FETAL AND NEONATAL ALLOIMMUNE THROMBOCYTOPENIA: IMMUNOLOGICAL MECHANISMS AND CLINICAL CONSEQUENCES

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Stockholm 2017
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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family
“The world is full of obvious things which nobody by any chance ever observes.”

Arthur Conan Doyle
ABSTRACT

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a rare condition that may occur in connection with pregnancy, when maternal antibodies directed against paternally inherited platelet antigens cross the placenta and mediate thrombocytopenia in the fetus and neonate. FNAIT puts the fetus at risk of severe bleedings, with an intracranial hemorrhage being the most feared outcome. Antibodies targeting Human Platelet Antigen 1a (HPA-1a) are the most common cause of FNAIT in Caucasians, and several mechanisms may potentially be involved. The overall aim of this thesis was to use both clinical and experimental data to investigate possible mechanisms of FNAIT, and to evaluate the impact of FNAIT on neonatal morbidity in Sweden by investigating the frequency of maternal platelet alloimmunization in newborns diagnosed with an intracranial hemorrhage (ICH).

In study I and II, the presence of anti-HLA class I antibodies in relation to FNAIT is investigated. Anti-HLA class I antibodies are a common occurrence after pregnancy, and the only serological finding in many cases of suspected FNAIT. Platelets express HLA class I antigen, and it has for long been debated whether anti-HLA class I antibodies could cause FNAIT. Based on national referrals in Sweden and Norway, we identified cases of suspected FNAIT where only anti-HLA class I antibodies could be detected. We compared anti-HLA class I antibody specificities and levels in these referred cases to controls, and found that the included cases in both studies had higher antibody levels compared to the control groups, and that especially antibodies against HLA-B antigens stood out with high levels. In study II, genotyping of the maternal and neonatal HLA class I alleles could untangle seemingly broad antibody specificity patterns, as mapping of epitopes revealed that the antibodies were in fact largely specific against paternal epitopes, expressed on multiple antigens. There was only weak correlations between anti-HLA class I antibody levels and clinical outcome, and especially in study I, a majority of cases were found to have other factors that could contribute to thrombocytopenia. In study III, we investigated platelet function in adult and neonatal blood, and the effects of anti-HPA-1a antibodies on platelet function. We found that umbilical cord blood was characterized by a pronounced decrease in platelet aggregation, a reduced change in expression of glycoproteins with activation, and an enhanced primary hemostasis, compared to adult peripheral blood. The presence of a monoclonal anti-HPA-1a antibody reduced fibrinogen binding to HPA-1a positive platelets, but with limited functional consequences, as measured by flow cytometry. For study IV, we investigated the frequency of maternal platelet alloimmunization in clinically recognized cases of neonatal ICH. Using the Swedish Neonatal Quality register, we identified 286 neonates registered with ICH born at or from 32 weeks of gestation, and ultimately included 105 maternal samples for analysis. We found two HPA-1a antigen negative (HPA-1bb) mothers, of which one had detectable anti-HPA-1a antibodies and a severely thrombocytopenic neonate. Two other mothers had detectable antibodies (anti-HPA-5b and anti-HPA-15a) and neonates with moderate thrombocytopenia. The available clinical data revealed a high frequency of other factors with a known association to ICH, and the study suggests a lower frequency of ICH associated with FNAIT than previously estimated in prospective studies. Overall, these studies confirm the notion of FNAIT as a heterogeneous condition, with findings that may inform future studies on frequency of severe FNAIT, and potential mechanisms.
LIST OF SCIENTIFIC PAPERS

I. Characterisation of Maternal Human Leukocyte Antigen Class I Antibodies in Suspected Foetal and Neonatal Alloimmune Thrombocytopenia

Transfusion Medicine 2017, 27:43-51

II. Unraveling the Role of Maternal Anti-HLA Class I Antibodies in Fetal and Neonatal Thrombocytopenia – Antibody Specificity Analysis Using Epitope Data

J Reproductive Immunology 2017, 122:1-9

III. Adding to the Complexity of Fetal and Neonatal Alloimmune Thrombocytopenia: Reduced Fibrinogen Binding in the Presence of Anti-HPA-1a Antibody and Hypo-Responsive Neonatal Platelets

Refsum E, Meinke S, Gryfelt G, Wikman A, Höglund P.
Manuscript

IV. Intracranial Hemorrhages in Neonates Born From 32 Weeks of Gestation – Low Frequency of Associated Fetal and Neonatal Alloimmune Thrombocytopenia: A Register Based Study

Refsum E, Håkansson S, Mörtberg A, Wikman A, Westgren M.
Manuscript, accepted for publication in Transfusion September 2017

Not included in the thesis:

Sensitive detection of platelet-specific antibodies with a modified MAIPA using biotinylated antibodies and streptavidin-coated beads
J Immunol Methods 2016, 434:9-15
LIST OF ABBREVIATIONS

26-4 Monoclonal anti-HPA-1 antibody 26-4
ADCC Antibody-dependent cell-mediated cytotoxicity
ADP Adenosine diphosphate
ANOVA Analysis of variance
APC Allophycocyanin
APC Antigen presenting cell
AUC Area under the curve
BCR B-cell receptor
BSA Bovine serum albumin
C3d Complement component 3d
CCL Chemokine (CC-motif) ligand
CD Cluster of differentiation
CDC Complement-dependent cytotoxicity
CFT Clot formation time
CHO cell Chinese hamster ovary cell
CPA Cone platelet analyzer
CPD Citrate phosphate dextrose solution
CR2 Complement receptor 2
CRP C-reactive protein
CT Clotting time
CXCL C-X-C-motif chemokine ligand
DIC Disseminated intravascular coagulation
EDTA Ethylenediaminetetraacetic acid
EVT Extravillous trophoblast cell
FCM Flow cytometry cross-matching
FcRn Neonatal Fc receptor
FITC Fluorescein isothiocyanate
FNAIT Fetal and neonatal alloimmune thrombocytopenia
FOXP3 Forkhead box P3
FSC Forward scatter
GP Glycoprotein
GT Glanzmann’s thrombasthenia
GW Weeks of gestation
HDFN Hemolytic disease of the fetus and newborn
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA Human leukocyte antigen
HPA Human platelet antigen
ICH Intracranial hemorrhage
IDO Indoleamine 2,3-dioxygenase
IgG Immunoglobulin G
IL Interleukin
ITP Idiopathic thrombocytopenic purpura
IUFD Intrauterine fetal death
IUGR Intrauterine growth restriction
IVH Intraventricular hemorrhage
IVIG Intravenous immunoglobulin
MAIPA Monoclonal antibody immobilization of platelet antigens assay
MBRN Medical Birth Registry of Norway
MCF Maximum clot firmness
MCV  Mean corpuscular volume
MESF  Molecules of equivalent soluble fluorochrome
MFI  Median fluorescence intensity
MK  Megakaryocyte
MoAb  Monoclonal antibody
MPV  Mean platelet volume
NEC  Necrotizing enterocolitis
NK cell  Natural killer cell
PAC-1  Platelet activation, clone number 1
PAR1  Protease-activated receptor 1
PBS  Phosphate buffered saline
PC7  Phycoerythrin-cyanine7
PIFT  Platelet immunofluorescence test
PIRCHIE-II  Predicted indirectly recognizable HLA epitopes presented by HLA class II
PTP  Post transfusion purpura
PVH  Periventricular hemorrhage
RANTES  Regulated on activation, normal, T-cell expressed, and presumably secreted
RT  Room temperature
RT-PCR  Real-time polymerase chain reaction
SFI  Standard fluorescence intensity
SGA  Small for gestational age
SNQ  Swedish Neonatal Quality register
SSC  Sideward scatter
TCR  T-cell receptor
TNF-α  Tumor necrosis factor-α
TPO  Thrombopoietin
TRALI  Transfusion-related acute lung injury
TRAP  Thrombin receptor activating peptide 6
TXA2  Thromboxane A2
U  Arbitrary units
uNK  Uterine natural killer cell
vWF  Von Willebrand factor
WT  Wild type
1 INTRODUCTION

1.1 A BIRD’S EYE VIEW OF FETAL AND NEONATAL ALLOIMMUNE THROMBOCYTOPENIA

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a rare condition with an estimated incidence of 1:1000-2000 live births[1-4]. During pregnancy, maternal alloantibodies targeting fetal antigens inherited from the father cross the placenta and mediate thrombocytopenia in the fetus and newborn, resulting in an increased risk of bleedings. The antibody specificities that cause FNAIT vary in different populations, and in Caucasians FNAIT is most commonly caused by antibodies targeting human platelet antigen 1a (HPA-1a)[5]. Cases of neonatal thrombocytopenia in the presence of these maternal alloantibodies were first described in the early 1960’s[6]. Today, the immunobiology and pathophysiology of FNAIT is still not completely understood. The exact incidence and natural history of FNAIT is also unknown because most screening programs have included interventions to avoid serious complications, such as an intracranial hemorrhage[7]. No country has implemented any prenatal screening program to prevent FNAIT due to the unknown true incidence of severe FNAIT, and lack of interventions such as targeted therapy and prophylaxis. However, large efforts are going into developing a prophylaxis[8], and screening studies are ongoing in Poland[9], and planned in the Netherlands (the HPA-screening In Pregnancy study), with the aims to determine the frequency of severe FNAIT, and pave the way for future screening programs. The large resources going into these efforts, together with the potential severe consequences of FNAIT, justifies further research on this condition. The main focus of this introduction is the possible immunological mechanisms of FNAIT, and what is learned from clinical data so far.

Figure 1: The Basic Concept of FNAIT. Recognition of fetal platelet antigens by the maternal immune system leads to transport of maternal anti-platelet antibodies across the placenta, mediating thrombocytopenia and increased risk of bleedings in the fetus and neonate. Adapted from [10].
1.2 AN OVERVIEW OF ANTIGEN RECOGNITION AND ANTIBODY PRODUCTION

An essential ability of the immune system is to distinguish self from non-self, and protect against foreign, and potentially damaging, antigens by eliminating or neutralizing them. At the heart of this ability lies antigen recognition and antibody production. To avoid launching an attack against the body’s own cells, each step in this process is controlled by the requirement of co-stimulatory signals, either from interaction with other immune cells, or by means of “danger signals” – which can be produced by cells under stress, or expressed by exogenous pathogens.

Lymphocytes, in particular B- and T-cells, orchestrate the antibody response against antigens, which are defined as molecules that can bind to an antibody or T-cell receptor. Briefly, an antigen will first be encountered and processed by antigen presenting cells (APCs). Depending on properties of the antigen and signals from the surrounding milieu, the APC can become activated, enhance its presentation of the antigen, and travel to the lymph node. The lymph node functions as a meeting point, where several APCs can present multiple antigens encountered throughout the body to mature T-cells. The APCs present the processed antigen on its surface to T-cells, which can have receptors specific for the encountered antigen. If a T-cell recognizes the same particular antigen, and also receives co-stimulatory signals from the activated APC, this can trigger activation of the T-cell. The T-cell will then in turn activate the antigen-specific B-cell, which will lead to an expansion of this B-cell clone. With help from T-cells, the clone expressing the B-cell receptor with the highest affinity towards the antigen will survive and develop into an efficient antibody-producing plasma cell. These cells reside for long periods of time in the body, producing antibodies directed against the antigen; in case of new encounters with the same antigen, the immune system is rapidly able to mount an efficient response.

T-cells are crucial for both controlling and enhancing the response against antigens. Antigens are presented to T-cells in two ways; endogenous peptides can be presented by any cell expressing HLA class I molecules, and “professional antigen presenting cells”, such as dendritic cells, B-cells and macrophages, can process and present peptide fragments of the antigen on HLA class II molecules. The T-cell receptor (TCR) can recognize a magnitude of antigens as it is encoded by genes assembled after somatic re-arrangement of germ-line encoded gene elements (known as V(D)J recombination). A few hundred germ-line gene elements can generate millions of different antigen receptors, with potentially equal number of specificities. The T-cells undergo positive and negative selection in the thymus, which ensures binding of self-antigens with an adequate affinity, to be functional and self-tolerant T-cells. The surviving “ naïve” (CD4+CD8+) T-cell can then develop into CD8+ T-cells or CD4+, depending on whether the T-cell receptor recognizes HLA class I or II, respectively. CD8+ T-cells are also known as cytotoxic T-cells, important in the defense against viruses and cancer. CD4+ T-cells can stimulate B-cells, and is part of the humoral response. Both require co-stimulatory signals, together with binding of TCR to antigen presented on their respective HLA molecule, to be activated, and signals from co-stimulatory molecules and cytokines will stimulate differentiation into various subtypes of T-cells.
For antibody production, antigen-presenting cells (APCs) that have encountered and processed an antigen, will typically travel to the lymph node and encounter a T-cell with a TCR for the specific antigen. During the process of developing from an “antigen-capturing” to an antigen-presenting cell, the APC will express cytokines and co-stimulatory molecules, to become an activated APC that can interact with the T-cell. The binding of the specific TCR to HLA class II-bound peptide on the APC, will together with binding of CD4, which binds to a different part of the HLA class II molecule, lead to phosphorylation and activate intracellular pathways of the T-cell (signal 1). A second stimulatory signal is necessary for the T-cell to become activated: CD80 or CD86, expressed on activated APC, bind CD28 on CD4⁺ T-cells, which alters the intracellular signals for the T-cells, ultimately allowing for activation. This two-step activation leads to proliferation of the T-cell, and in future encounters with the same antigen, only the first signal is necessary for activation of the T-cell. Activation leads to release of IL-2, which stimulates the T-cell in an autocrine manner, and leads to a clonal expansion of the T-cell.

A naïve B-cell, which have yet to encounter its specific antigen, will express a mature B-cell receptor (BCR), consisting of a trans-membrane protein, and a surface bound immunoglobulin, which can be shed as an antibody (IgM/D). Similarly to T-cells, the genes encoding the B-cell receptor also undergo V(D)J recombination, allowing for heterogeneity of the antigen-binding site. There are several thousands of copies of BCRs on the B-cell surface, and upon binding of the antigen, the BCRs can cluster together and signal for activation of the B-cell[11]. As mentioned earlier, B-cells can also present antigen to T-cells, by processing the antigen and present it on HLA class II, leading to further activation of both lymphocytes[12]. Different antigens can be divided by these two mechanisms for B-cell activation: T-cell independent antigens stimulate the B-cell by pattern recognition receptors, such as as toll-like receptors that recognize “danger signals” expressed on the antigen surface, or by cross-linking of the BCRs[13], while T-cell dependent antigens require co-stimulatory signals such as CD40-CD40L interaction, and subsequent release of IL-4 from CD4⁺ T-cells will further stimulate the differentiation of the activated B-cell into plasma cells. In addition, complement can enhance B-cell activation and antibody production[14], by signaling through the CR2 complex (which binds C3d bound to the antigen).

The production of antibodies induced by T-cell dependent antigens is a two-step process[15]: As a rapid, “extra-follicular”, response, B-cells receive an antigen-dependent signal that leads to the development of plasmablasts that divide, and may undergo immunoglobulin class switching. However, these cells are short-lived, and there is little somatic hypermutation. The second step ensures the production of high-affinity antibodies: The majority of mature B-cells reside in follicles of secondary lymphoid organs as follicular B cells, where they can be exposed to antigens by follicular dendritic cells. With support of follicular B helper T cells (T_{FH}), antigen-activated B-cells can form germinal centers and undergo further modification of the BCR genes by somatic hypermutation. Somatic hypermutation of the BCR genes changes the affinity to the antigen, and B cell clones with a high affinity will by the help of T_{FH} be selected to undergo class switching (as IgM to IgG), and ultimately differentiate into “effector B-cells”; plasma cells, which will secrete high-affinity antibodies and may persist as long-lived cells[16], and memory B-cells, both likely important for an efficient secondary immune response[17].
The body produces 10^{12} antibodies per day (Wermeling F, Basic Immunology lecture notes, 2015). IgG has a half-life of approximately 20 days, and is the most abundant in human serum of the five immunoglobulin isotypes (IgA, IgD, IgE, IgM, IgG)[18]. IgG can be divided into four subclasses (IgG1-4). The subclasses differ in their constant regions, which are involved in IgG binding (such as to FcγR and C1q); different subclasses of IgG can therefore have different effector functions, such as triggering FcγR expressing cells, leading to phagocytosis or antibody-dependent cell-mediated cytotoxicity (ADCC), and activating complement[19].

Figure 2: Overview of the generation of antibodies from antigen-activated B-cells. Activated B-cells differentiate into centroblast. Somatic hypermutation (SHM) introduces base-pair changes in the V(D)J segments that encode the variable regions of the immunoglobulin heavy and light chain. Centroblasts further differentiate into centrocytes, where they can be selected by T-cells on the basis of their improved receptor binding to the antigen. Centroblasts and centrocytes cycle between dark and light zone. Centrocytes with inadequate binding to the antigen undergo apoptosis, and only a subset of the centrocytes undergo class-switch recombination (CSR), and eventually differentiate into plasma cells or memory B cells. Adapted from[20].
1.3 MATERNAL TOLERANCE OF THE FETUS DURING PREGNANCY

The fetus is semi-allogeneic – meaning it inherits both antigens from the father, which the mother’s immune system can recognize as non-self, and antigens from the mother, which will be recognized as self. To allow for a developing semi-allogeneic fetus while simultaneously upholding responses required for immunity, the maternal immune system needs unique qualities, most of which are still unknown.

Likely, there is a site-specific inhibition of the maternal immune system at the placenta, that allows for maternal tolerance[21]. Briefly, mechanisms may include: Fetal expression of FasL, which can induce T-cell apoptosis[22]; expression of HLA-G and HLA-C on extravillous trophoblast cells (EVTs), which may respectively serve as ligand for inhibitory NK cell receptors (such as KIR2DL4)[23], and modulate decidual NK cell response to invading trophoblast[24]; release of immunosuppressive molecules such as indoleamine 2,3-dioxygenase (IDO), depleting T-cells of tryptophan and dampening their response [25]; entrapment of antigen-presenting dendritic cells in the decidua[26].

