte leiden. Om informatie over het natuurlijk beloop en deze risico-inschatting te verzamelen hebben we een grote, landelijke, prospectieve en non-interventie studie opgezet. Deze studie, genaamd de HIP-studie (HPA-screening In Pregnancy), wordt beschreven in *hoofdstuk 2*.

In het kort zal voor de HIP-studie bij een grote groep zwangere vrouwen de aanwezigheid van HPA-1a op hun trombocyten getest worden. Bloedplasma van de groep HPA-1a negatieve vrouwen zal worden bewaard, en voor elk HPA-1a negatieve sample zal ook een HPA-1a positieve controle worden geïncludeerd. Van alle vrouwen waar materiaal van is opgeslagen, zowel HPA-1a negatief als HPA-1a positief, zullen klinische gegevens worden verzameld over de algemene medische voorgeschiedenis, de obstetrische voorgeschiedenis, de zwangerschap, de bevalling en de gezondheid van het kind in de eerste levensweek. Vervolgens zal in het bewaarde plasma van de HPA-1a negatieve vrouwen een anti-HPA-1a antistofscreening verricht worden voor de detectie van antistoffen tegen HPA-1a. Deze resultaten geven niet alleen nieuwe kennis over het

natuurlijk beloop van FNAIT in Nederland, maar zorgen ook voor de identificatie van een nieuwe en unieke controle groep. Dit zijn geïmmuniseerde zwangerschappen waarbij er geen klinische ziekte is geweest. Door eigenschappen van deze antistoffen te vergelijken met antistoffen die wel tot ziekte hebben geleid, kunnen we er in slagen om (een combinatie van) factoren te identificeren die kunnen voorspellen welke immunisaties wel en niet tot ziekte leiden. Dit is nodig om tijdens een mogelijke screening te kunnen bepalen welke zwangere vrouwen met anti-HPA-1a antistoffen wel of niet baat hebben bij een behandeling, om zo overbehandeling te kunnen voorkomen.

De ziekte waarvoor een screening wordt geïmplementeerd moet een belangrijk gezondheidsprobleem representeren. Dit kan ofwel betekenen een ziekte die vaak voorkomt, ófwel een ziekte die een grote (maatschappelijke) impact heeft. In *hoofdstuk 3 en 4* bespreken we de ziektelast die door FNAIT wordt veroorzaakt. Deze wordt gekenmerkt door een verhoogde bloedingsneiging, waarvan een hersenbloeding de meest gevreesde complicatie is. Van de gevolgen op lange termijn wordt gezegd dat deze erg ongunstig zijn, maar gestructureerde en gedetailleerde studies, die de lange termijn follow-up van kinderen na een hersenbloeding door FNAIT beschrijven, ontbreken. Deze informatie is in onze ogen erg belangrijk in de voorlichting van ouders, iets wat bij eventuele implementatie van prenatale screening vaker nodig zal zijn. In *hoofdstuk 3* wordt een observationele cohort studie beschreven waarin we zowel naar de korte termijn als naar de lange-termijn uitkomst hebben gekeken van 21 kinderen die een hersenbloeding hebben gehad als gevolg van FNAIT. De sterftekans bleek hoog te zijn (48%) en van de overlevende kinderen hadden er zes een ernstige neurologische ontwikkelingsstoornis en één kind een matige. Alle zeven kinderen hadden een vorm van verlamming (enkelzijdig of dubbelzijdig), een ernstige cognitieve en een ernstige motorische achterstand. Vier van de tien levende kinderen had een gezichtsbeperking en/of waren gediagnosticeerd met epilepsie.

Naast hersenbloedingen kan FNAIT in principe bloedingen in elk orgaan veroorzaken. *Hoofdstuk 4* beschrijft de resultaten van een retrospectief dossieronderzoek en literatuuronderzoek hiernaar. Naast de longbloedingen en de gastro-intestinale bloeding die behandeld zijn in het LUMC, vonden we in de literatuur beschrijvingen van oog-, nier-, ruggenmerg-, urogenitale en subgaleale bloedingen. Met dit hoofdstuk willen we benadrukken dat ook al zijn deze bloedingen niet net zo sterk geassocieerd met FNAIT als een hersenbloeding, ze wel zeker net zulke ernstige gevolgen kunnen hebben. In de praktijk zou er bij een trombocytopeen, maar verder gezond, kind met een orgaanbloeding gedacht moeten worden aan de diagnose FNAIT. Dit zorgt niet alleen voor een adequate diagnose en behandeling van deze kinderen, maar is nog belangrijk voor adequate follow-up van de moeders en toekomstige zwangerschappen.

