Faculty of Health Sciences

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Maternal Anti-HLA Class I Antibodies in Connection with Pregnancy and Neonatal Thrombocytopenia – A Cause for Concern?

Clinical Characteristics and Antibody Analysis of Retrospective and Prospective Populations

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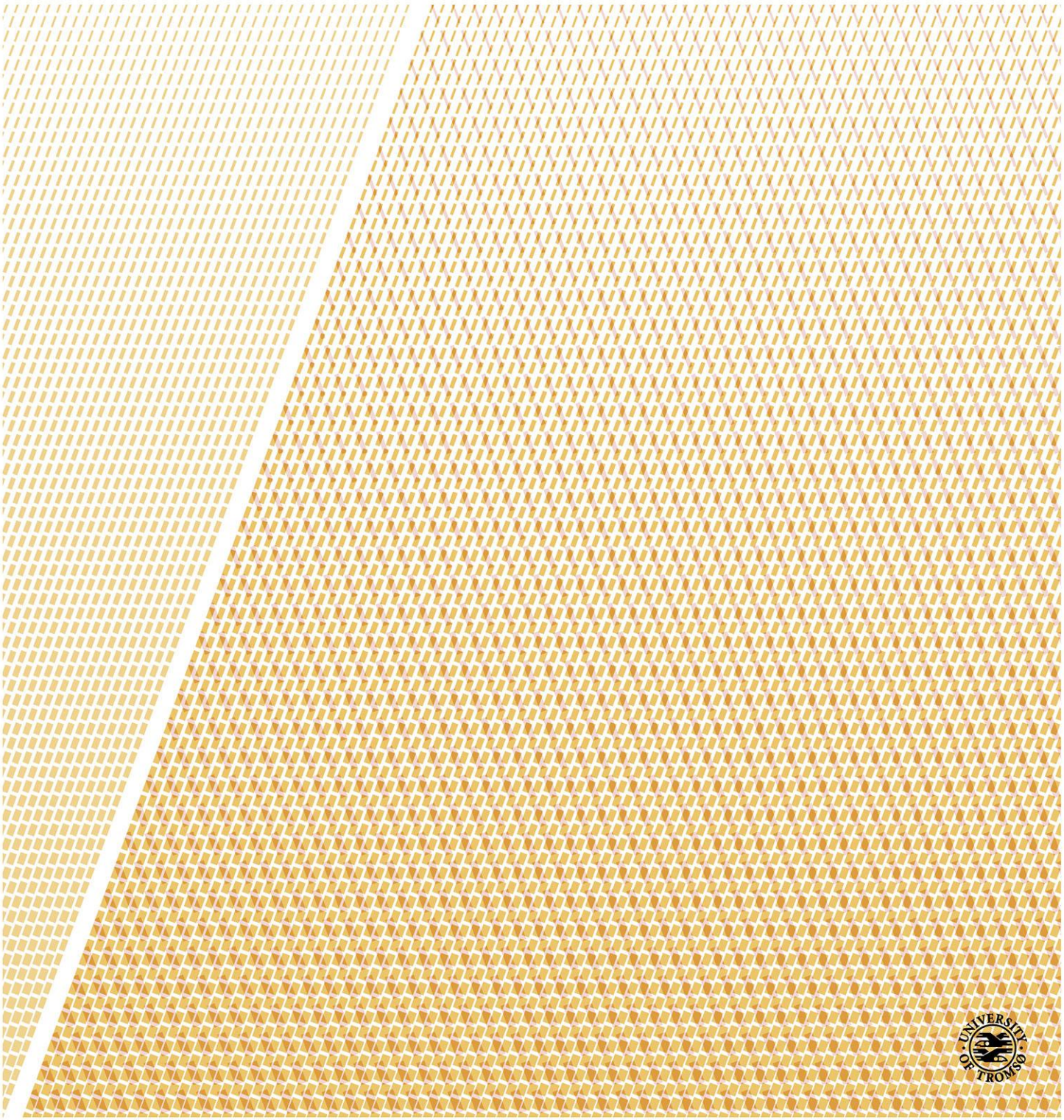

Time, alas, will make us sell it short.