However, the invading placenta is not the only site where fetal tolerance needs to be upheld: Fetal cells enter the maternal circulation and can persist for decades[27, 28], a phenomena known as fetal microchimerism. (Reciprocally, maternal cells enter fetal circulation (maternal microchimerism), where they may persist into adulthood[29]). The presence of these fetal cells likely requires more systemic changes to the maternal immune system: There is evidence that the maternal immune system generally is skewed towards a Th2 response[30], which is characterized by antibody production and inhibition of phagocytic cell functions[31], has an anti-inflammatory phenotype with a constant decreased pro-inflammatory cytokine expression (reduced CCL2, CXCL10, IL-18 and TNF-α), and increased anti-inflammatory expression with gestational age[32], and that there is an increased amount of T-regs (CD4+FOXP3’) during pregnancy[30, 33, 34], although their exact role is uncertain[35]. How these immunological changes and the presence of fetal microchimerism affects the mother’s immune system later in life, and in subsequent pregnancies, is unknown, but possibly it can reinforce fetal tolerance in future pregnancies[36].

To summarize, multiple, tightly regulated mechanisms are at play to secure a successful pregnancy. Breach of tolerance may however occur, and maternal alloimmunization against fetal antigens can lead to a condition such as FNAIT.
Figure 3: Possible Mechanisms of Fetal Tolerance. Anchoring villous trophoblasts attach to the decidua of the uterus and further differentiate into syncytiotrophoblasts, cytotrophoblasts and extravillous cytotrophoblasts (EVTs). Floating villous trophoblast, bathed in maternal blood, carry fetal blood vessels and can shed placental exosomes, microparticles and fetal cells. Decidual NK cells, induced by IL-15 and SCF from dHSCs, promote IDO production and induction of tolerogenic DCs. tDCs, monocytes, and dNKs promote T_regs generation and effector T-cell apoptosis. Pregnancy hormones modulate the decidualization. DSC=Decidual stromal cell. dHSC=Decidual hematopoietic stem cells. tDC=Tolerogenic dendritic cells. SCF=Stem cell factor. M1C-1=Macrophage inhibitory cytokine-1. hCG=Human chorionic gonadotropin. KYN=kynurenine. VEGF=vascular endothelial growth factor. Adapted from [37].
2  RECENT PATHOPHYSIOLOGICAL ASPECTS OF FNAIT

2.1 THE HPA-1a EPITOPE IS EXPRESSED ON THE PLATELET αIIbβ3 INTEGRIN

The HPA-1a epitope is situated on the glycoprotein (GP) IIIa (β3 integrin, CD61), which in complex with GPIIb constitutes the fibrinogen receptor on platelets (GPIIb/IIIa, αIIbβ3 integrin). The HPA-1a epitope is the result of a single amino acid substitution, from proline (HPA-1b), to leucine (HPA-1a)[38]. HPA-1a is also expressed on microparticles derived from platelets[39]. The fetal platelets express this HPA-1a epitope at least as early as 18 weeks of gestation[40], and exposure of the epitope by mixing of maternal and fetal blood may occur in a fetomaternal bleeding during pregnancy or in conjunction with delivery. Nearly all pregnancies have some degree of fetomaternal hemorrhage, but rarely larger volumes[41], and the small number of neonatal platelets could be too low to cause an maternal alloimmunization[42]. FNAIT may also occur in the first pregnancy. This could imply that the HPA-1a epitope is extremely immunogenic, and/or that other sources of the epitope than fetal platelets could be involved.

2.2 OTHER SOURCES OF ANTIGEN EXPOSURE – HPA-1a EPITOPE ON INTEGRIN αVβ3

The β3 integrin also forms a heterodimer with the αv integrin (CD51), to form the vitronectin receptor αvβ3. The expression of αIIbβ3 on platelets is at least 40-fold higher compared to αvβ3[43-45]. However, the αvβ3 complex is in addition to platelets, also expressed on placental syncytiotrophoblasts[46], and syncytiotrophoblast particles[47], which may enter maternal circulation during pregnancy[48], and hence represents another source of maternal exposure to the HPA-1a epitope.

The αvβ3 integrin complex is further expressed in endothelial cells, where it is involved in vasculogenesis[49] and angiogenesis[50]; in a study from 2009, antibodies targeting β3, both mouse anti-human CD61 monoclonal antibody, and purified patient-derived anti-HPA-1a IgGs, were shown to negatively affect endothelial cell layer spreading and integrity, indicating that defective vasculature could contribute to the bleedings seen in cases of FNAIT[51].

The binding of maternal antibodies to HPA-1a on β3 expressed on trophoblast has also been suggested to negatively affect trophoblast function: By using a human monoclonal anti-HPA-1a antibody (26-4) on a first trimester extravillous trophoblast cell line, adhesion and migration was reduced by 20%, while there was an inconsistent effect on invasiveness (reduced in 3/4 experiments, with 9-25%)[52]. Though with limited demonstrable in vitro effects, a reduced trophoblast function in the presence of anti-HPA-1a could theoretically be one explanation to the reduced birth weight observed in boys suffering from FNAIT[53], and the associated placental inflammation shown in cases of FNAIT[54, 55], but likely other mechanisms are also involved (see chapter 2.9.1.).
2.2.1 Lessons From Clinical Studies on Anti-αvβ3 and ICH

Antibodies from HPA-1a immunized mothers are likely to bind both variations of the β3 integrin (αIIbβ3 and αvβ3), and this has been demonstrated by surface plasmon resonance (SPR) technique using a monoclonal antibody derived from an HPA-1a immunized mother (26-4)[56]. Few clinical studies have tested for these αvβ3 antibodies until recently: In a cohort of FNAIT cases with and without ICH (n=18 per cohort), anti-HPA-1a antibodies reactive against αvβ3 from endothelial cells were only detected in the cases with ICH (in 17/18), and only HPA-1a antibodies of the αvβ3 type (and not anti-β3 type) disrupted adhesion to vitronectin, induced endothelial cell apoptosis (anoikis), and disturbed the endothelial cell proliferation (tube formation)[57]. This discrimination of subtype anti-HPA-1a antibodies into anti-αIIbβ3, anti-β3, anti-αvβ3, and associating them to clinical outcome is new, and has major implications for the understanding of the pathophysiology of bleedings in FNAIT. Testing for anti-αvβ3 could potentially be used to select pregnancies of high risk of ICH. How common these types of anti-HPA-1a antibodies are in an unselected (screened positive) population is not known, but likely this will be further investigated in larger materials in the future.

FNAIT is often thought of as the platelet equivalent to red cell alloimmunization during pregnancy, which causes hemolytic disease of the fetus and newborn (HDFN), and the two are often compared. The HPA-1a antibodies in FNAIT are not actually platelet specific, and have additional effects other than mediation of platelet destruction; they could also be the result of “trophoblast immunization”, and cause bleedings by affecting the function of vascular endothelial cells and the angiogenesis during fetal development. This suggests that FNAIT could be more different from HDFN than previously assumed, and opens up for a new understanding of the pathophysiological processes behind FNAIT. Furthermore, the presence of anti-αvβ3 antibodies in FNAIT, and not in maternal idiopathic thrombocytopenic purpura (ITP), could perhaps partly explain the lower frequency of ICH associated with this condition[58].

2.3 RECOGNITION OF THE HPA-1a ANTIGEN IS MHC CLASS II RESTRICTED

Generally, an efficient production of antibodies requires activation of antigen specific B- and T lymphocytes (see Figure 2). Perhaps surprisingly, little is known about these cellular interactions towards fetal antigens, and how they play a role in the pathophysiology of FNAIT. The HLA class II complexes are necessary for presentation of extracellular antigens, and overrepresentation of certain HLA class II alleles in immunized mothers has been described. About 10% of mothers that are HPA-1a negative (HPA-1bb) are immunized, and the majority of immunized mothers are positive for the HLA-DRB3*0101 allele[8]. The HLA-DRB3*0101 allele together with HLA-DRA, encodes the MHC class II molecule HLA-DR52a[59], which has been shown to bind β3 integrin from HPA-1a, but not HPA-1b alleles. It has been hypothesized that the switch from proline (HPA-1b) to leucine (HPA-1a) stabilizes the binding of the antigen to the peptide-binding groove of HLA-DR52a[60]. This subsequent increase in affinity leads to an anchoring of a peptide that otherwise would not bind, and could explain why immunization towards HPA-1b is so rare (so called
unidirectional alloantibody response)[61]. However, production of the so-called low-avidity anti-HPA-1a antibodies, which may be detected by SPR, is not associated with HLA DRBR3*0101. Mouse studies suggest that these antibodies do not necessarily mediate thrombocytopenia, but that they can still cause FNAIT with moderate to severe thrombocytopenia in some cases[66].

2.4 MATERNAL IMMUNE CELLS REACT TO THE HPA-1a ANTIGEN

Maternal CD4+ T-cells reactive towards allogeneic HPA-1a have been studied: One study used HPA-1a peptides with different truncations and substitutions to investigate the specific residues required for T cell activation, confirming the previous molecular modeling of the binding of HPA-1a to HLA-DR52a [62]. Another study [63] showed that HPA-1a specific clonal T cells only proliferated when stimulated by HLA-DRB3*0101-positive APCs presenting the L33 peptide, demonstrating HLA-DR52a restricted recognition of the HPA-1a antigen. One role of these T cells is probably to support B cells in antibody production. It is known from clinical experience that antibodies can persist in maternal circulation long after an HPA-1a incompatible pregnancy; one can therefore assume that there also are long-lived plasma cells specific to the HPA-1a antigen. However, there are (to my knowledge) no studies on B cell function and FNAIT.

2.5 ANTI-HPA ANTIBODY SPECIFICITIES INVOLVED IN FNAIT

As mentioned earlier, in Caucasians most cases of FNAIT are due to anti-HPA-1a antibodies. Nevertheless, platelets express over thirty different HPAs, and other anti-HPA antibodies can also cause FNAIT. The different HPAs are numbered after their discovery, and the high frequency alleles are denoted “a”, while the lower frequency alleles are denoted “b”. Besides anti-HPA-1a (68%), anti-HPA-1b (6%), anti-HPA-3a (1.3%), anti-HPA-5b (15%), and anti-HPA-5a (2%) antibodies are the most common antibodies implicated in FNAIT in Caucasians[64]. In addition to the well-known anti-HPA antibodies, new low frequency platelet antigens are continuously discovered to cause FNAIT[65, 66].
2.6 ANTI-HLA CLASS I ANTIBODIES IMPLICATED IN FNAIT

A different group of antibodies that is associated with FNAIT is anti-HLA class I antibodies. Whether these antibodies actually cause FNAIT, or if they are just an incidental finding, is still being debated. HLA class I antigens are present on extravillous trophoblast cells (HLA-G, HLA-C[68]), and on platelets and nucleated cells (HLA-A, -B, and -C). Platelets, EVTs, and other cells or cell particles may be shed into maternal circulation across the placenta during pregnancy, in conjunction with delivery, or by a fetomaternal hemorrhage. Maternal exposure to fetal HLA class I antigens is therefore likely, and anti-HLA class I antibodies are detected in 10-46% of women with a history of pregnancy, depending on detection technique[69-74]. As platelets are in abundance compared to other cells, they represent a major source of HLA class I antigen in blood[75, 76], and they may be susceptible to binding of anti-HLA class I antibodies. In suspected cases of FNAIT, anti-HLA class I antibodies are often the only detectable antibody.

Several case reports on FNAIT due to HLA class I antibodies exist[77-80], but most of these do not sufficiently rule out rare and difficult to detect anti-HPA antibodies. In addition, anti-HLA class I antibodies can often be detected together with anti-HPA antibodies. Possibly they could have a synergistic role in causing thromboctopenia[81], but this has to date not been investigated in larger, prospective materials. The presence of anti-HLA class I antibodies could also render the treatment of FNAIT due to anti-HPA antibodies less efficient because of reactivity against donor platelets[82].
To make a diagnosis of FNAIT caused by HLA class I antibodies, other reasons for thrombocytopenia need to be ruled out. With a frequency of ca. 1% in unselected newborns[83], and in approximately 22% of those admitted to neonatal intensive care units[84], neonatal thrombocytopenia is not uncommon. Other potential causes of thrombocytopenia include infections, placental insufficiency, asphyxia, aneuploidy, and other congenital disorders[85] (examples are listed in Figure 5). However, anti-HLA class I antibodies have been suggested to be a cause of thrombocytopenia in small for gestational age infants[86], and is associated with a reduced chance for live birth after secondary miscarriage[87]. Prospective studies[73], reviews[88], and meta-analyses[89] on anti-HLA class I antibodies and FNAIT (or pregnancy outcome[89]) have been inconclusive or negative. Anti-HLA class I antibodies can play a role in platelet transfusion refractoriness[90], transfusion-related acute lung injury (TRALI)[91], and organ transplantations[92]. It is therefore a conundrum that these antibodies can be harmful in other settings, but how and if they affect the fetus is still unknown; this warrants further studies on anti-HLA class I alloimmunization during pregnancy.

Figure 5: Directed Acyclic Graph (DAG) over possible mechanisms behind maternal HLA class I alloimmunization and neonatal thrombocytopenia. Created using www.dagitty.net
2.7 ANTIBODY COMPOSITION AND PLACENTAL TRANSPORT

There are different antibody specificities, and there are also different antibody isotypes and compositions. Antibodies involved in FNAIT are of the IgG isotype, the main isotype to cross the placenta. Concentrations of maternal IgG have been shown to rise from early in the 2nd trimester to term, where fetal IgG may exceed maternal levels[93]. The different isotypes have different transport capacity, with mostly IgG1 being transported, followed by IgG4>IgG3>IgG2[94]. The transport of IgG is an active process mediated by the neonatal Fc receptor (FcRn)[95], and is still not completely understood[96].

![Image of immunoglobulin structure](https://example.com/image.png)

Figure 6: Basic structure of an immunoglobulin. The unit consists of two identical light chains (L) and heavy chains (H), where each region is composed of variable (V) and constant (C) regions. Disulfide bonds maintain folding of the protein, and allow for flexibility for better antigen binding. Adapted from [97].

2.7.1 IgG1 and IgG3 Are the Dominating Isotypes in FNAIT

In an earlier study on IgGs involved in FNAIT, most maternal sera contained IgG1 alone (22/40), or in combination with IgG3 (10/40), while IgG4 was rare (3/40, together with IgG1 and IgG3) and IgG2 absent[98]. The study included alloimmunized mothers with and without thrombocytopenic children (n=36 and n=4, respectively), and there was no association between outcome and IgG isotype.

2.7.2 Transport of IgG Across the Placenta

No large studies have quantified in what extent anti-HPA antibodies are transported across the placenta and into the fetal circulation during pregnancy. Though it is possible to quantify anti-HPA-1a antibodies[99], fetal sampling during pregnancy is rarely performed due to risk
of complications, and sampling from thrombocytopenic neonates could also be problematic. One interesting report with regards to the transport of IgG in FNAIT concerns polymorphisms within IgG3[100]: Due to an amino acid difference (arginine for histidine), IgG3 usually has a shorter half-life compared to the other isotypes (7 days vs. 21 days), because of decreased binding to the FcRn, which protects the immunoglobulin from being degraded. However, rare allotypes of IgG3 (with the histidine substitution) can bind the FcRn with higher affinity, so that the half-life is at a level similar to IgG1[101], and it was shown that this IgG3 allotype could be transported across the placenta as efficiently as IgG1[100]. Higher levels of IgG3 in the neonate could be clinically relevant as IgG3 is thought to have a high capacity to bind FcR receptors and activate effector functions, especially FcγRIIIA (on macrophages, NK cells, and monocytes)[102]; though rare, the presence of this IgG3 allotype could potentially explain some of the heterogeneity in outcome of pregnancies with FNAIT, and there is some evidence towards associations between IgG3 concentrations and severity of FNAIT[103]. By comparing concentrations in the mother and neonate (cord blood) directly after delivery, the study also showed a correlation between maternal and cord blood total IgG levels in normal pregnancies, with a saturation of cord blood IgG concentration that was enhanced when the mothers were treated with IVIG[100]; this could be one possible mechanism as to why IVIG seems to protect against bleedings[104].