Volgens de wereld gezondheidsorganisatie is waarschijnlijk het meest belangrijke criterium voor screening de beschikbaarheid van een adequate behandeling. In *hoofdstuk 5 en 6* hebben we zowel gekeken naar de preventieve antenatale behandeling als naar de postnatale behandeling

van aangedane kinderen. In *hoofdstuk 5* beschrijven we een systematische review, waarin we alle studies hiernaar, zowel 4 gerandomiseerde als 22 niet-gerandomiseerde studies geanalyseerd hebben. Antenatale behandeling kan opgedeeld worden in invasieve en non-invasieve behandeling. De invasieve behandeling bestaat uit een foetale bloedafname (in Engels: fetal blood sampling, FBS) eventueel gevolgd door een transfusie met trombocyten (in Engels: intrauterine platelet transfusion, IUPT). We zagen dat dit een erg risicovolle behandeling is, waarbij we aantoonden dat er in 11% van de zwangerschappen, die op deze manier behandeld werden, een ernstige complicatie optreedt, waarbij zelfs 1 op 4 van deze complicaties fataal is (26%). Schokkend was dat van alle sterftegevallen, meer dan de helft werd veroorzaakt door de invasieve behandeling. Een niet-invasieve behandeling bestaat uit wekelijkse toediening van IVIg (doorgaans 1.0 mg/kg/week), met of zonder de toevoeging corticosteroïden. We zagen dat er onvoldoende bewijs was voor het toevoegen van corticosteroïden of het verlagen dan wel verhogen van de IVIg dosering (0.5mg/kg, 1.0mg/kg of 2.0 mg/kg). Behandeling met IVIg bleek, vergeleken met invasieve behandeling, even effectief in het voorkomen van hersenbloedingen, zonder het verhoogde risico op complicaties. Ons advies voor antenatale behandeling van geïmmuniseerde zwangerschappen is dan ook wekelijkse infusies met 1.0 mg/kg/week IVIg. Start van deze behandeling is tussen de 20 en 24 weken zwangerschap, tenzij er sprake is van een eerdere zwangerschap met een foetale of neonatale hersenbloeding. In dat geval dient er reeds tussen 12 en 20 weken zwangerschap gestart te worden met de behandeling.

In de cohort studie, die in *hoofdstuk 6* wordt beschreven, worden verschillende toegepaste postnatale behandelstrategieën en uitkomsten geanalyseerd. Hierbij zagen we in de eerste plaats, dat, ondanks een landelijke richtlijn (alleen transfusie bij een trombocytengetal onder de 20×10^9/L – 30×10^9/L en pas neonatale IVIg wanneer er na 2 transfusies geen trombocyten getal boven 50×10^9/L is), er veel verschillende behandelingen werden toegepast; geen behandeling, trombocytentransfusie met compatibele of random-donor trombocyten, of met beide, en als laatste neonatale toediening van IVIg (met of zonder trombocytentransfusie). In de tweede plaats concludeerden we dat, ongeacht de toegepaste behandeling, het trombocytengetal in alle gevallen binnen vier dagen tot een veilige waarde steeg, zonder dat er nieuwe bloedingen optraden. De snelste en hoogste stijging werd bereikt wanneer er een compatibele transfusie werd gegeven en de kleinste stijging na IVIg behandeling. Opvallend was dat de transfusie met random-donor trombocyten niet geassocieerd was met een extra aantal benodigde transfusies. Dit suggereert dat, wanneer er een indicatie voor transfusie is en er niet direct een compatibel product beschikbaar is, een transfusie met random-donor trombocyten een goede eerste-lijn behandeling is in FNAIT.