Philip K. Dick

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1. Acknowledgements

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2. List of Papers

Paper I:

J Dahl, A Husebekk, G Acharya, K Flo, T B Stuge, B Skogen, B Straume, H Tiller: Maternal anti-HLA class I antibodies are associated with reduced birth weight in thrombocytopenic neonates. J Reprod Immunol. 2016 Feb;113:27-34. doi: 10.1016/j.jri.2015.10.003. Epub 2015 Oct 29.

Paper II:

J Dahl, E Refsum, MT Ahlen, T Egeland, T Jensen, MK. Viken, TB Stuge, G Acharya, A Husebekk, B Skogen, H Tiller: Unraveling the Role of Maternal Anti-HLA Class I Antibodies in Fetal and Neonatal Thrombocytopenia – Antibody Specificity Analysis Using Epitope Data. Revised manuscript submitted to Journal of Reproductive Immunology 6th June 2017.

Paper III:

J Dahl, B Skogen, M Kjaer, A Husebekk, J Kjeldsen-Kragh, H Tiller: Maternal anti-HLA class I antibodies in addition to anti-HPA-1a antibodies influence severity of fetal and neonatal alloimmune thrombocytopenia (FNAIT) – data from a large prospective screening study. Unsubmitted manuscipt.

3. Abbreviations

APC - Antigen-Presenting Cell
CDC - Complement-Dependent Cytotoxicity
CREG - Cross-Reactive Group
CRP - C-Reactive Protein
DNA - Deoxyribonucleic Acid
ELISA - Enzyme-Linked Immunosorbent Assay
FcRn - Neonatal Fc Receptor
FNAIT - Fetal and Neonatal Alloimmune Thrombocytopenia
GP - Glycoprotein
HDFN - Hemolytic Disease of the Fetus and Newborn
HLA - Human Leukocyte Antigen
HNA - Human Neutrophil Antigen
HPA - Human Platelet Antigen
ICH - Intracranial Hemorrhage
Ig - Immunoglobulin
IUGR - Intrauterine Growth Restriction
IVIg - Intravenous Immunoglobulin
MAIPA - Monoclonal Antibody Immobilization of Platelet Antigens
MFI - Mean Fluorescence Intensity
MHC - Major Histocompatibility Complex
NAIT - Neonatal Alloimmune Thrombocytopenia
NAN - Neonatal Alloimmune Neutropenia
PW/BW - Placental Weight/Birth Weight
RhD - Rhesus D
SGA - Small for Gestational Age
TRALI - Transfusion Related Acute Lung Injury

4. Summary

Background: Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal antibodies that target fetal platelets during pregnancy. It can occur when fetal platelets express a paternally-inherited antigen that is not shared by the mother. Maternal antibodies that cause FNAIT are directed against human platelet antigens (HPAs) on fetal platelets, and the resulting fetal/ neonatal thrombocytopenia can range from non-symptomatic to life-threatening. The polymorphic human leukocyte antigens (HLA) class I are also expressed on platelets. It has long been hypothesized that anti-HLA class I antibodies could be a cause of FNAIT, since they are a well-known cause of platelet refractoriness following transfusion, and often appear in cases with suspected FNAIT, both alone and alongside anti-HPA antibodies. Anti-HLA class I antibodies are also a frequent occurrence in uncomplicated pregnancies, and have not been definitively tied to any adverse fetal-maternal outcome, although multiple associations have been reported.

Results: Using retrospective data from the Norwegian National Unit for Platelet Immunology in Tromsø and data from a previous large prospective screening study on FNAIT, we have described the antibody properties and clinical characteristics of cases with suspected FNAIT due to anti-HLA class I antibodies alone, as well as alongside anti-HPA antibodies, and compared these to data from control pregnancies. We found that newborns with suspected FNAIT due to maternal anti-HLA class I antibodies typically had severe thrombocytopenia, and a frequency of intracranial hemorrhage comparable to what is reported for anti-HPA antibody induced FNAIT.