2.7.3 IgG Composition May Dictate Antibodies’ Effector Functions

Immunoglobulins are glycoproteins, and lately the glycan composition of IgG1 in FNAIT has been more closely described[105, 106]: The Fc part of the IgG, which binds to Fc receptors (FcγR), and subsequently plays a large role in orchestrating the antibody effects, may have different sugar molecules (glycosylation), which affects this FcγR binding and subsequently the activity of antibody effects. These differences in antibody effects could help explain the varying association between antibody titer and clinical outcome[107-109]. Indeed, it was shown that decreased levels of fucose enhanced *in vitro* phagocytosis of platelets, and was associated with a worse clinical outcome with lower platelet counts and more severe bleedings (n=48); however, no sera from cases of ICH were investigated in the study. These effects can be explained by an enhanced binding to FcγRs with lower levels of IgG core fucosylation. Skewing in fucosylation was only seen for anti-HPA-1a antibodies formed during pregnancy; anti-HLA class I antibodies formed during pregnancy and antibodies from platelet refractory patients had higher levels of fucosylation[105]. Why such changes in fucosylation appear is not known, but it is likely tightly regulated, and could reflect a combination of the unique immunological environment during pregnancy and the HPA-1a antigen[105]. A later article from the same group demonstrated in a larger cohort of samples (n=166), including 26 longitudinal samples, that the glycosylation pattern changed with the first pregnancy, and then remained stable in subsequent pregnancies. By using ICH or fetal death as outcome in a multivariate model, the authors suggested that a combination of fucosylation (decreased levels), galactosylation (increased levels), together with antibody titer could be used to identify risk of severe FNAIT[106]. Such a model needs to be validated in larger cohorts, and could possibly be of use in future screening for FNAIT.
2.8 ANTI-HPA-1a ANTIBODIES MEDIATE PHAGOCYTOSIS IN VITRO

Without direct in vivo evidence, it has been assumed that the main mechanism behind the thrombocytopenia in FNAIT is phagocytosis of platelets; binding of antibody coated platelets to Fcγ receptors (FcγR) on mononuclear phagocytes (for instance macrophages in the liver and spleen) leads to destruction and removal of platelets from the fetal circulation. In vitro experiments have shown that platelets sensitized with anti-HPA-1a antibodies bind FcγRI and FcγRII on neonatal monocytes[110]. The same group later published a study using an in vitro platelet phagocytosis assay. Phagocytosis was found to be dependent on serum concentrations of anti-HPA-1a antibodies and time, and inhibited by IVIG, a monoclonal anti-FcγRI antibody, and cytochalasin D (which binds actin filaments on platelets and thereby hinder the engulfment needed for successful phagocytosis). However, two of the six anti-HPA-1a sera tested did not induce phagocytosis, despite coming from immunized mothers of thrombocytopenic children, which the authors suggest was due to a higher binding and phagocytosis potential in in vivo splenic macrophages compared to monocytes used in the in vitro assay. Anti-FcγRIII antibodies only inhibited phagocytosis mediated by one of the two anti-HPA-1a sera tested. Also, anti-FcγRII antibodies did not inhibit the phagocytosis, which was attributed to the expression of this FcγR on only a certain monocyte subset, suggesting this FcγR could still mediate phagocytosis in vivo[111]. These results suggest that the interplay between anti-HPA-1a and the different Fc-receptors is tightly regulated, and that the extent of phagocytosis can vary considerably between individuals.

2.8.1 Phagocytosis Can Be Affected by Neonatal Factors

A later study confirmed the findings that anti-HPA-1a antibodies could mediate phagocytosis, and showed that CRP could enhance this platelet destruction [112]. The study also showed an elevated CRP level in neonates diagnosed with FNAIT and children with ITP, compared to cord blood of healthy neonates. In a mouse model, high concentrations of CRP and anti-platelet antibodies lead to a decreased platelet count compared to antibodies alone, whereas CRP alone had no effect. This suggests that CRP can enhance phagocytosis mediated by anti-HPA-1a antibodies. As an elevated CRP can be present in neonates for many different reasons (including non-infectious causes such as a prolonged delivery[113]), this could be clinically relevant for FNAIT.

2.9 OTHER POTENTIAL MECHANISMS INVOLVED IN FNAIT

2.9.1 NK Cells

There are other mechanisms than phagocytosis of platelets that can cause thrombocytopenia and bleedings in FNAIT. Firstly, other mechanisms of antibody-mediated platelet destruction have not been thoroughly investigated in FNAIT, and it cannot be ruled out that for instance antibody-dependent cell-mediated cytotoxicity (ADCC) through NK cells (which usually express FcγRIII) is involved. ADCC is an important mechanism of hemolytic disease of the newborn, and in an earlier study ADCC lysis was shown to correlate with anti-A/B IgG3 titers, number of A/B antigen per red cell, and clinical severity of the newborn[114]. It is not
unlikely that NK cells can also kill anti-HPA-1a sensitized platelets, and the degree to which it contributes to the thrombocytopenia in FNAIT has not been investigated.

A recent study demonstrated another possible role of NK cells in FNAIT: Using a murine model of FNAIT, uterine NK cells were implicated in fetal loss and growth restriction[115]. The authors hypothesized that maternal anti-β3 IgG antibodies binding to fetal alloantigen expressed on trophoblast could trigger ADCC and subsequently trophoblast cell death, ultimately leading to pregnancy failure. Immunized mice had splenomegaly with pro-inflammatory Th17 cells, increased plasma levels of pro-inflammatory cytokines, and a skewed profile of pro- and anti-angiogenic factors. The fetuses had impaired placental exchange functions, and the majority died mid gestation. Decidual numbers of uNK cells were increased, with higher perforin and NKp46 expression, and increased degranulation (CD107^+ NK cells), suggestive of cytotoxicity. Interestingly, treatment with anti-NK antibodies reduced number of miscarriages and protected against the placental changes.

There are several drawbacks to the use of this study model (also discussed in chapter 2.9.4). Notably, the mice were only immunized after transfusion of wild type (WT) platelets, not after the first or second pregnancy after breeding with a WT mouse, which was used as control. This is in contrast to a human setting, where immunization after transfusion of HPA-1a positive platelets is rare[116]. Immunization after transfusion can differ from that after pregnancy, considering the different immunological environments. However, this study highlights intrauterine growth restriction as an important aspect of FNAIT, and could pave the way for further studies on human materials. Furthermore, it demonstrates that NK cell mediated ADCC indeed could be implicated in FNAIT, which possibly opens up for new treatment strategies.

2.9.2 Immune Destruction of Platelets May Be Independent of B-cells

Although it might be a stretch to compare with FNAIT, destruction of platelets in platelet refractoriness was recently shown to be antibody and B-cell independent, and rather mediated by CD8^+ T cells in mice[117]: Platelet refractoriness is defined by the lack of an increment in platelet counts after transfusion, and although it occurs in patients without anti-HLA class I antibodies (and vice versa), efforts are going into matching donor and recipient platelets by their HLA class I expression to treat refractory patients. The study immunized mice with mismatched MHC antigens, and found that B cell deficient mice cleared transfused MHC mismatched platelets, and that depletion of CD8^+ T cells in vivo improved the survival of mismatched platelets. This could have implications for the treatment of platelet refractoriness, which often focuses on the antibody component, and perhaps could also be a mechanism behind platelet clearance in FNAIT[118]. Although in many ways a different condition, another hint towards the potential role of cytotoxic T cells in FNAIT comes from idiopathic thrombocytopenic purpura (ITP), where CD8^+ T cells have been shown to be involved through different mechanisms[119, 120]. How and if maternal CD8^+ T cells could lead to destruction of fetal platelets is an open question, but maternal CD8^+ T cells can cause placental villitis[121], which can be found in cases of FNAIT[55] (described in chapter 2.12).
2.9.3 Anti-HPA-1a Antibodies Can Negatively Affect Megakaryocytes

Going back to effects mediated by anti-HPA-1a antibodies, both increased platelet destruction and reduced platelet production could lead to a low platelet count: Megakaryocytes (MKs) are bone marrow progenitors of platelets, regulated by thrombopoietin (TPO) in a homeostatic manner[122, 123], and also express the HPA-1a antigen. In a cell culture model, 14/17 selected anti-HPA-1a sera from FNAIT cases was shown to reduce megakaryopoiesis, and induced apoptosis and cell death of early megakaryocytes (progenitors), while the surviving MKs had a normal maturation. The MK growth in culture correlated with neonatal platelet counts from women undergoing IVIG therapy during pregnancy, and the authors even suggested this to be a potential predictive factor for the effect of maternal IVIG therapy[124]. It is an interesting notion that anti-HPA-1a antibody suppression of MKs could have an additive effect on decreased platelet counts, possibly limiting the body’s normal response to an increased demand for platelets.

2.9.4 Blocking of the β3 Integrin and Bleedings – Differences in Mice and Men

There’s not always a strict correlation between platelet counts and the severity of the bleeding in cases of FNAIT, suggesting a low platelet count alone is not the sole reason for these bleedings. This has recently been elucidated by two studies: The first is based on a mouse model where platelet antigen (β3 or GPIbα) deficient mice were immunized by transfusing wild type platelets, and then bred with wild type mice. It was shown that, although giving similar levels of thrombocytopenia, only anti-β3 sera and not anti-GPIbα sera negatively affected angiogenesis and induced ICH in the pups. The impaired angiogenesis could be rescued by IVIG[125]. A drawback in this study is that the immunization after platelet transfusion and during pregnancy might differ: There will be no exposure of the antigen in the placenta when immunizing with platelets, and the immune response to antigen may differ during pregnancy. Also, the β3 deficient mice were shown to have placental defects and postnatal hemorrhages in the first paper introducing the mouse model, originally developed to study Glanzmann thrombasthenia[126], suggesting that the absence of the β3 integrin itself could also negatively affect the neonatal outcome, even without alloimmunization. More importantly, anti-β3 antibodies may have different effects than anti-HPA-1a antibodies, in that the binding sites and subsequent potential to block the β3 integrin would be different. Further, the mice were injected with anti-β3 sera, which may contain several different types of anti-β3 antibodies. However, following this study came the work on anti-αvβ3 antibodies from cases of FNAIT with ICH previously mentioned[57], demonstrating the clinical relevance of these striking mouse model findings. Although contrasting to the mouse study, anti-β3 antibodies did not negatively affect human endothelial cells (only anti-αvβ3 antibodies had this effect). Together, these studies suggest that negative effects on endothelial cells, and subsequent vascular impairment, likely play an important role for bleedings in FNAIT.
2.9.5 Effects of Anti-HPA-1a Antibodies on Platelet Function

Platelets are essential for successful coagulation, which can be summarized in three overlapping stages: Initiation, where platelets bind vWF, collagen or tissue factor exposed on a cell surface; amplification, with activation of platelets and co-factors; and propagation, where large amounts of thrombin are generated on the activated platelet surface [127]. It has been hypothesized that another possible explanation for the occasional dissociation between neonatal platelet counts and presence of severe bleedings in FNAIT could be the antibodies’ effect on platelet function, as the anti-HPA-1a alloantibodies target their epitope on the fibrinogen receptor - an important functional platelet glycoprotein.

![Figure 7: Simplified overview of platelet activation and thrombus formation.](image)

Fibrinogen Binding and Platelet Activation

Fibrinogen is a large molecule with multiple interaction sites that can bind to several receptors, and among these, integrin αIIbβ3 [129] and αvβ3 [130] are the best characterized. Apart from its role in maintaining hemostasis by mediating platelet aggregation, clot formation, and wound healing, it can, together with fibrin, also function as a component of extracellular matrix in other processes, supporting placental development, angiogenesis, atherosclerosis, and metastasis [131].

On resting platelets, only immobilized fibrinogen binds to the GPIIb/IIIa, but upon activation induced by ADP, thrombin, or collagen, an active conformation of the receptor will allow for
increased binding of fibrinogen. Fibrinogen binds to the active GPIIb/IIIa with a dissociation constant (Kd) of 100 nM, 100 times lower than the concentration of fibrinogen in plasma, which suggests immediate binding to the receptors – the binding activity of GPIIb/IIIa therefore needs to be tightly regulated[132].

On the integrin αIIbβ3, fibrinogen has two binding sites, and two signaling events act in synergy to activate the platelet and promote cell spreading to surface-bound fibrinogen [131]: An initial binding to the αIIb subunit generates signaling events that lead to clustering of αIIbβ3 in focal complexes, as well as intracellular signals (FAK tyrosine phosphorylation and Rac1 activation). The second signaling event is the binding of fibrinogen to β3 (RGD-β3 interaction), which relies on receptor activation. Conformational changes following this interaction allows for a second wave of intracellular signals (such as RhoA activation, which is required for cell spreading).

In addition to cell spreading, fibrinogen binding initiates outside-in signaling that ultimately leads to activation of the platelet with the release of granules (Figure 8). These granules contain adhesion proteins, nucleotides, and enzymes (such as ADP, ATP, vWF, fibrinogen, coagulation factors, cytokines, serotonin, histamine) in different compositions, depending on granule type. The release of these mediators will together with platelet-platelet interactions amplify the activation of the platelets, recruit circulating platelets into aggregates, and stabilize the thrombus[133].

Considering the distal location of the HPA-1a epitope in relation to the fibrinogen binding site on GPIIIa[134], any inhibitory effect on platelet function could likely result from a form of allosteric hindrance of fibrinogen binding. Decreased fibrinogen binding could decrease the release of granules, which can be reflected by decreased CD62-P expression, and interfere with platelet-platelet interaction, which can be reflected by decreased aggregation. Such effects have been evaluated in a handful of studies, described below.
2.9.6.1 Functional Effects of Recombinant Anti-HPA-1a Antibodies

In a study from 2004, effects of a recombinant human IgG1 anti-HPA-1a antibody (B2GI), designed as a potential therapy for FNAIT, were tested on adult platelets using several assays[135]: For HPA-1ab platelets, the antibody decreased aggregation measured by the PAP-4 aggregometer (which measures alterations in light transmission) with ADP as stimulus (and not collagen) by 50%, and in a whole blood perfusion assay, platelet adhesion to slips covered with fibrinogen was inhibited by about 20% (again, no effect on collagen-coated surface). Closure time was not affected for HPA-1ab platelets, while HPA-1aa platelets were inhibited. In a whole blood flow perfusion assay, αvβ3- and αIIbβ3-mediated cell adhesion to fibrinogen was also measured, using transfected Chinese hamster ovary (CHO) cells: αIIbβ3-Leu33 or αvβ3-Pro33-mediated cell spreading was not affected by pre-incubation with the antibody, but the antibody affected αβ3-Leu33 (HPA-1a) mediated cell spreading and attachment.

In summary, while the study showed pronounced effects on HPA-1aa platelets in some of the assays, few and mild effects were demonstrated on HPA-1ab platelets, and the most pronounced effects was for aggregation with a weak stimulus (ADP), and for assays testing fibrinogen binding to platelets. Although this study thoroughly investigated antibody effects
on platelet function (and possibly also laid some of the foundation for the later anti-αvβ3 discoveries), it is limited by the use of adult platelets only, and recombinant antibodies may have different effects than (polyclonal) alloantibodies in vivo.

2.9.6.2 Human Anti-HPA-1a Antibodies

Another study, where the primary aim was to produce a human monoclonal anti-HPA-1a, also described inhibitory effects on platelet aggregation[56]. The investigators incubated adult whole blood anti-coagulated with hirudin with the antibody, and measured aggregation using the Multiplate (which measures changes in electrical resistance with aggregation of platelets). HPA-1ab platelet aggregation was reduced 20% compared to the control (no antibody).

In a study from 1994[136], binding of radiolabelled fibrinogen to washed and stimulated adult HPA-1aa platelets was investigated in the presence of sera from mothers of children with FNAIT, and patients with post transfusion purpura (PTP), both containing anti-HPA-1a antibodies. Surprisingly, while 6/7 PTP sera inhibited fibrinogen binding, none of the nine maternal sera affected fibrinogen binding. The use of sera and not purified IgG may have affected the results, and anti-HPA-1a titers were low for the FNAIT sera.

In an interesting study from 1984[137], plasma from two immunized mothers were used to investigate effects on platelet function. Effects of plasma, isolated anti-HPA-1a IgG, and the epitope-binding F(ab) and F(ab')2 fragments of IgG were investigated. Aggregation, measured by changes in light transmission, was inhibited in a dose-dependent manner by plasma when induced by ADP and collagen, but not by ristocetin (a more potent stimulus compared to ADP and collagen), while change in platelet-shape with stimulation was observed to be normal. Anti-HPA-1a plasma also inhibited ADP-induced clot retraction. Interestingly, binding of radiolabelled fibrinogen was reduced down to similar levels as patients with Glanzmann’s thrombasthenia (GT), who lack an effective fibrinogen receptor. The authors suggested that indeed these anti-HPA-1a antibodies could induce a similar platelet phenotype as in GT patients, and could ascribe these changes to the epitope binding of anti-HPA-1a antibodies by using F(ab) and F(ab’2) fragments of IgG. The authors reported similar effects on HPA-1a heterozygous and homozygous donors. One of the major findings of the study at the time was actually the association of HPA-1a antibody binding to the fibrinogen-binding site, which suggested that fibrinogen bound to the GPIIIa.

Since we now can assume that there are different types of anti-HPA-1a antibodies, this could perhaps also explain some of the heterogeneity in studies examining the effect of these antibodies using maternal sera. In a study from 2005, sera from FNAIT cases (n=43) and PTP cases (n=8) were used to examine inhibition of cell adhesion to fibrinogen[138]. Of the FNAIT sera, only two specifically inhibited HPA-1a cells. In both of these cases the children had severe bleedings, but this also occurred in sera without inhibitory effects. Also, two other sera blocked both HPA-1a and HPA-1b cells. The authors suggested that these different effects could be the result of alloantibody maturation, starting from unspecific inhibition to allospecific inhibition, and finally to no inhibitory capacity, and that the timing of sampling could explain the low number of inhibitory alloantibodies. It is difficult to know whether such
maturation would follow this pattern with regards to inhibitory capacity or not; the HPA-1a epitope is distal to the fibrinogen binding site[134], but possibly there can still be a form of allosteric hindrance with the increased binding affinity of “matured” anti-HPA-1a antibodies. Moreover, differences in the composition of polyclonal anti-HPA-1a antibodies in the sera could possibly affect the level of this allosteric hindrance.

It is evident from these studies that anti-HPA-1a antibody likely can affect platelet function, and that a monoclonal anti-HPA-1a antibody (or antigen-binding fragments of such antibodies) is most suitable for further mechanistic studies of these effects, although this approach is more difficult to translate into the in vivo setting.

2.9.6.3 Activating Effects of Anti-HPA-1a Antibodies

Platelet antibodies may also have activating effects, by clustering and cross-linking of Fc receptors (demonstrated for anti-GPIb moAbs[139]), antibody-mediated complement activation [140], or direct binding to and triggering of a signal-transducing antigen. However, few studies on this are performed, and the antibody effects have shown great heterogeneity, possibly due to differences in platelet activation, and presence of other antibodies (such as anti-HLA class I antibodies) in the sera [141]. In a study from 2001, anti-HPA-1a sera from immunized mothers and PTP patients were incubated with HPA-1aa platelets, and the release of RANTES (regulated on activation, normal, T-cell expressed, and presumably secreted/CCL5 – a chemotactic) measured; only two out of eleven FNAIT sera induced release of RANTES in HPA-1aa platelets, which could be blocked by anti-FcγRII antibody[142]. Only HPA-1aa and HPA-1bb platelets were tested in the study, so the relevance to FNAIT is difficult to ascertain, but theoretically this effect could be present in a lesser degree in FNAIT.