In *hoofdstuk 7* beschrijven wij een enzyme-linked immunosorbent assay (ELISA) als geschikte test voor screening. Bij een mogelijke perinatale screening zal je eerst vanuit alle zwangere vrouwen de groep vrouwen willen identificeren die HPA-1a negatief zijn. Omdat dit slechts

2% van de totale populatie is, zullen hierna 49 van de 50 zwangere vrouwen afvallen en geen vervolgtest meer nodig hebben. De test die gebruikt wordt HPA-1a typering zal daarom enorm bepalend zijn voor de logistieke haalbaarheid en de kosteneffectiviteit van een eventueel screeningsprogramma. De ELISA die beschreven wordt in dit hoofdstuk is grotendeels geautomatiseerd en maakt gebruik van buizen bloed die niet voorbehandeld hoeven te worden en van matige kwaliteit zijn. Dit maakt het assay geschikt voor het screenen op grote schaal en vermindert de arbeidsuren en –kosten. Belangrijk bij screening is dat er geen samples die HPA-1a negatief zijn gemist worden. Hiervoor moet de test een 100% sensitiviteit hebben. Deze werd bereikt met nog steeds een erg hoge specificiteit van 99.9% en een fout-HPA-1a negatieve ratio van 0.03 (d.w.z. 3/100 samples geïdentificeerd als HPA-1a negatief zullen een HPA-1a positief genotype hebben).

In de huidige zorg, waarbij consumentisme steeds prominenter aanwezig is en de patiënt centraal staat, is het des te belangrijker dat een potentieel screeningsprogramma acceptabel is voor de doelgroep. In *hoofdstuk 8* beschrijven we een cross-sectionele vragenlijststudie bij gezonde zwangere vrouwen, om hun mening over een eventueel toekomstige HPA-screening naar FNAIT te onderzoeken. Hierbij maakten we gebruik van een gevalideerd model (Multidimensional Measurement of Informed Choice), waarbij zowel de mening/houding als de kennis en de uptake, en daarmee de geïnformeerde keuze, werden bepaald. Het bleek dat de houding van zwangere vrouwen ten opzichte van eventuele HPA-screening enorm positief was (91%) en gebaseerd was op adequate kennis bij 94%. In totaal werd er door 87% van de vrouwen een geïnformeerde keuze gemaakt, wat inhield dat hun keuze om wel of niet deel te nemen aan de screening gebaseerd was op adequate kennis en overeenkwam met hun houding (wel/niet positief). We zagen dat er significant minder geïnformeerde keuzes werden gemaakt in een kleine groep vrouwen die van niet-Europese afkomst waren. Dit benadrukt dat het in de toekomst belangrijk is om voorlichting te laten aansluiten bij de achtergrond, cultuur en taal van de zwangere vrouw.

In *hoofdstuk 9* beschrijven we de voorlopige resultaten na 10 maanden HIP-studie. Gedurende deze periode werden er 40.945 zwangere vrouwen getypeerd voor HPA-1a, waarvan er 986 (2.4%) HPA-1a negatief bleken te zijn. Van alle HPA-1a negatieve zwangere vrouwen gaven 263 (27%) toestemming voor deelname aan de HIP-studie. De antistofscreening in deze groep leverde de detectie van 24 samples met anti-HPA-1a antistoffen op (9.2%), waarvan er 4 uiteindelijk leidden tot klinisch relevante FNAIT. Hierbij was er sprake van een ernstige hersenbloeding die op een echo bij 29 weken zwangerschap werd ontdekt en uiteindelijk leidde tot het besluit om een late zwangerschapsafbreking bij 34 weken te verrichten. De andere drie gevallen hadden als uiting van FNAIT milde bloedingssymptomen, één pasgeborene had een cefaalhematoom (een onderhuidse bloeduitstorting op het hoofd) en de andere twee kinderen hadden diffuus verspreidde blauwe plekken en petechiën.