A surprisingly high number of these children were first-borns, and many of the children were also small for gestational age (SGA). An increasing anti-HLA class I antibody level was found to be associated with a decreasing birth weight. Maternal immunization was not tied to any particular HLA class I antigen, but the detected maternal anti-HLA class I antibodies were specific towards paternally-inherited fetal epitopes. Anti-HLA class I antibody levels were higher in cases with suspected FNAIT compared to control pregnancies with detectable anti-HLA class I antibodies. When detected together with anti-HPA-1a antibodies, the presence of anti-HLA class I antibodies was associated with an increased risk of neonatal thrombocytopenia as well as more severe thrombocytopenia, particularly in pregnancies where the mother was nulliparous.

Conclusions: We found that neonates with suspected FNAIT due to maternal anti-HLA class I antibodies not only had severe thrombocytopenia, but that the combination of anti-HLA class I antibodies with neonatal thrombocytopenia was associated with significant perinatal morbidity. Furthermore, we have demonstrated that the additional presence of anti-HLA class I antibodies in HPA-1a alloimmunized pregnancies was associated with an increased risk and severity of neonatal thrombocytopenia. These findings were particularly evident for first-borns. However, since our studies could not address the central question of causality, it remains unclear whether or not the presence of anti-HLA class I antibodies are an epiphenomenon in pregnancies where the neonate develops thrombocytopenia. This question would need to be addressed in a larger prospective study.

5. Introduction

5.1 Alloimmunization and Pregnancy

A person's immune system typically does not induce immune responses towards antigens that are expressed by their own cells. These self-antigens are also called autoantigens. Antigens that are not expressed by a person's own cells, but are expressed by other individuals of the same species, are referred to as alloantigens. Alloantigens may be potent immunogens, and can become a significant clinical problem in settings where one person's cells are introduced into another, for example during transplantation, transfusion or pregnancy. This thesis will focus on alloimmunization during pregnancy, where the mother produces antibodies that target paternally-inherited antigens in the fetus/newborn.

There are several conditions that can arise from such a maternal alloimmune response towards fetal/neonatal antigens, and the resulting symptoms will depend on which fetal/neonatal cells that express the targeted antigen. The most well known of these conditions is hemolytic disease of the fetus and newborn (HDFN). During HDFN, maternal IgG antibodies are directed towards fetal red blood cell antigens, most commonly the RhD . When antibodies targeting fetal red blood cells cross the placenta this can cause antibody-mediated hemolysis in the fetus/newborn, in turn leading to the condition hemolytic disease of the fetus and newborn (HDFN). In a similar manner both fetal/neonatal neutrophils and platelets can also be the targets for maternal antibodies, resulting in the conditions neonatal alloimmune neutropenia (NAN) and fetal and neonatal alloimmune thrombocytopenia (FNAIT), respectively. The primary focus of this thesis will be on FNAIT.

The fetus is semi-allogenic, since it shares around half of its genetic code with each parent. The fetus will therefore always express multiple paternally-inherited antigens that is foreign to the mother. These mismatched alloantigens are generally - and fortunately - well tolerated by the mother during pregnancy, even though the exact same antigens can cause significant clinical problems in other settings (1). This fetal-maternal tolerance during pregnancy has been an emphasized topic of research for decades (2), since it presents a unique immunological environment in which foreign cells are tolerated by the host. Understanding how this fetal-maternal tolerance is established, and what events that can cause a breach, could provide valuable insight into basic aspects of the human immune response – but our understanding of these mechanisms is still incomplete (3).

5.2 Platelet Antigens

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal alloantibodies that target paternally-inherited human platelet antigens (HPAs) on fetal/neonatal platelets. Since maternal IgG can cross the placenta and enter the fetal circulation by binding to neonatal Fc receptors (FcRn) (4, 5), anti-HPA antibodies developed by the mother can potentially cause the destruction or removal of fetal platelets - rendering the fetus/neonate thrombocytopenic and at risk of hemorrhage.