2.10 NEONATAL HEMOSTASIS

Another important aspect in FNAIT is the neonatal platelet function itself, which may differ from adults, and render them more vulnerable for potential antibody effects. Newborn hemostasis is thought to evolve with gestational age, so called “developmental hemostasis”: Concentrations of coagulation factors and proteins increase during pregnancy, and while levels of vitamin K-dependent factors (II, VII, IX, X), contact-dependent factors (XI, XII), and thrombin are lower than adult values, neonates also have lower levels of inhibitors of coagulation and fibrinolysis, which ensures hemostatic balance[143]. With higher levels of hematocrit, presence of large nucleated red cells and increased levels and function of von Willebrand factor (vWF), primary hemostasis is thought to be enhanced in neonates [143, 144].

2.11 NEONATAL PLATELET FUNCTION

As opposed to quantifying coagulation factors, functional studies of platelet function require relatively large volumes of blood, and so there are a limited number of such studies on
neonatal platelet function. However, when using flow cytometry very small amounts of blood are needed, and in a study from 2009, in vitro reactivity of neonatal platelets was shown to be decreased compared to adults: Neonatal platelets had a lower expression of CD62-P (P-selectin, released from α-granules upon stimulation) and PAC-1 (activated GPIIb/IIIa) after stimulation with thrombin receptor activating peptide (TRAP). Neonatal platelets had a higher CD62-P and a lower PAC-1 expression on resting platelets. They also had a lower GPIIb/IIIa (CD41), GPIIIa (CD61), and GPIb (CD42a) expression, and these tendencies were more pronounced with lower gestational age. In term neonates, the expression of CD62-P in response to TRAP increased during 12 days after delivery (from less than 20% to ca. 50% positive platelets)[145].

Another type of more functional studies is platelet function tests such as platelet function analyzer (PFA-100), cone platelet analyzer (CPA) and rotating thromboelastogram (ROTEM). Neonatal (n=17) blood has been shown to give a shorter closure time than adults (n=31) with the PFA-100[146], while platelet function measured by CPA was shown to correlate with gestational age (total n=231)[147]. Recently neonatal reference ranges for ROTEM were established, and using cord blood from 184 term and 47 pre-term neonates, clotting time (CT) and maximum clot firmness (MCF) was found to be lower in neonates compared to adult reference values[148].

To summarize, although there is a limited number of studies, it appears that neonatal platelet function in vitro is reduced compared to adults, and that compensatory mechanisms such as enhanced primary hemostasis could explain why newborns normally don’t have an increased bleeding tendency. However, this balance could be vulnerable, and may perhaps be affected by conditions such as FNAIT, where anti-HPA-1a antibodies may interfere with hemostasis.

2.12 CLINICAL MANIFESTATIONS OF FNAIT

In the absence of screening programs, most cases of FNAIT are diagnosed when a newborn presents with petechiae or other signs of bleedings. In addition to thrombocytopenia and potentially severe bleedings, presence of anti-HPA-1a antibody has been shown to be associated with fetal growth restriction in boys [53], which also could give long-term consequences for the child[149]. FNAIT is also likely to cause miscarriages and fetal loss[150], but it is unknown how often this is caused by FNAIT.

Smaller, retrospective cohorts of untreated FNAIT have revealed placental findings characterized by chronic inflammatory reactions, such as chronic chorioamnionitis and villitis[54, 55]. Chronic villitis is associated with IUGR, and suggested to be a mark of breach of fetal-maternal tolerance[121, 151]. In light of the recent study on uNK cells mediating IUGR in mice suffering from FNAIT [115], such mechanisms should be further addressed in a human setting, with larger cohorts of patients.
2.12.1 Incidence of Intracranial Hemorrhages Associated with FNAIT

Intracranial hemorrhage has been considered the most feared complication of FNAIT, as it can be life threatening and give lifelong impairment. In contrast to the focus on risk of ICH in FNAIT, the true incidence of ICH due to FNAIT is unknown, as most screening studies have included interventions to avoid bleedings[7]. A systematic review from 2014 on incidence of FNAIT based on prospective screening studies of low-risk unselected newborns, found a pooled incidence of ICH due to FNAIT at 6 per 100,000 newborns. In total, almost 60,000 newborns were screened for thrombocytopenia, and all cases of ICH appeared to have occurred in utero[152].

2.12.2 Retrospective Studies on ICH and FNAIT

The largest study describing cases of FNAIT with ICH to date is based on an international multicenter registry, and reviewed all diagnosed cases of FNAIT with ICH over a nine-year period[153]. Most of the bleedings occurred early, before 28 weeks of gestation (gw) (23/43, 54%), and affected the first-born child (27/43, 63%). Where the types of bleedings could be described, 15 were classified as intraparenchymal, and 13 as intraventricular/periventricular hemorrhage (IVH/PVH). Some cases had multiple bleeding episodes, or a mix of different types of bleedings. In the cases with bleedings before 28 gw, all except one were classified as IVH/PVH. The bleedings in this study were associated with a severe outcome; only 5 (12%) were discharged alive and well, 6 (14%) were stillborn, 9 (21%) died after delivery, and 23 (53%) were discharged with neurological impairment. The study has the limitation of being a retrospective study; without any screening for platelet alloimmunization, neonatal thrombocytopenia, or presence of intracranial hemorrhage (which may be asymptomatic[154]), only clinically recognized cases are included, and so probably only the most severe cases of ICH due to FNAIT were described. The study shows these bleedings may occur early in pregnancy, which could be of importance for any future screening and intervention program, and could also fit with the development of the fetal brain - perhaps the neonates would be more vulnerable for antibodies targeting endothelial cells earlier in gestation. The fact that most of the cases were premature, could also point towards multifactorial causes of bleeding.

2.12.3 Predictors of ICH

As maternal platelet alloimmunisation can manifest as anything from a healthy newborn without thrombocytopenia, to asymptomatic thrombocytopenia without bleedings, symptomatic thrombocytopenia with minor bleedings, and to thrombocytopenia and life-threatening bleedings, it is important to predict in which cases a severe bleeding such as an ICH is more likely. Unfortunately, the strongest predictors for an ICH is number of gestations[155] and a previous child with ICH[156]. For primiparous women, antibody levels is of limited use[107], but they may together with HLA class II typing help predict a severe ICH and response to IVIG therapy[155]. Factors that potentially could be used to predict severe FNAIT are summarized in Table 1.
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<th>Factors That Could Predict Severe FNAIT (ICH)</th>
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<tr>
<td>Previous ICH</td>
<td>Important predictor in subsequent pregnancies [155, 156]</td>
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<tr>
<td>Presence of anti-HPA-1a subtypes (anti-αvβ3)</td>
<td>Probably of great importance, but only one clinical study [57]</td>
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<tr>
<td>Glycosylation pattern</td>
<td>Strong evidence, but needs to be shown in larger, prospective cohorts of cases [106]</td>
</tr>
<tr>
<td>Antibody titer</td>
<td>Variable effect [107, 109, 157], could be useful in subsequent pregnancies</td>
</tr>
<tr>
<td>HLADRβ3*0101 positive mother</td>
<td>Probably necessary for efficient immunization, not useful for predicting severity of FNAIT [158, 159]</td>
</tr>
<tr>
<td>Number of gestations</td>
<td>Uncertain variable, and not useful in the first pregnancy</td>
</tr>
<tr>
<td>IgG3 polymorphism</td>
<td>Rare polymorphism, could lead to increased exposure of anti-HPA-1a antibodies to the fetus</td>
</tr>
<tr>
<td>IVIG response in MK/anti-HPA-1a culture</td>
<td>Likely not feasible, and uncertain</td>
</tr>
</tbody>
</table>

*Table 1: Potential predictors of severe FNAIT.*
2.13 SUMMARY

FNAIT is a complex condition. Exposure of the antigen to the maternal immune system and its response, the different types of antibodies, and the fetal development and reaction to alloantibodies all likely determine the neonatal outcome. Still, central immunological aspects remain uncertain, and the potentially severe consequences of FNAIT calls for further research.

Figure 9: Overview of possible mechanisms involved in FNAIT. Created using www.dagitty.net.
3 AIMS OF THE THESIS

The two main aims of the thesis was to use both clinical and experimental data to investigate possible underlying mechanisms of FNAIT, and to evaluate the impact of FNAIT on neonatal morbidity in Sweden by investigating the frequency of maternal platelet alloantibodies in newborns diagnosed with an intracranial hemorrhage (ICH). More specifically, the aims were:

• To further describe anti-HLA class I antibody specificities and levels in cases of clinically suspected FNAIT, to shed light onto whether anti-HLA class I antibodies could cause FNAIT in some cases (paper I and II)

• To describe clinical characteristics of cases of suspected FNAIT where only anti-HLA class I antibodies are detectable (paper I and II)

• To determine whether certain fetal-maternal mismatches in HLA class I genotype is associated with neonatal thrombocytopenia (paper II)

• To investigate the HPA-1a epitope expression on neonatal platelets, and further investigate neonatal platelet function with regards to FNAIT (paper III)

• To investigate whether anti-HPA-1a antibody could affect platelet function, and generate hypotheses that can inform future studies investigating such effects (paper III)

• To investigate the prevalence of platelet alloantibodies in mothers of newborns born from 32 weeks of gestation and diagnosed with an ICH in Sweden (paper IV)

• To describe clinical characteristics of neonates diagnosed with an ICH born from 32 weeks of gestation (paper IV)
### 4 RESULTS AND DISCUSSION STUDY I AND II

Both study I and II spring from the same underlying research question of whether anti-HLA class I antibodies could be a cause of FNAIT. As a means to elucidate this underlying question we aimed to describe the antibody response and clinical data associated with cases of suspected FNAIT with only HLA class I antibodies detectable. The two studies share similarities in approach and case selection, and while the results are mainly in consonance, study II also provides a potential resolve to some of the issues from study I.

#### 4.1 SUMMARY OF MAIN FINDINGS STUDY I

In paper I, the main findings can be briefly summarized as follows: Twenty-three cases from 260 national referrals for suspected FNAIT from January 2007 to March 2012 were included (Fig. 1). The cases had an increased antibody reactivity towards HLA class I antigens compared to both control groups – representing that of a normal pregnancy (control mothers, n=33), and background levels after previous immunizations (blood donors, n=19). This was quantified by comparing the number of HLA class I antigen coated beads with SFI > 9999, and by comparing median SFI levels for all reactive antigen beads (HLA-ABC SFIs). For cases and control mothers, the SFI levels were highest for antibodies targeting HLA-B antigens, compared to HLA-A and -C, although only the HLA-A vs. -C comparison reached statistical significance. Blood donors had a tendency towards increased reactivity against HLA-C antigens. For the particular antibody specificities, only two were more frequent among cases (A*03:01 and A*66:02), and reactivity against all antigens were seen in both cases and control mothers. Most of the specificities were directed against very rare HLA class I antigens. The available clinical data revealed a case population with a high morbidity, and the majority of selected cases actually had other factors that could contribute to thrombocytopenia.

#### 4.2 SUMMARY OF MAIN FINDINGS STUDY II

The results from paper II are in many aspects similar to paper I: The cases were based on national referrals over an eleven-year period (n=537), and selected on the basis of anti-HLA class I antibodies and available clinical data. The final study population consisted of 50 cases (Fig. 1). Controls were samples from normal pregnancies (n=60). Similarly to paper I, the cases had higher antibody levels compared to controls, as quantified by comparing number of specificities with MFI > 10 000. Broad reactivity patterns were also apparent in this study population, but could be resolved by applying data on HLA class I epitope expression; the reactivity proved to be mainly specific against paternally-inherited epitopes, with little reactivity against maternal self or third-party antigens (n=33 for genotyping data). As in paper I, cases and controls reacted against the same antigen beads, there were no particular mismatches that were associated with immunization among the cases. However, with the addition of genotyping data, it could be demonstrated that cases with a more severe outcome had a tendency towards higher reactivity against paternal antigens, and that multiparous mothers had significantly higher MFI levels of paternal-specific antibodies. Comparing to the
background population, the case population had similar HLA class I allele frequencies, though with a higher frequency of HLA-A*31 and HLA-B*27, which should be interpreted with caution considering the disparate sample sizes. The clinical data revealed a population with severe thrombocytopenia, and a high frequency of ICH at 10%. Although compared to the population described in paper I, this case population apparently had fewer factors that also could explain thrombocytopenia, there was a high frequency of caesarean sections (50%), preeclampsia (13%) and small for gestational age neonates (48%).

4.3 DISCUSSION

4.3.1 Increased Reactivity Against HLA-B Antigens

One of the main findings in both studies is the increased level of antibodies directed against HLA-B antigens. In study II, 97% of cases had at least one mismatched HLA-B antigen between mother and neonate (likewise, 79% had a HLA-A antigen mismatch). These two findings may be explained by the high degree of polymorphism in the HLA-B alleles; to date, almost 5,000 alleles have been described (hl.alleles.org, retrieved 17-09-10), which means a mismatch between mother and neonates is almost inevitable. Furthermore, there is some evidence that HLA-B antigen is expressed earlier than HLA-A on fetal villous stromal cells in human placenta, demonstrated by use of immunohistochemistry[68], which could imply that immunization against HLA-B may occur earlier, and therefore could give rise to a more potent immune response. In both papers, the reactivity against HLA-C antigens were much lower than that directed against HLA-A and -B, and in paper II, most of the reactivity against HLA-C could be ascribed to epitopes shared with HLA-A and -B antigens. This is in line with the low expression of HLA-C antigens on most cells[160], including platelets[161], but in contrast to the HLA class I antigen expression in the placenta – where HLA class I is mainly expressed as HLA-C, -E, and -G on extravillous trophoblast (EVT)[162]. This implies that other cells than EVTs are the likely source of immunization.

4.3.2 Nulliparity

If EVTs were not the source of immunization, this would indicate that also other cells were available to the maternal immune system during pregnancy. Many of the case mothers (9/16 in paper I, and 21/47 in paper II) were nulliparous – it is therefore likely that the immunization against cells other than EVTs occurred during pregnancy, and not due to a delivery or a secondary immune response in a subsequent pregnancy. Transfusion and transplantation can also lead to HLA class I immunization, but is rare in women of fertile age, and seems an improbable explanation to the presence of anti-HLA class I antibodies with high MFI/SFIs in these cases. We selected our cases based on the presence of anti-HLA class I antibodies, which are known to occur more frequently with increased parity[74], the high frequencies of nulliparous mothers is therefore somewhat unexpected. On the other hand, an increased morbidity among neonates of immunized nulliparous mothers is seen in RhD immunization[163], indicating that a breach of maternal tolerance may be more severe in the first pregnancy. Nulliparity is also a risk factor for preeclampsia[164, 165], and associated with increased neonatal mortality and SGA neonates[166]. The passage of fetal cells into
maternal circulation may enhance tolerance in subsequent pregnancies[36], and lack of such an effect might be one possible explanation for the high frequency of nulliparous mothers in our materials, and could also be in line with the high antibody reactivity.

4.3.3 Higher Levels Rather Than Unique Specificities Compared to Controls

Another important finding in these studies is that both case groups had antibody specificities that were also present in the control groups, implying that the antibody levels, reflected by the high SFI/MFI values, and not certain specificities, could be associated with a worse outcome among the cases. This is in line with a retrospective study of FNAIT cases from 2016[155], where the group with ICH had higher levels of both anti-HPA-1a and neonatal antigen-specific anti-HLA class I antibodies compared to the anti-HPA-1a immunized group without ICH (although the anti-HLA class I antibody comparison was not statistically significant). In paper I, the antibody levels were quantified as a median value of all reactive beads with SFI>999. In paper II, we could further distinguish beads with paternal-specific epitopes, and compare the MFI values on beads coated with such epitopes versus those without; we found that beads without mismatched epitopes had lower MFIs (Fig. 4), higher paternal-specific MFI levels in multiparous mothers (Fig. 5b), and a tendency towards a increased paternal-specific MFI associated with lower platelet counts in the neonate (Fig. 5a). These findings strengthened the relevance of the higher MFI values in cases compared to controls.

4.3.4 Correlation to Clinical Data

In paper I, both cases with and without signs of bleeding had severe thrombocytopenia, and there was no correlation between platelet count and SFI values. Though not significant, there was a tendency towards lower SFI values in the cases with bleedings excluded, but this analysis was hampered by the fact that 9/23 cases had missing or inadequate data available on bleeding outcomes (Table 2). SFI levels correlated with increased maternal age, but there was no statistical significant difference in levels comparing nulliparous and multiparous mothers. In paper II, such a difference could be demonstrated, as multipara had higher fetal/paternal-specific MFI values. There was also a tendency towards higher specific MFI values in cases with ICH, but again, missing data made the comparison flawed, since the diagnosis could not be ruled out in 13/50 cases, and only 2 of the ICH cases had genotyping data available (Fig. 5). One interesting observation was the decreased platelet counts in cases with mismatched HLA B-27, and the high frequency of the HLA-B27 antigen among the case mothers; HLA-B27 is associated with several autoimmune diseases[167], and this finding should also be tested in larger materials.

4.3.5 Translating High Reactivity in Luminex to Biological Relevance

An important question is how these increased antibody levels as measured using Luminex can be translated into effects in vivo. One major limitation of the Luminex assay is that the antigens expressed on the beads may have undergone conformational changes, and antibody
binding in vitro may be affected by both loss of epitopes and unnatural expression of cryptic epitopes; there is therefore a chance that antibody binding in the Luminex will not reflect the true binding of these antibodies in vivo[168]. This is also suggested to be one explanation to the poor correlation between IgG MFI levels and complement binding measured in Luminex[169, 170], an assay that can be used in transplantation settings. To confirm the biological relevance of the reactions in Luminex, one could also add other, cell-based, methods of measuring anti-HLA class I antibody binding, such as the complement-dependent cytotoxicity assay (CDC)[171], and flow cytometry cross-matching (FCM)[172]. However, in CDC only complement-activating antibodies are detected, and FCM may have “false” positive results of no relation to outcome (as investigated in a transplantation setting)[173].