In dit proefschrift wordt belangrijke bewijs geleverd dat gebruikt kan worden bij het beoordelen van de W&J criteria voor screening. Een discussie en interpretatie van dit bewijs wordt gegeven in *hoofdstuk 10*. Daarnaast beschrijven we de mogelijkheid van specifieke interactie van anti-HPA-1a antistoffen met endotheelcellen en syncytiotrophoblastcellen van de placenta. Deze relatief nieuwe inzichten leiden mogelijk tot een vergroting van het klinische spectrum van FNAIT en tot een mogelijke nieuwe marker en voorspeller van het identificeren van zwangerschappen met een hoog risico op bloedingen. Als laatste stellen we een mogelijk screening scenario voor en bespreken welke informatie er nog benodigd is in het debat over het implementeren van HPA-screening tijdens de zwangerschap in Nederland, om de klinische gevolgen van FNAIT te voorkomen.

Appendix

List of abbreviations

AE	Adverse event
AUC	Area under the curve
CI	Confidence interval
CP	Cerebral palsy
CRF	Case report form
CS	Cesarean section
CV	Coefficient of variation
ECIS	Electric cell-substrate impedance sensing
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal blood sampling
FcRn	Neonatal Fc-receptor
FNAIT	Fetal and neonatal alloimmune thrombocytopenia
GA	Gestational age
GMFCS	Gross motor function classification system
GP	Glycoprotein
HDFN	Hemolytic disease of fetus and newborn
HIP	HPA-screening in pregnancy
HLA	Human leukocyte antigen
HPA	Human platelet antigen
HUVEC	Human umbilical vein endothelial cell
ICH	Intracranial hemorrhage
IgG	Immunoglobulin G
IQR	Interquartile range
ITP	Immune thrombocytopenic purpura
IUFD	Intrauterine fetal demise
IUGR	Intrauterine growth restriction
IUPT	Intrauterine platelet transfusion
IVH	Intraventricular hemorrhage
IVIg	Intravenous Immunoglobulins
LUMC	Leiden university medical center
MMIC	Multidimensional measurement of informed choice
NCTS	Near-term cesarean section
NDI	Neurodevelopmental impairment
NOS	Newcastle-Ottawa scale
OD	Optic density
PCR	Polymerase chain reaction

PLT	Platelet
PSIE	Prenatal screening for infectious diseases and erythrocyte immunization
PTx	Platelet transfusion
RBC	Red blood cell
RCT	Randomized controlled trial
SD	Standard deviation
SE	Side effect
SGA	Small for gestational age
SNP	Single nucleotide polymorphism
TOP	Termination of pregnancy
W&J	Wilson and Jungner
WHO	World health organization
WISC-III	Wechsler Intelligence Scale for Children third edition
WPPSI-III	Wechsler Preschool Primary Scale of Intelligence third edition