To date, 35 different HPAs have been described, and these are expressed on six different platelet surface glycoproteins (6). All HPAs, except for HPA-15 (7), are the result of single-nucleotide polymorphisms that are expressed as amino acid substitutions on the main platelet receptors (8, 9). In a Caucasian population the vast majority of FNAIT cases are due to maternal antibodies that target fetal HPA-1a (10, 11). The HPA-1a antigen is expressed on the β3-integrin (12), also known as glycoprotein IIIa (GPIIIa) or CD61. There are five different heterodimeric transmembrane receptors, or integrins, expressed on platelets (13): αIIβ1, αVβ1, αvIβ1, αVβ3 and αIIbβ3. This means that there are two receptors on the platelet surface that express the HPA-1 antigen: αVβ3 and αIIbβ3. There are 40-fold (14), or perhaps even >400-fold (15), as many αIIbβ3 molecules per platelet as there are αVβ3 molecules.

The αIIbβ3 receptor is known to bind fibrinogen, von Willebrand factor, fibronectin and vitronectin, and is an essential part of in vivo platelet function (16). The αIIbβ3 is only expressed by platelets and megakaryocytes. It is unclear if αVβ3 (vitronectin receptor) has any effect on in vivo platelet function, but it is also expressed on endothelial cells (17), where it is known to be involved in vasculogenesis (18) and angiogenesis (19). Furthermore, αVβ3 is also expressed on the surface of syncytiotrophoblasts in the placenta (20, 21), and the β3 integrin has been detected on syncytiotrophoblast microparticles (22).

5.3 Platelet Alloimmunization

FNAIT was first described in the 1960s (23), and has been found to occur in 1:1000-2000 live births (24-28). There are many HPAs known to cause FNAIT. In a Caucasian population, anti-HPA-1a and HPA-5b antibodies are reported to be the cause of more than 90% FNAIT cases in Caucasians, with anti-HPA-1a constituting more than 80% of cases (10, 29, 30). Anti-HLA class I antibodies are also a common finding in cases with suspected FNAIT (29,

31-34), and have been suggested as a possible cause of FNAIT by multiple case reports (35-45).

Mothers that produce anti-HPA-1a antibodies are themselves HPA-1a negative, or rather homozygous for the HPA-1b allele (HPA-1bb). The most likely case of FNAIT in a Caucasian population is therefore an HPA-1ab child born to an HPA-1bb mother and an HPA-1aa/ab father. HPA-1b is the minor allele in Caucasian populations, and the frequency of women who are HPA-1bb (HPA-1a negative) has been estimated to be around two percent in a Caucasoid population (6, 28, 46).

Around 10% of HPA-1a negative mothers carrying an HPA-1a positive fetus will have detectable anti-HPA-1a antibodies (28). The low frequency of immunization is partly explained by the presentation of HPA-1a to the maternal immune system being dependent on the mother's HLA class II expression. A large screening study found that 90% of the mothers that produced anti-HPA-1a antibodies were HLA-DRB3*01:01 positive (28). This strong association has also been observed by others (26, 47, 48). The HLA-DRB3*01:01 allele, along with the HLA-DRA*01:01 allele, encodes the HLA-DR52a molecule (49), an HLA molecule which has been shown to bind peptides with a leucine in position 33 of integrin β3 (HPA-1a) but not peptides with a proline in the same position (HPA-1b) (50). In summary, this means that the immune response in most FNAIT cases is HLA class II restricted (51, 52). The generation of clonal HPA-1a-specific CD4+ T-cells that were restricted to recognition of HPA-1a antigens presented by HLA-DR52a further emphasizes this point (53).

HPA-1 alloimmunization can occur both during pregnancy and delivery (54), and even in a first pregnancy (26, 27, 29, 54). Maternal anti-HPA-1a antibodies can bind the HPA-1a antigen both when expressed by platelets and throphoblasts (55). Maternal anti-HPA-1a antibody levels have been reported to correlate with the severity of the neonatal thrombocytopenia in larger prospective studies (26, 54, 56).

5.4 Clinical Features of FNAIT

The most typical clinical presentation of FNAIT is an otherwise healthy term newborn that presents with skin bleedings shortly after birth, with FNAIT being the most common cause of severe thrombocytopenia in an otherwise healthy term newborn (57, 58). The clinical picture

of FNAIT range from non-symptomatic to a life-threatening bleeding, with intra-cranial hemorrhage being the most feared complication.