Aside from different ways of measuring the presence of antibodies, another approach to confirm the relevance of such findings is to predict the binding affinity of the fetal-paternal HLA class I peptides to maternal HLA class II molecules. This is attempted by the “predicted indirectly recognizable HLA epitopes presented by HLA class II” (PIRCH-II) algorithm, but is only available for the HLA-DRB1 alleles. Increasing PIRCHE-II numbers were associated with an increased percentage of mismatched HLA class I antigens that had elicited antibody production, but highly immunogenic HLA molecules had similar PIRCHE-II numbers as less immunogenic molecules[174].

Another possibility is to investigate other effector functions of HLA class I antibodies, in addition to complement binding. In anti-HPA-1a alloimmunization, decreased fucosylation has been shown to be associated with enhanced phagocytosis of platelets[105], and while this was not shown for anti-HLA class I antibodies formed after pregnancy, it has to date not been investigated in other cohorts. Along that line, it could be interesting if certain anti-HLA class I antibody specificities could also bind endothelial cells, and if this could mediate a higher risk of ICH, similar as with anti-αvβ3 antibodies. There were several neonates with ICH among our cases: 2/23 neonates in paper I, and 5/50 in paper II. Although these cases were also severely thrombocytopenic, it is known from the transplantation setting that anti-HLA class I alloantibodies may induce vascular damage[175], and it would be interesting if also maternal anti-HLA class I alloantibodies could have similar effects in some cases.

A central question that arises from studies on anti-HLA class I antibodies and FNAIT is why only platelets are affected by the presence of these antibodies. In contrast to HPA antigens, HLA class I is expressed on near all nucleated cells. If present in the fetal circulation, anti-HLA class I antibodies should therefore be able to bind fetal cells and tissues indiscriminately. There is considerable intra- and interindividual variation in the platelet HLA class I antigen expression [176-180], but due to the relative abundance of platelets compared to other cells in the circulation, it is estimated that two thirds of the HLA class I antigen in blood is carried by platelets[75], and one possible explanation is that they may function as a “buffer” for the circulating anti-HLA class I antibodies.
4.3.6 Limitations

The major limitation to both papers is the relevance of the controls groups. Neither of the control groups was matched with regards to age or parity. In paper II, we only had access to genotyping data for 5 controls, which hindered an adequate comparison of antibody binding to beads expressing fetal/paternal specific epitopes. Furthermore, the study would have benefitted from genotyping data on the fathers, as unresolved cases could have been due to antibodies directed against paternal antigens that the fetus did not inherit. There is probably also an effect of selection bias in both studies, and different sampling times between cases and controls could possibly have affected the comparisons, and also hinders any causal inference (as discussed in the chapter on methodology). Another limitation is that the Luminex assay is only a semi-quantitative method, although it is often interpreted in a quantitative manner.

4.4 IMPLICATIONS

These two studies demonstrate that although rare, suspected FNAIT associated with anti-HLA class I antibodies are cases with severe clinical outcomes. However, both studies suffer from significant limitations, and to be able to answer the underlying research question of causality, prospective studies are needed. Genotyping data and the epitope mapping approach used in study II proved essential to evaluate the antibody reactions, and would be useful to implement in any prospective studies, as it ensures that the antibodies evaluated are in fact specific against paternally inherited antigens. We could demonstrate such paternal specificity for our cases in study II, and this supports the hypothesis that FNAIT could be caused by anti-HLA class I antibodies. However, it is important to keep in mind both the high frequency of these antibodies in healthy pregnancies, and that our cases also had other factors that could mediate thrombocytopenia.
5 RESULTS AND DISCUSSION STUDY III

To investigate a possible role of reduced platelet function in FNAIT, this project consisted of two main parts - to compare neonatal platelet function to adult, and to investigate the potential effects of maternal anti-HPA-1a antibodies on fetal platelets. The comparisons of neonatal and adult platelet function confirm and expand upon notions from previous studies, and is an important backdrop to the potential effects of anti-HPA-1a antibody on platelets in FNAIT. The antibody effects were tested using different approaches, and when utilizing a monoclonal anti-HPA-1a antibody, we could demonstrate a decreased fibrinogen binding, which could affect the neonatal hemostatic balance.

5.1 SUMMARY OF MAIN FINDINGS

Functional assays on platelet function in adult and cord blood revealed differences in aggregation, as measured in Multiplate, a reduced change in expression of glycoproteins with activation, as measured in flow cytometry, and discrete differences in hemostatic functions, as measured in Rotem. We also evaluated the HPA-1a epitope expression, by comparing MFI's from an anti-CD61 antibody specific for the HPA-1a allele; we found that there was a tendency towards increased expression in neonates as compared to adults, and that the expression increased with activation of the platelets. Together, these data point towards a decreased in vitro platelet reactivity of neonatal platelets, while the limited differences in Rotem, which evaluates the hemostatic function more as a whole since it depends on both the platelets and coagulation factors, hint towards compensatory mechanisms and enhanced neonatal hemostasis. In the presence of a monoclonal anti-HPA-1a antibody, fibrinogen binding was reduced, and the effect corresponded to the HPA-1a antigen expression as expected. However, this reduction in fibrinogen binding seemed to have limited functional consequences, as measured by CD62-P up-regulation.

5.2 DISCUSSION

5.2.1 Hypo-Responsive Platelets and Enhanced Primary Hemostasis in Neonatal Blood

The decrease in platelet reactivity observed here and in other studies[181] could be due to multiple mechanisms. Neonatal platelets have recently been shown to have a decreased number of dense granules[182], with a lower concentration of ADP and serotonin[183]. The release of these dense granules upon stimulation with thrombin has also been shown to be lower in neonates compared to adults[184]. Reports have also shown impaired signal transduction pathways in neonatal platelets, affecting the ability to mobilize intracellular calcium, G-protein mediated responses, and the pathways that involve thromboxane A2 (TXA2) synthesis[185]. The impairment of these signaling pathways can result in a reduced up-regulation of adhesion receptors (such as p-selectin), and a lower production of TXA2 with activation, which again can contribute to the hypofunctionality[186].
Despite these differences in in vitro platelet reactivity, neonates normally have no increased bleeding tendency, and actually have an enhanced primary hemostasis, as measured in PFA-100[187] and Rotem[148]. The enhanced primary hemostasis has been attributed to higher levels of vWF, presence of high-molecular weight vWF multimers, higher hematocrit levels[143], and higher mean corpuscular volume (MCV)[186, 188]; most other hemostatic factors are decreased in neonates compared to adults, except for increased levels of FVIII immediately after birth, and unchanged levels of FXIII[189]. The high vWF levels may enhance adhesion capacity, and an all together more efficient primary hemostasis may compensate for decreased platelet reactivity[186].

Our samples of adult and neonates also reflect these previous findings: Though with decreased platelet reactivity and aggregation (Fig. 1, Fig. 2, Table 1), we found enhanced primary hemostasis reflected by shorter clotting times in Rotem (Supp. Fig. 1). We did not evaluate the concentrations of coagulation factors, but the neonates had statistically significant higher MCV and hematocrit levels (Supp. Table 1). The neonates also had higher platelet counts (not statistically significant), and higher lymphocyte counts, which may enhance aggregation[190]; this might have helped balanced out differences in platelet reactivity. Conversely, red blood cell counts and mean platelet volumes (MPV) were reduced in the cord blood. In our data, there was no correlation with MPV and aggregation (Suppl. Fig. 2), although such correlations have been suggested in older studies[191, 192].

5.2.2 Results From Experiments with Maternal Plasmas

To investigate whether anti-HPA-1a antibodies could affect platelet function, we tried several approaches. For set-up experiments, we first tested the standardized, pooled anti-HPA-1a plasma (NIBSC) using Multiplate on healthy adult donors and cord blood, with ADP and TRAP as stimuli: This gave variable results, with even increased aggregation in some samples. AB serum induced variability in aggregation with a different kinetic in the aggregation curves (Figure 10), and proved inappropriate as a control (CV% 1-21% vs. 3-6% without AB serum). Overall the trend was a decreased aggregation with NIBSC compared to normal tests (no serum, PBS), but with less effect than AB serum (Figure 11). Rotem analysis showed a shortening of clotting time with NIBSC, for both adult and cord blood (data not shown).
Due to the inappropriate controls and variable effects, we next tried maternal plasmas containing anti-HPA-1a antibodies at different concentrations (sample B1, 43 IU/mL; sample B2, 130 IU/mL; sample B3, 258 IU/mL), with AB plasma from a single donor as control. Readouts were aggregation in Multiplate, Rotem parameters (CFT, CT, MCF), and CD62-P up-regulation in flow cytometry. Three adult donors of each HPA-1a genotype were tested (Figure 12). Compared to the controls (AB plasma and PBS), there was a decrease in aggregation, but the effect was unspecific with regards to HPA-1a type, as both HPA-1a positive and negative platelets were affected. For two donors we also added the donor’s own EDTA plasma as an extra control, which did not affect aggregation (data not shown). The decrease in aggregation with maternal plasmas did not correlate with binding of the antibodies, as HPA-1a positive platelets had a tendency towards higher anti-IgG stainings than HPA-1bb platelets, and one plasma with low anti-IgG staining (B1) also severely affected aggregation. In anti-CD62-P stainings, we found increased expression of CD62-P on unstimulated platelets, but a lower CD62-P increase with TRAP stimulation, on both HPA-1a positive and negative platelets.

Unpurified plasma may contain IgM, anti-HLA class I antibodies, different levels of platelet activators such as ADP and fibrinogen, and other factors that could have affected these results. We therefore also tested purified IgG from maternal plasmas, that were screened negative for anti-HLA class I antibodies, with the elution buffer as control. In these experiments the anti-IgG staining was also unspecific; the purified IgG bound to both HPA-1aa and HPA-1bb platelets, and there was a reduced CD62-P up-regulation with TRAP stimulation on both platelet types (data not shown). For these experiments, the total IgG concentration was ca. 120 μg/mL (measured by spectrophotometry[193]), but the final anti-HPA-1a IgG concentration was unknown.
To summarize, results from these experiments indicated that while there was some inhibitory effect on the platelets with anti-HPA-1a antibodies, the selected controls were not appropriate, and the different plasmas containing anti-HPA-1a antibodies gave unspecific effects with regards to HPA-1a type. This is in line with previous studies on platelet function performed with plasmas[138]. The unspecific effects may possibly be explained by anti-HPA-1a antibodies with lower affinity; in addition to the presence of IgG with other specificities, this might be enough to block high affinity antibodies from binding to HPA-1a, while simultaneously the binding may be too weak or unspecific to elicit a substantial blocking effect restricted to HPA-1a positive platelets. Furthermore, undisclosed factors present in plasma may have affected the results. Although not reflecting the (patho-) physiological presence of anti-HPA-1a antibodies in vivo, it was apparent that a monoclonal anti-HPA-1a antibody would be the best approach to investigate the potential effects of anti-HPA-1a antibodies on platelet function.
Figure 12: Results from experiments with adult donors, n=3 for each HPA-1a type. Left upper panel: Multiplate aggregation after ADP stimulation with maternal plasmas of different anti-HPA-1a titers (B1-B3), AB plasma from a single, random donor, and PBS. Right upper panel: Anti-IgG MFIs using the same samples showed an inconsistent tendency towards HPA-1a specific binding of the IgG present in the maternal plasmas. Left lower panel: CD62-P MFIs from samples without stimulation, relative to AB plasma, indicating an up-regulation of CD62-P on unstimulated platelets. Right lower panel: CD62-P MFIs from TRAP stimulated samples relative to AB plasma, indicating a lower up-regulation of CD62-P in the presence of anti-HPA-1a antibodies. For all figures: Full circle=HPA-1aa, half circle=HPA-1ab, empty circle=HPA-1bb.
5.2.3 Effects Mediated by a Monoclonal Anti-HPA-1a Antibody

When incubating with the monoclonal anti-HPA-1a antibody (26-4), we found decreased fibrinogen binding to washed platelets (Fig. 3A,B), and a reduced anti-PAC-1 binding in whole blood, indicating a blocking of the active confirmation of the fibrinogen receptor GPIIb/IIIa by 26-4 (Suppl. Fig. 5). These effects were consistent with regards to HPA-1a type, with increased inhibition for HPA-1aa platelets compared to HPA-1ab platelets, and with little effect on HPA-1bb platelets, which also fit with the HPA-1a specific binding of 26-4 to platelets (Suppl. Fig. 4).

Considering the earlier published data on the 26-4 antibody and reduced aggregation[56], these findings are not unexpected, but this is still the first study to use a monoclonal anti-HPA-1a antibody derived from an immunized mother to demonstrate decreased fibrinogen binding as a potential aspect of FNAIT. However, in a study from 1984[137], similar results were actually demonstrated using polyclonal IgG and Fab fragments isolated from two immunized mothers. The fibrinogen binding was quantified as association of radiolabeled fibrinogen to a platelet pellet, and similar results were observed for HPA-1a homozygous and heterozygous platelets, indicating a less sensitive assay compared to flow cytometry, where the fluorochrome-conjugated fibrinogen is measured on a single cell-level. Given the possible implications of these findings, and the potential to further decipher underlying mechanisms using a monoclonal antibody, it may be worth repeating and expanding these experiments from the 1980s.

5.2.4 Does Anti-HPA-1a Antibody Mediate Signaling or Blocking Effects?

After incubation in whole blood, we found a marginal effect on CD62-P up-regulation with the 26-4, and the effect was most pronounced on platelets with a low stimulation (TRAP 10 μM) (Fig. 3C). This could indicate that the blocking effect of 26-4 may be overridden by a strong stimulus, which could fit with a form of allosteric hindrance. This is in line with a study from 2009, investigating the effect of anti-HPA-1a on endothelial cell spreading[51]: purified anti-HPA-1a IgG fractions affected cell spreading, but as opposed to anti-CD61 antibodies, the anti-HPA-1a antibodies had no effect on cell adhesion; the authors suggest that this could be due to a signaling, rather than blocking effect of the human anti-HPA-1a antibodies. It would be useful to include an anti-β3 antibody (such as anti-CD61) as comparison to the 26-4 antibody in future analyses; this could help discriminate effects of different levels of blocking of the β3-integrin.

Another hint towards signaling effects mediated by anti-HPA-1a antibodies is results from GPIIb/IIIa inhibitors such as Abciximab (chimeric mouse-human anti-β3 Fab-fragments), which has been shown to decrease fibrinogen binding while simultaneously lead to a transient increase in agonist-induced CD62-P expression, without effect on resting platelets[194]. The authors postulated an effect of increased mass of protein in α-granules with uptake of Abciximab leading to increased release of granules, while others have also suggested an activation of GPIIb/IIIa by Abciximab, with subsequent increased fibrinogen binding and aggregation[195]. GPIIb/IIIa inhibitors may exert their effects depending on half-life and affinity to their epitope on the GPIIb/IIIa receptor[196], extrapolating results from such
studies to effects of anti-HPA-1a antibodies in FNAIT is therefore not ideal as both half-life, epitope, and binding affinity differ. However, some anti-HPA-1a antibodies have been shown to have activating effects \cite{142}, and possibly there can be a combination of \( \beta_3 \)-blocking and signaling effects, depending on the affinity of the anti-HPA-1a antibodies, and activation status of the platelet.

5.2.5 Limitations

If anti-HPA-1a antibodies can increase CD62-P expression, this marker may not be suitable as the only read-out when investigating the effects of these antibodies on platelet function. One could consider other flow cytometry platelet activation markers, such as CD63, which is expressed upon lysosome release \cite{197}, monocyte-platelet aggregates, which forms after degranulation of platelets \cite{198}, and other functional assays, such as Rotem. Although neonatal peripheral blood and umbilical cord blood have been shown to be comparable with regards to activation \cite{145}, another limitation of the study is the different sampling techniques for neonatal and adult blood. There will be different flow conditions when sampling from the cubital vein and from the placenta, and this may affect platelet activation. For the last sets of experiments (Fig. 3), we also used citrate tubes for adults, and bags containing citrate buffer for the cord blood, which potentially also could affect activation.

5.3 IMPLICATIONS

The limited and variable effects of plasma containing anti-HPA-1a antibodies, both in our hands and in previous studies \cite{138}, indicate that the \textit{in vivo} effects of polyclonal anti-HPA-1a antibodies are variable, and may depend on binding affinity to the HPA-1a antigen. The monoclonal 26-4 used in our study is selected by its high affinity to the HPA-1a antigen, while \textit{in vivo}, several antibody clones, also with reduced binding affinity, will be present, possibly reducing any effect by competitively blocking the binding of high-affinity anti-HPA-1a antibodies to the HPA-1a antigen. Monoclonal antibodies are probably best for deciphering effects on platelet function in relation to FNAIT, but the effects of other human monoclonal antibodies should also be tested. Furthermore, it is important to use umbilical cord blood or neonatal platelets for such studies, since the GPIIb/IIIa expression is lower \cite{145}, and the neonatal hemostatic balance differs from that in adults.
6 RESULTS AND DISCUSSION STUDY IV

In this study we focused on the most feared complication of FNAIT, an intracranial hemorrhage. There is a continuous debate on whether screening for FNAIT to prevent ICH should be implemented, and one of the arguments against screening is that the true risk of ICH in index cases of FNAIT is unknown. To get an indication of how often an ICH is associated with FNAIT, we used the Swedish Neonatal Quality register (SNQ) to assess the frequency of maternal platelet alloimmunization in a population of neonates with intracranial hemorrhage.