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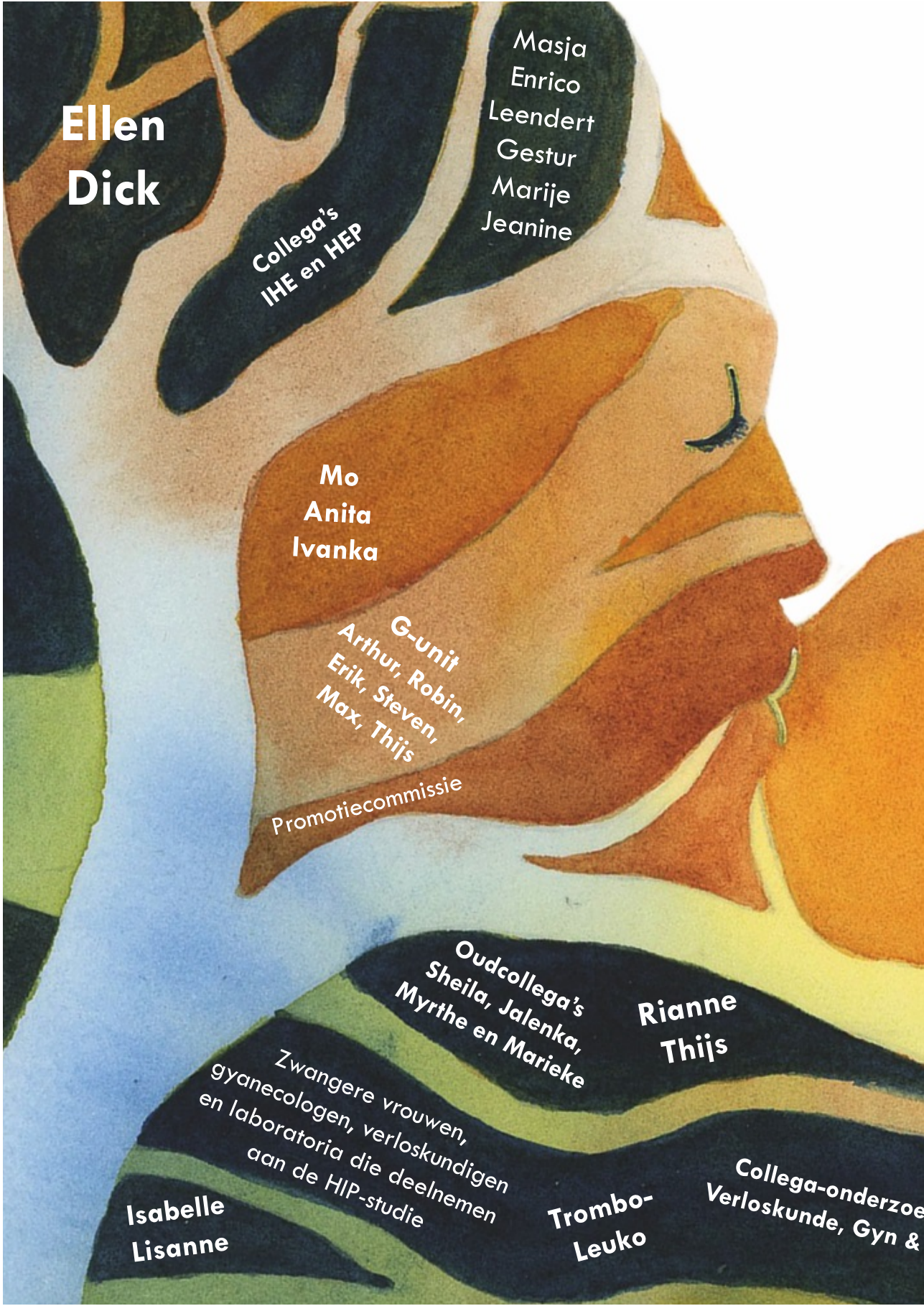
Curriculum Vitae

Dian Winkelhorst, author of this thesis was born at home in Rekken on the 3rd of July 1989. She grew up in the Achterhoek, in the east of the Netherlands together with her two sisters Minoe and Tess. In 2006, she obtained her gymnasium diploma at R.K.S.G. Marianum in Groenlo.

After her graduation, she moved to Utrecht and started medical school at Utrecht University. After her Obstetrics and Gynecology internship at St. Antonius Hospital in Nieuwegein she developed special interest towards this specialty. During her scientific internship in 2013, she studied reasons for RhD alloimmunization despite adequate prophylaxis with the guidance of Dr Lieve Page-Christaens and Prof. C. Ellen van der Schoot. After she attained her medical degree in March 2014, she started working as a physician (ANIOS) at the department of Obstetrics and Gynecology at St. Antonius Hospital in Nieuwegein.

In 2015, she was offered a PhD position by Prof. C. Ellen van der Schoot to study Fetal and Neonatal Alloimmune Thrombocytopenia. This project was a collaboration between the division of Fetal Therapy (Prof. D. Oepkes), department of Obstetrics at Leiden University Medical Center (LUMC) and department of Experimental Immunohematology at Sanquin (Prof. C. Ellen van der Schoot). As part of her PhD, Dian set up the HIP-study, a nationwide prospective observational study. In 2017, Dian received the 'De Snoo van 't Hoogerhuijs Stichting - Junior Research Award' for her research proposal "Can we identify HPA-1a alloimmunized pregnancies at high risk for developing fetal or neonatal bleeding complications?". In October 2019, she started her residency at St. Antonius Hospital under the supervision of Dr G.C.M. Graziosi.

Dian lives in Utrecht together with Wouter and their son Rein (2018).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It always seems impossible until it's done

- Nelson Mandela -

Thank you!