It has been reported that mothers who have delivered children affected by FNAIT also have a history of repeated miscarriages (59), and similarly that maternal anti-integrin β3 antibodies can promote fetal miscarriage in a murine model (60, 61).

Anti-HPA-1a antibodies have also been shown to be associated with reduced birth weight (62, 63). Whether or not binding of anti-HPA-1a antibodies to throphoblasts can impair placental function is a hypothesis that is currently being investigated. Members of our research group recently published a pilot study demonstrating that a monoclonal anti-HPA-1a antibody could partially inhibit adhesion and migration of extravillous trophoblasts (64). In support of this idea, a recent report described that FNAIT was associated with an increased frequency of chronic chorioamnionitis, basal chronic villitis and chronic intervillositis – different types of chronic inflammation in the placenta that are otherwise associated with reduced fetal growth (65).

The largest study of FNAIT-induced ICH was an observational cohort study of all registered cases from the international No IntraCranial Haemorrhage (NOICH) registry over a nine-year period, that identified 43 confirmed cases of ICH due to FNAIT (63). In the majority of these cases the ICH occurred in a first-born child before week 28 of gestation. The bleedings were typically classified as either intraparenchymal or intraventricular/periventricular. Only 5 of the 43 (12%) neonates were alive and without severe neurological sequelae upon discharge. Since the identification of these cases were based on clinical referrals, and a smaller neonatal ICH can be asymptomatic (66), they are likely to represent the more severe cases of FNAIT-induced ICH. Retrospective studies have estimated ICH to occur in 7-26% of severe FNAIT cases (29, 67-69), while a review of prospective studies on FNAIT estimates this frequency to be 10% (46).

5.5 Treatment and Prevention of FNAIT

First and foremost, children with severe and/or symptomatic thrombocytopenia will commonly be treated with platelet transfusions irrespective of the underlying cause (70). Mothers who are known to be at an increased risk of delivering a child affected by FNAIT, which will typically be mothers who have previously delivered an FNAIT-affected child, are

in most Western countries treated with antenatal IVIg (30, 71, 72). Some suggest systemic corticosteroids as a supplement to IVIg, but this has not been definitively shown to provide additional protection (73). Repeated cordocentesis with intrauterine platelet transfusions has been abandoned by most due to the high risk of procedure-related complications (74, 75), and a non-invasive treatment strategy is generally recommended (76). Delivery by caesarean section has been suggested to reduce the risk of ICH, but this potential effect also remains uncertain (46, 73). Case reports have suggested that pre-implantation genetic diagnosis (77) and in vitro fertilization (78) could be useful to avoid a fetal-maternal HPA mismatch althogether.

As it stands, treatment and prevention of FNAIT is generally dependent on prior knowledge of an increased risk in the mother, which means that a first-born child with FNAIT will mostly not benefit from these strategies. However, it has been reported that the majority of maternal immunizations occur in connection with delivery (54). This observation, along with the demonstration of an induced antibody-mediated immune suppression (AMIS) effect through administration of anti-HPA-1a antibdies in mice (79), potentially opens up the possibility for a prophylactic approach to FNAIT. This would in theory be similar to how RhD-associated HDFN is prevented, by administration of anti-RhD (or HPA-1a) antibodies to RhD negative (HPA-1a negative) women following the birth of an RhD positive (HPA-1a positive) child. The production and testing of a prophylactic treatment for FNAIT is ongoing (80). Such an approach would nevertheless be dependent on antenatal HPA-1 screening.

5.6 Neonatal Thrombocytopenia in General

Neonatal thrombocytopenia occurs in 1-5% of all newborns (57, 81, 82), and is even more frequent among neonates admitted to neonatal intensive care units (83). Some of the more common causes of neonatal thrombocytopenia include infections, prematurity, antibody-mediated platelet destruction, perinatal hypoxia/asphyxia and chromosomal abnormalities, although neonatal thrombocytopenia is associated with a multitude of complicating factors (70, 84, 85).