6.1 SUMMARY OF MAIN FINDINGS

Between 2003 and 2012, 286 neonates with an ICH born at or after 32 weeks of gestation were registered in the SNQ. Of the mothers, 278 could be contacted, and we received samples from 120 mothers. We excluded cases where the ICH had occurred after the neonatal period (n=2), and cases where the neonatal ICH diagnosis was uncertain when reviewing hospital records (n=15) (Fig.1). When comparing hospital records to data in the SNQ (n=93), we found that the diagnosis of ICH could be verified in 84% of reviewed cases. Of the 105 relevant samples, we found two HPA-1a antigen negative (HPA-1bb) mothers, of which one had detectable anti-HPA-1a antibodies and a severely thrombocytopenic neonate. The other HPA-1bb mother had delivered a boy with a large infarction and secondary bleedings in the CNS, and he was not thrombocytopenic. Two other mothers had detectable antibodies, anti-HPA-5b antibodies, and anti-HPA-5b antibodies together with anti-HPA-15a antibodies; both of these two neonates had moderate thrombocytopenia (Table 3). When reviewing the clinical data from the SNQ register and hospital records, we found that thrombocytopenia in general was rare; 5.3% (n=14/262) were diagnosed as thrombocytopenic, and 8.5% had platelet counts <50x10^9/L (n=5/59) noted in hospital records. Furthermore, we found a rather heterogenous case population with regards to other clinical data, and a high frequency of factors with a known association with ICH, such as asphyxia, trauma, and infections.

6.2 DISCUSSION

6.2.1 Possible Limitations that Could Explain the Low Frequency of Platelet Alloimmunization

This study is the first of its kind, and we did therefore not have any strong preconceptions on the frequency of anti-HPA immunized mothers among the selected cases of neonates with ICH. Still, compared to prospective studies on FNAIT[104], the frequency is lower than expected, and several limitations could explain this finding:

6.2.1.1 The SNQ Register

The basis of the study was the SNQ register, and one apparent possibility is that not all relevant cases were registered. The registry was implemented gradually, and we estimate that
about 75% of all births should be covered in the study period. Although there is a possibility that some cases were not included due to low coverage of the registry, there is no reason to suspect that cases with platelet alloimmunization should be less likely to be registered. The registry covers neonates admitted to a neonatal intensive ward (NICU), and the chances that a live neonate should be diagnosed with an ICH without being admitted to NICU, or that a neonate with ICH should succumb after birth but before admission to NICU, are small.

6.2.1.2 Intrauterine Fetal Death

We also reviewed cases of intrauterine fetal death (IUFD) in the Stockholm region in the study period, and found a low frequency of signs of ICH among the investigated cases (n=9/745), born from 22 weeks of gestation. We expect cases of IUFD in Stockholm to be representative of the rest of Sweden. Although the frequency of ICH in cases of IUFD can be higher than the frequency of ICH among live born neonates, the frequency of IUFD in itself is low in Sweden, and it is not likely that we missed a majority of the FNAIT cases in Sweden due to IUFD. However, in future screening studies on FNAIT, cases of IUFD should also be included.

6.2.1.3 Higher Frequency of Platelet Alloimmunization in ICH in Very Premature Neonates?

The selection criteria of gestational age of at least 32 weeks at birth could have affected our results, as ICH due to FNAIT can develop early in gestation. In a retrospective study of ICH and FNAIT, 67% (29/43) were born before week 37, and while only four were born before week 28[153], excluding cases with ICH born before week 32 could be a possible explanation for the low number of cases associated with FNAIT in our study population. On the other hand, a study on frequency of anti-HPA in a cohort of suspected fetal ICH without demonstrated thrombocytopenia confirmed FNAIT in 2/33 cases[199]; this low frequency is in line with our findings, and indicates that platelet alloimmunization is rare also in premature neonates with ICH.

6.2.1.4 Asymptomatic Cases

The most likely reason for the low frequency of FNAIT in our selected population of neonates could be that neonates without symptoms of their ICH would not be included. The clinical relevance and impact of such ICHs are not known, and probably very variable. The frequency also would depend on detection techniques; an MRI examination can detect signs of ICH in up to 26% of presumably healthy term neonates without clinical signs of ICH[154], while with less sensitive techniques such as ultrasound, the frequency is much lower[200]. It would be important to also include asymptomatic cases of ICH in screening studies on FNAIT, to get the full clinical spectrum of this condition. Retrospective investigation of clinical data might lead to an underestimation of frequency, with an overrepresentation of the most severe cases.
6.2.1.5 Sampling Interval and Lack of Cross Matching

The mothers were sampled during 2015, while the oldest child was born 2003. Since we serotyped all mothers for the HPA-1a antigen, it is highly unlikely that we missed cases with anti-HPA-1a immunization, but due to the long interval, there is a possibility that we missed cases with other anti-HPAs than anti-HPA-1a, as antibodies may disappear over time. Furthermore, we only tested for antibodies against HPA-1, HPA-2, HPA-3, and HPA-15 antigen systems, and antibodies against rare, private HPAs would not be identified without cross matching with paternal platelets. Alloantibodies against low frequency human platelet antigens is rare, also in cases of suspected FNAIT [201], and is as such not an apparent reason for the low frequency of anti-HPA in our study.

6.2.2 High Morbidity in Cases of ICH

By comparing to the background population, it was evident that our cases of neonates with ICH had a high mortality and morbidity, and the register data also revealed a multitude of different factors that can be associated with an ICH. The frequency of mothers with an HPA-1bb genotype at approximately 2% suggests that FNAIT was not overrepresented among these neonates with ICH. Although neonatal ICH due to FNAIT has received attention due to its potential severity, and might seem easy to prevent, or at least anticipate, investigation of and attention to other preventable causes of ICH are also justified.

6.3 IMPLICATIONS

This study suggests that FNAIT is rare in cases of clinically recognized ICH. However, it also demonstrates that cases of neonatal ICH with maternal platelet alloimmunization are difficult to identify retrospectively. Well-designed prospective screening studies of sufficient size are necessary in order to answer the main underlying research question of the occurrence of ICH in FNAIT, and to gain new knowledge on a potentially severe condition. The results from this study indicate that such screening studies would require thorough monitoring of fetal and neonatal health to get the full clinical picture of FNAIT.
7 METHODOLOGICAL APPROACHES AND CONSIDERATIONS

7.1 PLATELET ALLOANTIBODY DETECTION USING MAIPA

Study I, II and IV all depended on precise detection of maternal anti-HPA antibodies.

The Monoclonal Antibody-specific Immobilization of Platelet Antigens (MAIPA) assay has for long been the cornerstone for detection of platelet antibodies, and has since its introduction in 1987[202] undergone several modifications to increase sensitivity, and reduce variation between laboratories.

MAIPA is a glycoprotein-specific assay, and its properties are dependent on a reliable panel of platelet antigens, some of which are rare in a general population. Another limitation is that plasma antibodies and monoclonal antibodies may compete[203]; these limitations may be overcome by selecting monoclonal antibodies that target different epitopes, and by substituting platelets with target cell lines[204], recombinant proteins[205] or purified platelet peptides (as used in SPR to detect low-avidity anti-HPA-1a antibodies [206, 207]) [208].

Some anti-HPAs are difficult to detect, such as antibodies targeting the HPA-3 and HPA-15 antigen systems. In the case of anti-HPA-3a, this can be due to unstable epitopes, that require an intact three-dimensional structure of the αIIbβ3 integrin, which may be lost after solubilizing[209], fixation[210], and storage of platelets[211]. The presence of anti-HLA class I antibodies may also interfere with detection[209]. For anti-HPA-15 antibodies, the CD109 protein, which carries the epitope, is lowly expressed compared to other HPAs, the expression varies among donors[212], and is dependent on proper storage conditions [212].

EDTA was recently shown to decrease sensitivity of MAIPA[213, 214]. EDTA is a chelating agent, and may disrupt the αIIbβ3 integrin as the heterodimer complex is dependent on calcium. The effects of EDTA depend on time of exposure and temperature, with little dissociation at temperatures below 25°C [215]. Though EDTA was shown to only marginally affect the beads-based MAIPA assay used in study IV, where calcium and magnesium is added to the assay buffer[216] (Figure 13), this highlights the need to be aware of different properties of buffers and reagents used in assays to detect anti-HPA antibodies.

As part of the routine investigations for FNAIT, samples on both parents are collected, so that paternal platelets may be used in the MAIPA. This ensures that antibodies against rare and private platelet antigens may be detected. Cross-matching against paternal platelets was performed for most cases in study I and II, as the cases were referred due to clinical suspicion. In study III, we only collected samples from the mothers; we could exclude antibodies against HPA-1a (as all were serotyped for the antigen), which was the alloantibody of focus for the study, but antibodies against rare and private epitopes could not be excluded.
Though MAIPA is considered the gold standard to detect platelet antibodies, each laboratory has its own protocol, and it is constantly renewed to improve sensitivity and specificity. This is also reflected by the studies included in this thesis: In study I, a conventional MAIPA was used to rule out anti-HPA immunization, and the same laboratory updated to a beads-based version in study IV for increased sensitivity to detect anti-HPA antibodies[216]. For study II, the analyses were performed in Tromsø, Norway, where the laboratory has other protocols for detection and quantification of antibodies[99, 217]. In addition to taking advantage of the high affinity between biotin and streptavidin (SA) to capture antigen/antibody complexes (Figure 13), and using PE-conjugated anti-human-IgG for detection, the methods also differ in the capture antibodies used, and by separate incubation of patient plasma samples and GP-specific antibodies[216]. Although it can potentially be seen as a drawback that different methods were used, it is the result of constant improvement in the laboratories, and the best methods available at the time were applied.

### 7.2 OTHER METHODS FOR ALLOANTIBODY DETECTION

With MAIPA’s limitations, multiple methods are needed to diagnose the presence of anti-HPA antibodies in sera with high sensitivity and specificity. Platelet immunofluorescence test (PIFT) is another established method to detect anti-platelet antibodies[218]. Here, antigen-positive platelets are incubated with sera, before anti-IgG antibodies, labeled with fluorescent molecules such as PE, are used to detect antibody-antigen complexes (by microscopy or flow cytometry). Although fast and easy to perform, as a whole-platelet method it does not allow for discrimination between different anti-platelet antibodies (such as anti-HLA and anti-HPA), and possibly has an insufficient specificity for anti-HPA alloantibodies[208]. It is primarily used as a screening test together with MAIPA or other more anti-platelet antibody specific methods (as in study I).

Another assay that can be used for detection of anti-platelet antibodies is enzyme-linked immunoassay (ELISA). There are many versions of ELISA, and depending on the capture antigens, antibodies of different specificities may be detected. Pak-12 (Immucor GTI Diagnostics, Waukesha, WI, USA), for detection of anti-HPA antibodies, and Quikscreen (Gen-probe GTI Diagnostics Inc, Norcross, GA, USA)[219] for detection of anti-HLA class I antibodies were used in study I to identify mothers with only anti-HLA class I antibodies. The basic principle of the assay used in the studies in this thesis is as follows: Patient plasma (or serum) is added to a microwell plate, with wells coated with HLA class I (or HPA) glycoproteins to which antibodies in the plasma can bind. Anti-IgG conjugated to ALP is then added and incubated, before a substrate (PNPP, p-nitrophenyl-phosphate) is added. Washing is necessary between each step to remove unbound materials. The use of secondary (anti-IgG) antibody amplifies the signal as each primary antibody contains multiple epitopes that can be bound by the secondary antibody (known as an indirect ELISA assay). The optical density of the developing color is measured in a spectrophotometer.
An example of a screening method for detection of anti-HLA class I antibodies, used in study II, is FlowPRA (OneLambda, CA, USA). Here, the basic principle is HLA class I antigens coated on several beads, covering the most common antigens; antibodies directed against the non-polymorphic region of the HLA class I molecule is used to immobilize the antigens, leaving the polymorphic domains for antibody binding. Patient serum is then incubated with the mix of beads, before anti-IgG, conjugated with fluorescent molecules (FITC), is added. Fluorescence is subsequently measured in a flow cytometer, and the assay gives an overall MFI. Both FlowPRA and Quikscreen/ELISA are sensitive methods, primarily used to detect the absence or presence of HLA class I antibodies.

7.3 DETECTION OF ANTI-HLA CLASS I ANTIBODY SPECIFICITIES

In study I and II, anti-HLA class I antibody specificities from screened positive mothers were investigated in detail. A Luminex microbead assay was applied in both studies (LABScreen Single Antigen, One Lambda, CA, USA). Here, different HLA-class I antigens are coated on fluorescent beads, to cover the most common antigens in the population (in total 97 antigen beads). Fluorescent anti-IgG (PE) is used to detect antibodies, allowing for detection of an anti-IgG signal that can be ascribed to a particular bead. Although each bead is coated with a specific antigen, epitopes can be shared between antigens, which explain the often seen reactivity to multiple beads, even though the tested antibody has arisen from immunization against a single antigen. In study II, these patterns were confirmed by dilution experiments, where after diluting the samples at 1:10, 1:50, and 1:100, the reactivity remained consistent. To compare between different samples over time, Quantiplex beads allows for standardization of MFI values (into SFIs). Though not validated as a quantitative assay, results are often interpreted in a semi-quantitative manner[220-222]. The binding of antibody to their respective epitopes will be reflected by the MFI values, and though different epitopes may be expressed at different frequencies, this should not affect the MFI signals as long as the binding sites are not completely saturated.

Cut-off for the Luminex is often set to an SFI/MFI >999. Though somewhat arbitrary, values below this threshold are traditionally considered unspecific. In study I, this was the cut-off initially used, but a higher cut-off was also applied, to visualize the differences in high levels of anti-HLA class antibodies between the groups. In study II, no cut-off was applied. In both studies, the statistical analyses provided similar results when comparing antibody levels between the groups, independent of the MFI>999 cut-off.

Median MFI for all beads (with MFI>999) was used as a measure of HLA class I antibody level in study I. Without genotyping data, we considered this measure as the best indicator of reactivity against immunizing HLA class I antigens, as most reactivity was thought to be directed against foreign/paternal HLA class I antigens. This overall median MFI was also used to compare antibody levels between cases and controls in study II. Here, additional genotyping data and epitope mapping (described below) also allowed for comparison of
MFIs for beads with paternal/immunizing epitopes versus beads without, and correlation of paternal-specific alloantibody levels to clinical data within the case group.

7.4 HPA GENOTYPING

To support alloantibody findings, and to confirm serotyping of HPA antigens, genotyping of relevant HPAs is performed as part of the diagnosis of FNAIT. Genotyping of HPA is generally considered reliable and precise with different PCR-based techniques, where the particular DNA of interest is amplified and can then be measured. Both in-house and commercially available methods are constantly developed to allow for genotyping of new antigens and high-throughput testing[223]. For study I: IDHPA (Progenika, Biscay, Spain), a multiplex genotyping platform based on Luminex technology was used; for study II the technique is described in the following references: cases referred until year 2000[224], cases referred later[225]; and for study III and IV: In-house Taqman SNP Genotyping assay (Applied Biosystems, MA, USA)[226].

7.5 HLA CLASS I GENOTYPING AND EPITOPE MAPPING

In study II, maternal and neonatal HLA class I genotyping was performed. The analyses were done by an in-house sequence-based typing, and analyzed using the Assign Software (Conexio Genomics, Fremantle, Australia)[227]. If genotyping indicated two or more likely alleles, the most frequent allele according to data from the Norwegian Bone Marrow Donor Registry[228] was chosen for the subsequent analyses.

Genotyping of HLA class I alleles in study II allowed for assignment of apparently broad and unspecific antibody patterns to particular immunizing epitopes, expressed on multiple beads. This was made possible by combining the genotyping data with data on HLA class I epitope expression. Epitopes not expressed by maternal antigens, and labeled as “antibody reactive” in the epitope registry, were assigned as immunizing. The MFI values from the antibody assay could then be interpreted as representing reactivity against paternal/immunizing antigens, third-party (for instance previous pregnancies), or self-antigens. Epitope data was retrieved from HLAMatchmaker (http://www.epitopes.net) and the HLA Epitope Registry (http://www.eregistry.com.br/index/databases/database/ABC/) in February 2016[229].
Figure 13: The beads-based MAIPA used in study IV. A) Overview of the beads-MAIPA method. 1) Platelets are incubated with plasma containing anti-HPA antibodies. 2) Incubation with biotinylated glycoprotein-specific antibody. 3) Platelets are lysed. 4) Incubation with streptavidin-coated beads. 5) Incubation with anti-human IgG-PE. B) Example raw data from flow cytometry analysis. Median fluorescence intensity on 1000 beads gated in FSC and SSC plot is used as read-out. Representative histograms for negative (filled) and positive (unfilled) control samples for anti-HPA-1a, anti-HPA-3a, anti-HPA-5b and anti-HPA-15b are shown. C) Sensitivity and reproducibility of beads-MAIPA. The same negative and positive samples were analyzed at different occasions with test platelets from different blood donors. Median fluorescence intensity for negative (unfilled) and positive (filled) control samples for anti-HPA-1a (HPA-1aa platelets), anti-HPA-3a (HPA-3ab or -3aa platelets), anti-HPA-5b (HPA-5ab platelets) and anti-HPA-15b (HPA-15ab and HPA-15bb platelets). D) Comparison of signal-to-noise (positive control divided with negative control) for beads-MAIPA and MAIPA. For beads-MAIPA the median fluorescence intensity is used as read-out and for MAIPA the optical density at 450 nm is used. Heavily adapted from[216].
7.6 ASSAYS FOR EVALUATING PLATELET FUNCTION

7.6.1 Multiplate

We used Multiplate (Roche Diagnostics, IN, USA) as one measure of platelet function. Multiplate measures change in electrical resistance as the platelets aggregate, known as impedance aggregometry. Whole blood (anticoagulated with citrate or hirudin) is added to a cuvette containing electrodes, and incubated with a calcium-containing solution, before different stimuli are added (such as ristocetin, TRAP, ADP, collagen). Change in impedance is measured in arbitrary units over time, in total eight minutes (Figure 14). The assay reflects interactions between platelets, red cells, and leukocytes, and has been shown to be sensitive for platelet count levels, but not age, gender, ABO blood group, smoking, or use of oral contraceptives[190]. For study III, we chose to use ADP and TRAP as stimuli, to investigate effects of a weak and more potent stimulus, respectively[230].

Figure 14: Exemplary Multiplate output. Change in impedance is measured in two electrodes simultaneously, represented by the red and blue curves, and combined into one average measure as area under the curve. AU=arbitrary units. The curves show ADP-stimulated adult platelets.

7.6.2 Rotem

As the blood clot forms during coagulation, fibrin networks increase the viscosity of the blood. Viscoelastic methods evaluate this process, by measuring the initiation of the clot formation, its elasticity development, and finally the fibrinolysis in real time. The elasticity depends on the platelets’ contractile force, hematocrit levels, fibrinogen concentration, FXIII (which crosslinks fibrin), and the thrombin generation[230]. For rotational thromboelastometry (ROTEM, Tem International GmbH, Munich, Germany) used in study III, citrated whole blood is placed in a cup with a rotating pin. Once the blood starts to clot and fibrin strands are formed, the rotational force (torque) between the pin and the cup increases. Dissociation of the clot decreases the torque. In Rotem, the changes in torque are measured optically, and presented as a tracing of the clot formation[231] (Figure 15). A variety of inhibitors and activators can be used in Rotem to estimate different parts of the
coagulation process. We used EXTEM, which uses tissue factor as an activator of the extrinsic pathway, and reflects the clot strength dependent on platelet and fibrin interactions[232]. In this way Rotem complements Multiplate, as it is more dependent on coagulation factors (in particular fibrinogen), compared to Multiplate.

Figure 15: Exemplary Rotem graph, adapted from[233]. CT=Clotting time, representing the latency time from adding the start reagent to when the clot starts to form. CFT=Clot formation time, the time from CT until a clot firmness of 20 mm is formed. Alpha-angle=Angle of the tangent between 0 mm and the curve when the clot firmness is 20 mm. A10=Amplitude, representing clot firmness, at 10 mins (starting from CFT). MCF=Maximum clot firmness, the greatest vertical amplitude, reflecting the maximum strength of the clot. L130=Lysis index at 30 min, the percentage of remaining clot stability in relation to MCF. ML=Maximum lysis, the percentage of lost clot stability at any time point or at the end of the assay.

7.6.3 Flow Cytometry

We used flow cytometry to study the expression of platelet surface receptors in a quantitative manner, as the expression of certain surface markers can correlate with platelet function[234]: CD41 represents the αIIb part of the GPIIb/IIIa receptor dimer, which functions as the receptor for fibrinogen, von Willebrand factor, fibronectin, and vitronectin, when activated. PAC-1 recognizes the active conformation of GPIIb/IIIa. CD42a, which represents glycoprotein IX (part of the GPIb-IX-V von Willebrand receptor complex), was used as a platelet marker in most experiments, and can also be affected by activation status of the platelets[235]. However, CD62-P is the most sensitive marker for platelet activation, as it is expressed on α-granules, which are released upon activation and degranulation of platelets; the CD62-P expression is therefore very low on unstimulated platelets. We also quantified CD61/GPIIIa, using an antibody clone that recognizes GPIIIa derived from the HPA-1a allele, as an indirect way of quantifying the HPA-1a epitope expression.
We used whole blood to compare platelet function in cord blood and adult blood to avoid unnecessary manipulation of the platelets, and to analyze the platelets in a more physiological milieu (as compared to washed platelets). For study III, citrated whole blood was diluted with modified HEPES/Tyrode’s buffer (HEPES 10 mM, NaCl 137 mM, KCl 2.8 mM, MgCl₂ 1 mM, NaHCO₃ 12 mM, Na₂HPO₄ 0.4 mM, BSA 0.35 w/v, glucose 5 mM) 1:50, and stained with the following antibodies: Anti-CD42a (eFluor 450, GR-P clone, eBioscience/Affymetrix), anti-CD62P (APC, AK4 clone, BD Pharmingen), anti-CD41 (PC7, P2 clone, Beckman Coulter), and anti-CD61 (FITC, SZ21 clone, Beckman Coulter). All antibodies were titrated to be well saturated, to ensure that variation in MFI values reflected changes in antigen expression.

To evaluate changes associated with platelet activation, samples were incubated with either thrombin receptor activating peptide 6 (TRAP) or PBS, together with the antibody mix. After incubation, samples were fixed (1% paraformaldehyde, BD Cytofix) to prevent further activation, before dilution with PBS. Samples were kept at 4°C in the dark and analyzed on BD LSR Fortessa within 48 hours. Ideally all samples should be analyzed without delay, but this was not possible logistically. Initial set-up experiments demonstrated that the MFI values remained relatively stable for 48 hours (data not shown).

To reduce intra-assay variability, samples were run on a low speed, to ensure a flow of single cells. For CD61 MFI, expression was standardized by using Quantum beads (Bangs Laboratories, Inc., IN, USA). For other fluorochromes, compensation beads (OneComp beads, eBioscience/Affymetrix, CA, USA) were used for monitoring of fluorescence over time. For all acquisitions, the same gains/voltages and gating strategies were applied. At least 10,000 events were recorded to ensure a reliable measure of median MFI values.

Isotype controls for the antibodies were not used, as only platelets should express the relevant epitopes, and larger cells are excluded based on FSC/SSC properties, although unspecific Fc-binding on platelets cannot be ruled out. We did not include any live/dead staining, as previous experiments performed in the group showed up to 95% viability 24 hours after sampling, and all samples were stained within 4-6 hours.

![Figure 16: Flow cytometry gating strategy. Platelets were selected based on FSC/SSC properties (SSC-A/FSC-A), positive anti-CD42a staining (V450-A/SSC-A), and single cell discrimination (FSC-A/FSC-H). MFI values were calculated from single cells.](image-url)
7.7 BLOOD SAMPLING

Platelets are sensitive for sampling methods, mechanical stress, and manipulation. To avoid artifactual *in vitro* activation and reduce variability between donors, we had a consistent protocol for sampling of donors, where the first 2 mL was discarded before collecting whole blood into citrate tubes. All adult donors were healthy subjects, not smoking or using anticoagulants within the last 24 hrs. For neonatal blood we used umbilical cord blood. Comparing to peripheral blood, this is of no consequence to the child, and larger amounts can be acquired. Neonatal umbilical cord blood and peripheral blood has been shown to be comparable with regards to glycoprotein expression[145], although we cannot completely exclude that different sampling of adults and neonates could have affected our comparisons.

7.8 FIBRINOGEN BINDING EXPERIMENTS

To investigate the binding of fibrinogen to platelets, we used washed platelets to minimize competition of Alexa-488-conjugated and endogenous fibrinogen. Washing of platelets may lead to aggregation, which can alter the expression of relevant antigens, and also needs to be avoided to ensure that single platelets are investigated in the flow cytometer. Centrifugation on low speeds and the right type of tube (for instance polypropylene) is necessary to minimize the risk of aggregation. Although increased levels of platelet activation after centrifugations cannot be ruled out, we used a “gentle” protocol for isolating platelets, without using platelet inhibitors: Platelet rich plasma was first separated from whole blood (200 g, 15 min), and platelets separated from platelet poor plasma by a second centrifugation step (400 g, 15 min).

We evaluated the binding of fibrinogen to platelets by using fibrinogen conjugated with fluorescent dye molecules [236, 237]. Washed platelets were simultaneously incubated 1:1 with anti-HPA-1a/26-4 and Alexa-488 conjugated fibrinogen (ref. no. F13191, Thermo Fischer Scientific, MA, USA) for 30 min in the dark at 37°C. Tyrode’s buffer was used as control for 26-4. The platelets were then stained and activated before analysis.

7.9 DIFFERENT APPROACHES TO MEASURE ALLOANTIBODY EFFECTS ON PLATELET FUNCTION

To investigate potential effects of anti-HPA-1 antibodies on platelet function, we initially tested NIBSC, an international anti-HPA-1a antibody standard, containing pooled, purified plasma with anti-HPA-1a IgG from alloimmunized donors (National Institute for Biological Standards and Control, product code 03/152, Hertfordshire, UK). As a control, commercial pooled AB serum was used (Human serum type AB, Cat# 14-490E, BioWhittaker, Lonza, MD, USA). This was to be able to have sufficient plasma for all set-up experiments. We later tested (unpurified) maternal plasma from HPA-1a immunized mothers, collected from the biobank in the Stockholm laboratory. These samples had different levels of anti-HPA-1a, and
also other antibodies (IgM, anti-HLA class I antibodies) could be present in the plasma. As control for these experiments, AB plasma from a single donor was used. In a final round of experiments, purified IgG from immunized mothers (screened negative for anti-HLA class I antibodies) were used, with purified IgG from AB plasma as control. The IgG was purified from plasma using affinity chromatography (MAbTRAP kit with HiTrap Protein G HP columns, GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

For these experiments we tested platelet function in Multiplate (with ADP and TRAP as stimuli), and CD62-P up-regulation using flow cytometry. We hypothesized that the anti-HPA-1a antibodies could interfere with aggregation by blocking fibrinogen binding. Integrin outside-in signaling may stimulate the release of α-granules and CD62-P up-regulation[133], and we hypothesized that in the presence of anti-HPA-1a antibody, this could be reduced.

When using monoclonal anti-HPA-1a antibody (26–4), we decided to investigate the effects of anti-HPA-1a antibodies on fibrinogen binding, CD62-P up-regulation, and hemostatic effects in Rotem (as this assay depends more on fibrinogen, and reduction of aggregation in Multiplate had already been published).

7.10 STATISTICS
Data was analyzed using different versions of SPSS software (SPSS Inc., Chicago, IL, USA). Figures were created in SPSS, Microsoft Excel for Mac 2011, Prism 6 for Mac OS X, Plotly (plot.ly), and Adobe Photoshop for Windows.

Normality of distribution was evaluated graphically, and tested by Shapiro-Wilks and Kolmogorov-Smirnov tests, depending on size of the data. For continuous variables, independent samples t-test was used to compare means for data with normal distribution, while for variables without a normal distribution, Mann-Whitney U test was used. Similarly, One-Way ANOVA with Bonferroni post-hoc test or Kruskal-Wallis with Dunn-Bonferroni post-hoc test was used for comparison of continuous variables between groups. For comparisons within groups, Wilcoxon signed rank test was used. Chi-square and Fisher’s exact tests was used for comparing frequencies for categorical variables, depending on size of the data. For correlations, we report Pearson’s or Spearman’s correlation coefficients, depending on distribution of the data. Missing data was treated by pairwise deletion when comparing unadjusted data, and by listwise deletion for regression analyses. Level of significance was set at a p-value of <0.05.

Binary logistic regression models were used to examine associations of different clinical variables. Similar ICD-10 SE codes were categorized into groups, while continuous variables were for the most part kept continuous. In study IV, the reported model investigating increasing odds of seizures with increasing gestational age, birth-weight was included as an independent variable. For the odds of trauma, birth-weight, asphyxia, and vacuum delivery were set as independent variables.
7.11 STUDY DESIGN

Study I and II can both be classified as comparative cross-sectional studies. Cross-sectional studies are characterized by simultaneous measure of exposure (anti-HLA class I antibodies) and outcome (thrombocytopenia) in a given period, although the point of measurement does not need to be at the same time point for all cases. Cases in study I and II were based on selected referrals for FNAIT, over a six and eleven-year period, respectively, but exposure and outcome were measured at the same time for each case. The lack of latency period between exposure and observed outcome makes any causal inference problematic (although it can be assumed that the antibodies were present during pregnancy).

The control groups in both studies were collected prospectively, and were used to compare to the case groups. In study I, no clinical data was available on the controls, but the samples were from mothers with healthy pregnancies, where neonatal thrombocytopenia would not be expected. In study II, the control samples were collected from a prospective study [238, 239], and 45 of the 60 controls included in the study were randomly selected and checked; none of these control neonates were thrombocytopenic.

Since FNAIT due to anti-HLA class I alloantibodies likely could be considered a subgroup of a rare condition, a cross-sectional study design based on national referrals for FNAIT is an efficient approach. A cohort study, for instance comparing data on all deliveries with and without maternal anti-HLA class I antibodies, would be better suited to answer the main research question of the effect of anti-HLA class I antibodies during pregnancy, but would require large resources. Although flawed with regards to inference of causality, the selected study design allowed for a thorough description of a rare case group.

Study IV is a register-based study, where cases were identified by the presence of an outcome (ICH), and then checked for exposure (maternal anti-HPA antibodies) at a later time-point. This is at first glance similar to a case-control study, where study subjects are classified by their outcome, before checking for exposure. However, we lack data on maternal platelet alloantibodies (exposure) in a control group of neonates without ICH; the registry-approach doesn’t yield any control group since all cases were selected due to the presence of the outcome. An alternative approach would have been to also sample mothers who had given birth to healthy children in the same period, and use this data as a control group in a case-control study.

In a cohort study, study subjects are classified according to exposure, and then investigated for the outcome. To perform study IV as a cohort study, we would have had to investigate all mothers in a study population for anti-HPA antibodies (exposure), and then compare the frequency of neonatal ICH (outcome) among exposed and non-exposed. For instance, by using routine samples taken during pregnancy for anti-HPA antibody testing, and connecting this data with register data on neonatal ICH. However, this would be more time consuming and require a lot of resources, as both maternal anti-HPA antibodies and neonatal ICH are rare events.
The final study design is most similar to a comparative cross-sectional design, since it describes both outcome and exposure in a certain population at a given time: We use clinical data for the background population (all deliveries in Sweden) as a comparative control. Although exposure and outcome were not registered simultaneously, one could argue that the risk of immunization is mainly determined by maternal HPA-1a antigen status, which will remain unchangeable over time.

Assuming all cases of neonatal ICH in Sweden were registered, the register-based approach could give an indication of prevalence of neonatal ICH in the study period, and the prevalence of ICH associated with FNAIT. However, this assumption does not hold, as asymptomatic cases of ICH were likely missed. Moreover, we could not assess incidence of ICH due to FNAIT, as the mothers were investigated for alloantibodies years after delivery, and as such there was no assessable latency time between exposure and outcome. But, given these limitations, we could give a good estimate of the frequency of FNAIT in clinically recognized cases of ICH. Since all samples were typed for the HPA-1a antigen, we could at least exclude the most common cause of FNAIT in Sweden in the majority of investigated cases. Both neonatal ICH as outcome and maternal platelet antibodies as exposure are difficult to assess, especially retrospectively.

The “ideal” approach to assess incidence of ICH due to FNAIT would have been to prospectively sample the mothers during pregnancy and in connection with delivery, and screen all neonates for the presence of ICH and thrombocytopenia. This would however demand significant resources, and comes with considerable ethical considerations.

7.12 SELECTION BIAS

Both study I and II have an inherent selection bias, in that cases were selected based on national referrals for FNAIT. In the absence of screening for thrombocytopenia, only neonates with clinical symptoms (which could be related to other causes, as revealed in study I), were available for study. Different practices and clinical awareness of FNAIT at the hospitals could also affect the referral of cases. Mild cases of transient thrombocytopenia could perhaps be less likely to be referred, as compared to cases with severe thrombocytopenia in more complicated cases. Without screening, FNAIT is thought to be underdiagnosed[240], and the low platelet counts in our cases (medians 30 and 24x10^9/L in study I and II, respectively) could perhaps reflect a selection bias towards the more severe cases. This kind of bias is one of the common pitfalls of the chosen retrospective study design, but a retrospective study was necessary considering FNAIT due to anti-HLA class I antibodies is a rare entity.

The control groups were prospectively collected without any criteria for selection, and were consequently not affected by selection bias. However, the cases were referred nationally, and both control groups were recruited locally. In study II, the control group was therefore validated against the Medical Birth Registry of Norway (MBRN), to ensure that the controls
were representative of the general Norwegian population in the study period. The geographical location of these studies is worth considering, since allele frequencies vary across different populations, and the results should therefore be generalized to other populations with caution.

Study IV could also suffer from selection bias. Mothers of neonates registered with ICH were contacted and asked to provide samples, and it is difficult to know who would be more or less inclined to participate, and how this affected our results. While some expressed gratitude for the study, others declined to participate due to previous negative experiences with the health services. Comparing the clinical data available, there was a tendency towards less response from cases with more severe morbidity, and so there is a possible bias towards less complicated cases.

7.13 SAMPLING TIME

In study I, controls where sampled in conjunction with delivery, while the time from delivery to sampling varied considerably for cases. The median was 4.5 days after delivery, with two cases sampled years after, in the subsequent pregnancy. In study II, the controls were sampled during pregnancy, at 22-24 weeks of gestation. For the cases, similarly to study I, samples were taken after delivery as part of investigation for FNAIT, with median 6 days after delivery, and only four cases longer than 15 days after delivery (max 401 days). In study IV, the mothers were sampled several years after delivery, with the oldest child born 2003, while the sampling occurred during 2015.

There are three main problems with different sampling times in study I and II: Firstly, one can question whether the antibodies present in maternal circulation early in gestation really reflect immunization due to the current, and not any previous pregnancy. Secondly, antibodies detected after delivery could have higher levels due to the delivery itself. Anti-HLA class I antibodies have been shown to be detectable earlier than week 20[241], so samples taken at 22-24 weeks should reflect immunization in the ongoing pregnancy. On the other hand, later sampling could have led to more antibody-positive controls, due to an increased exposure of fetal antigens to the maternal immune system. As we only detected IgG, it is likely that the antibodies arose from a primary immunization due to a pregnancy, rather than due to delivery, since isotype switching from IgM to IgG usually takes longer than a week. However, a secondary immunization due to the delivery could theoretically increase the antibody levels rapidly[242].

The third problem with different sampling times is that levels of maternal alloantibodies may fluctuate during pregnancy. They may both decrease with increased placental transfer of antibody, and increase with increased exposure of fetal cells later in pregnancy, or after the loss of the placenta as an absorptive surface after delivery. Differences in sampling time may therefore affect the comparisons of antibody levels in unpredictable ways. Since all controls were sampled at the same time, we could not adjust for the differences in sampling time, but
antibody levels and time after delivery for the cases were not significantly correlated (study I). In study II, longitudinal samples were available for seven controls, and samples taken during pregnancy showed 66% higher MFI levels than those taken after delivery (mean ratio 1.66, 95% CI 0.94-2.38), suggesting that variation in sampling time actually lead to an underestimation of the differences in antibody levels. It is however important to note that a higher level may be due to both increased antibody production, and decrease in placental transfer, and ideally, anti-HLA class I antibody levels should also be quantified in the neonate, for instance in umbilical cord blood. To conclude, it is unlikely that differences in sampling time affected the comparison of antibody levels between cases and controls, but it is important to keep these considerations in mind if one were to try and draw any causal inference from these studies.

In study IV, the mothers were sampled several years after delivery. Anti-HPA-1a antibodies have been shown to increase postpartum [109], and can persist for years [106]. The study focused on anti-HPA-1a immunization, and all mothers were typed for the HPA-1a antigen. A late sampling was therefore not a concern for the detection of anti-HPA-1a antibodies in this study. It is not known how levels of the other anti-HPA change over time, but with sensitive detection methods, and the assumption that also other anti-HPAs can persist long after pregnancy, a late sampling of mothers does not seem a major concern, although it cannot be excluded that some cases were missed due to the long interval from delivery to sampling.

7.14 OTHER CAUSES OF THROMBOCYTOPENIA AS CONFOUNDING FACTORS IN STUDY I AND II

A confounding factor is defined as a factor affecting both the exposure (for instance anti-HLA class I antibodies) and outcome (neonatal thrombocytopenia). When selecting cases with suspected FNAIT based on referrals, there is a risk of including cases with neonatal thrombocytopenia caused by other factors, as evident in study I. In study II, such factors were to a certain extent excluded, but many of the children were small for gestational age (SGA), and had suffered from asphyxia. Both asphyxia[243] and SGA[244], which can be a result of IUGR, has been associated with thrombocytopenia. SGA and asphyxia are in themselves not confounding factors, as they don’t lead to anti-HLA class I immunization, but they could point towards another underlying condition, such as inflammation during pregnancy, which could be associated with both increased levels of anti-HLA class I antibodies[245] and thrombocytopenia[112, 246]. Unfortunately, we could not assess maternal inflammatory markers in our studies. Any future prospective studies should consider including such measures.
CONCLUDING REMARKS

FNAIT is a heterogeneous condition, where multiple mechanisms may be involved. The outcome likely depends on the alloantibodies involved, which may exert different effects depending on affinity, composition, titer, and specificity, but neonatal factors likely also play a role. FNAIT is as such not one single entity, which hampers any prediction of outcome based on one particular parameter.

Study III, on anti-HPA-1a antibodies and neonatal platelets, indicate that platelet function could be affected in FNAIT, by a decreased ability to bind fibrinogen in the presence of anti-HPA-1a antibody. However, any consequences on neonatal hemostasis need further studies to be ascertained. A better understanding of the neonatal platelet function in FNAIT could impact treatment strategies.

The two studies on anti-HLA class I antibodies and FNAIT (study I and II) thoroughly describe a rare group of cases, and although limited by the retrospective study design, the studies show that these cases can have a severe outcome, and that clinical data also needs to be thoroughly investigated in such cases. In both studies we found that the referred cases had higher levels of anti-HLA class I antibodies compared to controls, and a high frequency of nulliparous mothers, which could imply an aberrant breach of maternal tolerance with negative effects on the fetus. We were able to disentangle the anti-HLA class I antibody patterns, which will be a useful tool in future studies, and may also be of use in other settings. Nonetheless, it is obvious from these studies that large, prospective investigations are necessary to study any causal association between anti-HLA class I antibodies and FNAIT.

The study on intracranial hemorrhages and FNAIT (study IV) showed a vast number of factors associated with ICH in neonates born close to term, and very few identified cases with maternal platelet alloimmunization. This indicates that cases of FNAIT, even when manifested as an ICH, are difficult to identify retrospectively, and could perhaps indicate a lower frequency of ICH due to FNAIT than previously assumed.

Study of referred cases of FNAIT need to continue; without screening studies these cases are the only way to gain deeper knowledge on the clinical course of FNAIT. Clinical awareness of FNAIT is therefore important, not only for the individual case, but also because there is still much to learn and explore.
9 FUTURE PERSPECTIVES

In order to better understand the impact of anti-HLA class I antibodies on pregnancy, a large, international, prospective study is needed. Such a study should include genotyping of mother, neonate, and preferably also father, in addition to extensive clinical data on mother and neonate. From laboratory data, it would also be helpful to include not only hematological parameters, but also inflammatory markers, both from maternal samples during pregnancy, and from neonatal or cord blood samples, to see how and if inflammation relates to both anti-HLA class I alloimmunization and thrombocytopenia. If using routine samples taken during pregnancy and cord blood samples, such a study would not be physically invasive, although it would require extensive resources. Most likely a large number of cases would have to be included, considering the high frequency of anti-HLA class I antibodies in normal pregnancies, as compared to the rare occurrence of FNAIT.

It would also be useful to test for anti-\(\alpha\)v\(\beta\)3 antibodies in a large cohort of unselected pregnancies, to learn more about the frequency of these antibodies, and how their presence relates to clinical outcome. Furthermore, testing for these antibodies should already be implemented in routine work-ups of anticipated and suspected cases of FNAIT, as their presence could be associated with an ICH. If the ratio between anti-\(\alpha\)v\(\beta\)3, anti-\(\beta\)3, and anti-\(\alpha\)IIb\(\beta\)3 types of anti-HPA-1a antibodies could be determined in a prospective setting, by for example longitudinal sampling of mothers during pregnancy and after delivery, this could indicate whether platelets or trophoblast represent the immunizing source in FNAIT. Effects of anti-HPA-1a antibodies on placental function should also be investigated in this regard; through histopathological investigation of placentas, one could see if inflammatory changes, as previously reported in cases of FNAIT, corresponded with the presence and level of maternal anti-\(\alpha\)v\(\beta\)3 antibodies.

Investigation of anti-HPA-1a antibody glycosylation patterns and relation to clinical outcome should also be performed in a prospective setting. This is a promising diagnostic marker, but the current studies are performed on retrospectively identified cases. In addition, further studies on the B-cell machinery responsible for the antibody glycosylation would be interesting, to better understand the mechanisms involved and how they are regulated.

The possibility that neonatal platelet function could play a role in FNAIT should also be investigated further, both by investigating the available monoclonal antibody in other functional assays, and by developing and testing other monoclonal anti-HPA-1a antibodies. Testing neonatal platelet function in cases of suspected FNAIT could be problematic, especially with regards to sampling from a thrombocytopenic neonate, but flow cytometry based assays require only small volumes of blood, and could possibly be used to guide treatment.
10 POPULAR SCIENCE SUMMARY

Picture a baby in the womb, which have inherited its unique traits from both the mother and father. Similar to how a kidney transplant is sometimes rejected, your mother’s immune system might reject the parts of you that are inherited from your father. In fetal and neonatal alloimmune thrombocytopenia (FNAIT), the maternal immune system reacts to proteins inherited from the father that are expressed on fetal platelets. Maternal antibodies directed against fetal platelets are then carried across the placenta to the baby, where they can cause destruction of platelets.

Platelets, or thrombocytes, are small cells in your blood that are necessary for a successful clot formation. When a baby is born with a low number of platelets, it therefore runs the risk of bleedings.

Nearly a third of all mothers produce antibodies during pregnancy; in rare cases these antibodies target particular cells in the baby, such as platelets, and lead to their destruction, but in many cases we don’t know their exact role or consequence, as they target surface proteins found on almost all cell types. We don’t know if these more commonly detected antibodies also can lead to destruction of a baby’s platelets, or if they are just a normal finding. If these antibodies in fact also can cause harm, it would change the way we monitor and treat these mothers and their newborns.

We studied this common type of antibodies found after pregnancy (HLA class I antibodies). Based on cases referred due to low platelet counts in the newborn, we compared antibodies in mothers of children with low platelet numbers to antibodies in control mothers with healthy babies. In the studies, we found that the mothers with sick babies had higher levels of antibodies (study I and II), and that the antibodies were specific against cell surface proteins inherited from the father (study II). We also found that many of these babies had other complications that could contribute to a low number of platelets. A high concentration of antibodies could be associated with a worse pregnancy outcome, but we need larger studies to decide if the antibodies actually cause a low platelet count number, or if they are a sign of another underlying condition.

In an experimental study, we investigated how a type of platelet-specific antibodies (HPA-1a antibody) affected the platelets’ ability to form a blood clot (study III). The antibody inhibited binding of fibrinogen, a protein central for successful clot formation as it binds activated platelets together and further enhances their activation. We also compared adult and newborn blood platelets (using umbilical cord blood), and found that newborn platelets reacted less to stimuli. Together, this could in theory mean that less reactive platelets in the newborn are
particularly susceptible to the effects of anti-platelet antibodies, which could aggravate the bleedings in FNAIT; this possibility needs to be tested further.

Finally, we looked for antibodies directed against fetal platelets in mothers of newborns who had been diagnosed with a bleeding in the brain, or intracranial hemorrhage (ICH), which is the most feared type of bleeding complication (study IV). These types of bleedings are rare in newborns born near term, so the babies were identified using a register on neonatal health in Sweden. Between 2003 and 2012, 286 babies with ICH were registered, and we contacted the mothers for samples during the year 2015. Out of 105 included samples, we found only three mothers with platelet-specific antibodies. The most common antibody known to cause a low platelet number (anti-HPA-1a), was only found in one mother. Due to the long interval between the birth of the children and sampling of the mothers, the more rare antibodies could not be excluded. Still, we could exclude the most common cause of FNAIT (anti-HPA-1a) in the majority of cases investigated by analyzing the mothers’ own platelet type. We also reviewed clinical data from the register and hospital records, and found that low number of platelets in general were rare. This could mean that FNAIT is a rare cause of ICH, or that we missed cases of FNAIT due to how we conducted the study – for instance only symptomatic children would be diagnosed and registered, and we excluded those born very prematurely (before week 32). This could have implications for any future screening programs for FNAIT.

Together, these studies confirm the notion that FNAIT is a complicated condition, and that outcome likely depends on the different kinds of antibodies involved, in addition to factors in the newborn. Due to the potential severity of FNAIT, further studies are needed, and results from these studies can be used to inform future investigations.

*Figure 17: Janeway’s Immunobiology, 8th edition [247].*

Blodplater, også kalt trombocytter, er små celler i blodet som er nødvendige for at blodet skal kunne støre, eller koagulere. Når et barn fødes med et lavt antall blodplater, har det derfor en økt risiko for blødninger.

Nesten en tredjedel av alle mødre produserer antistoffer i forbindelse med svangerskapet. I sjeldne tilfeller er disse antistoffene rettet mot bestemte celler i barnet, som blodplater, men i mange tilfeller vet vi ikke nøyaktig betydningen av disse antistoffene, ettersom de kan være rettet mot proteiner som finnes på nesten alle kroppens celler. Vi vet altså ikke om de vanligste detekterte antistoffene også kan føre til ødeleggelse av blodplater, eller om de bare er et tilfeldig funn. Hvis disse antistoffene faktisk kan forårsake skade, vil det ha betydning for måten vi behandler og følger opp disse mødrene og deres nyfødte.

Vi undersøkte den vanlige typen av antistoffer som man finner etter graviditet (HLA klasse I antistoffer). Basert på et utvalg av nyfødte som ble henvist på grunn av lavt antall blodplater, sammenlignet vi antistoffer hos mødre til barn med lavt antall blodplater med antistoffer hos mødre med friske barn. I to studier fant vi at mødrene med syke babyer hadde en høyere konsentrasjon av antistoffer (studie I og II), og at antistoffene var spesifikk mot proteiner arvet fra far (studie II). Vi fant også at mange av disse babyene hadde andre komplikasjoner som kunne bidra til et lavt antall blodplater. En høy konsentrasjon av antistoffer kan være forbundet med risiko for sykdom, men vi trenger større studier for å bestemme om antistoffene faktisk forårsaker et lavt antall blodplater, eller om de er et tegn på en underliggende tilstand.

I en eksperimentell studie undersøkte vi hvordan en type blodplate-spesifikke antistoffer (HPA-1a antistoff) påvirket blodplatenes evne til å koagulere (studie III). Antistoffet hemmet binding av fibrinogen til blodplatene, et protein som hjelper blodplatene til å klistre seg sammen og aktiveres, og som dermed er viktig for en vellykket koagulering. Vi sammenlignet også blodplater fra voksne og nyfødte (ved bruk av navlestrengsblod), og fant at nyfødtes blodplater reagerte mindre på stimuli sammenlignet med voksne. Dette kan i teorien bety at blodplater hos nyfødte er spesielt utsatt for virkningsene av antistoffer rettet mot blodplater, noe som kan forverre blødninger. Denne muligheten bør undersøkes videre, ettersom det kan påvirke behandlingen av blødninger ved FNAIT.

Sammen bekrefter disse studiene at FNAIT er en sammensatt tilstand, og at utfallet sannsynligvis avhenger av hvilken type antistoff som involvert, i tillegg til faktorer hos den enkelte nyfødte. FNAIT er potensielt veldig alvorlig, og det er fortsatt mye man ikke vet. Resultater fra disse studiene kan være til nytte for fremtidige studier.

Figur 17: Janeway’s Immunobiology, 8th edition [247].
It seems like these four years have passed with exponential speed, but at the same time it feels like a lifetime since I first moved to Stockholm and started to navigate the PhD life. I am grateful and humble to have experienced the ups and downs of a PhD, and during this time I have met many wonderful people, who have all contributed in one way or another to make this such a memorable period. An acknowledgement statement will never be sufficient to convey the gratitude I feel, but in any case I want to express my thanks to some particular people, and hope that those who are not mentioned know that they are certainly not forgotten.

First of all, thank you, Agneta Wikman, for being my main supervisor. I have learnt a lot from you, and over the years you have been a reliable source of encouragement, inspiration, and support. You have taught me to be independent, and always made me feel like my contribution mattered. Thank you for investing your time and energy in me, and for always being available.

Thank you, Anne Husebekk, for inspiring me to do research in the first place. Your energy, generosity, and optimism are qualities I strive for, and I am forever grateful that you opened up this opportunity for me.

Thank you, Magnus Westgren, for being such an excellent co-supervisor! Your experience, enthusiasm, and confidence in me always left me feeling supported and motivated after our meetings, which was much needed on more than one occasion.

Thank you, Petter Höglund, for being my co-supervisor, and for generously including me in your fine research group. I am truly grateful for all the challenging discussions and your relentless optimism.

Thank you, Ganesh Acharya, for being my mentor! Your positive energy and hard work continue to inspire me. I am grateful for all the times we met, where you always managed to lift my spirits.

Thank you, Stephan Meinke, for being my favorite lab and fika partner. You have been like a supervisor in all but title, and I have learnt so much from you. Thank you for your immense patience, perseverance, and all the fun times in the lab – I couldn’t have done it without you!

Thank you to all my collaborators and co-authors throughout the years! Especially the staff at TRoLL; Anette Mörtberg, Paul Ratcliffe, Doris Hugoson, and Anette Johnsson – you have all provided invaluable support and help to the projects. Thank you, Gunilla Gryfelt, for helping me with both logistics and experiments, and for always being kind and enthusiastic. Thank you, Harjeet Kaur Malhi, Cecilia Götherström and the umbilical cord blood bank, for your excellent help with samples. Thank you, Marie Reilly, for both your help and inspirational encouragement with biostatistics. Thank you, Stellan Håkansson, for all your consistent support with the SNQ register, and Nikos Papadogiannakis, for help with the Stockholm IUFD register. Thank you, Iyadh Douagi, for generously sharing your expertise in all things flow cytometry related.
Thank you to all my wonderful colleagues at HERM! I am forever grateful to have been part of such an inspiring, brilliant, and fun environment. You are too many to mention, but I am grateful to each and every one of you. Special thanks to Monika Jansson for keeping everything together, to Eva Hellström-Lindberg for your leadership, to my fantastic office roommates for making each day a little bit more enjoyable, and to members of the Petter Höglund research group for including me – I have learnt so much from you, and already miss our fikas.

Thank you to all those outside the lab that made the years in Stockholm unforgettable and wonderful: Thank you, Aline Pfefferle, Martha-Lena Müller, and the rest of the dedicated climbers, for giving me a space where you can simultaneously fail and feel like you’re winning. Thank you, Helena Silva Cascales, for all the wonderful talks, your support and friendship, I already miss having you around. Thank you, Michael & Sabrina Chrobok, for all the good times together, and to Sabrina for being toastmaster! Thank you, Sandra Petrus Reurer, for being my neighbour, always in the mood for spontaneous visits. Thank you, Ferdi Zhong and Lizan Swan, for introducing me to Stockholm in the very beginning - I hope we can stay in touch! Thank you, Vladimir Pabón Martínez, for brightening any day when meeting you in the corridors.

Thank you to my family, for your everlasting love and support, and for always reminding me that it’s who you are, and not what you do, that matters. Thank you mamma, for teaching me that any dream is within reach, as long as there’s a list and a plan. Thank you Randi and Thoralf, for always being there. Thank you, Liv and Svein, for including me in your family, and for all your support. Thank you to all my friends in Oslo for being my base and helping me stay sane, and Astrid, for always being my bestest friend.

Thank you Jesper, for being my inspiration and constant source of joy, love and support. I feel lucky everyday for having you in my life.