Neonatal thrombocytopenia is generally defined as a platelet count $< 150 \times 10^9$/L, and severe neonatal thrombocytopenia as a platelet count $< 50 \times 10^9$/L. Clinical signs of bleeding are rare outside of cases with severe thrombocytopenia, and it is therefore generally recommended to

not transfuse platelets unless the neonatal platelet count is < 50×10^9/L (70). In Norway the recommended threshold is < $35x10^9$/L (86).

5.7 Human Leukocyte Antigens

Human leukocyte antigens (HLA) are the human variant of the major histocompatibility complex (MHC), a highly polymorphic cell surface molecule present in all jawed vertebrates (87). MHC was first described by Gorer and Snell in the mid-20th century, primarily based on observations of how certain genes in mice resulted in resistance to allogenic tumor growth (88). The first HLA, then called MAC and later identified as HLA-A2, was identified by Dausset in 1958. Dausset made his discovery by observing how sera from multitransfused patients caused agglutination of leucocytes from a high number of other individuals, but never with the patient's own leukocytes. These discoveries and the general history of HLA are well-described in Thorsby's *A short history of HLA* (89). The HLA gene loci is situated on the short arm of chromosome 6 and is the most polymorphic loci in humans, with 16,755 HLA alleles identified as of June 2017 (6).

The genes within the HLA loci that encode the leukocyte antigens are differentiated into class I and class II. Of the 16,755 described HLA alleles, 12,351 are class I while 4,404 are class II (6). The primary function of HLA class I is to present peptides that have been processed from intracellular antigens to CD8+ T-cells. The expression of HLA Class II molecules are usually limited to professional antigen presenting cells (APCs), where they present peptides that have been derived from extracellular proteins to CD4+ T-cells. Class II will not be further discussed here. The structure and function of HLA is described in detail in the two part review *The HLA System* by Klein and Sato (90, 91).

Class I is further divided into three major antigens that are significantly polymorphic and widely expressed (HLA-A, HLA-B and HLA-C), three minor antigens that have a low degree of polymorphism and are not widely expressed (HLA-E, HLA-F and HLA-G), and certain non-expressed pseudogenes (6).

5.8 Anti-HLA Class I Alloimmunization

Since HLA antigens are highly polymorphic, it is very unlikely that any two unrelated persons will have identical HLA antigens. This is illustrated by the need for large international registries of hematopoietic stem cell donors to facilitate HLA compatible bone marrow transplantations between unrelated donors (92). Along with the wide expression of HLA class I on virtually all nucleated human cells, this means that there is a high chance that introduction of tissue or cells from any one person to another will expose the recipient to foreign HLA antigens, and potentially cause a humoral alloimmune response with production of anti-HLA class I antibodies. Although there is some evidence that these antibodies may also be found in apparently non-alloimmunized individuals (93), they are generally detected in situations where it is well known that exposure to allogenic cells have occurred, such as transplantation, transfusion and pregnancy.

The extent to which anti-HLA class I antibodies could impede successful organ transplantation was something that became more and more evident as the sensitivity of detection methods increased (94, 95). Today, it is well known that anti-HLA class I antibodies can cause rejection of kidney allografts (96, 97), and that awareness of these antibodies are important for risk-stratification of solid organ transplantation in general (98), as the increased risk of rejection is likely not restricted to renal grafts (1).

Platelet refractoriness following transfusion refers to "a lack of adequate post-transfusion platelet count increment" (99), and this can be due to both immunological and non-immunological factors (100). Isolated immunological factors are estimated to be responsible for 18-25% of unsuccessful platelet transfusions (101, 102), but they can also be present alongside non-immunological factors. Anti-HLA class I alloimmunization is the most common immunological cause (103, 104), and is a particular problem in patients that have had repeated exposure to allogenic HLA by for example multiple transfusions, pregnancy and/or transplantation.

Anti-HLA class I antibodies are also believed to be involved in the development of transfusion-related acute lung injury (TRALI), which is a non-cardiogenic lung oedema that occurs secondary to transfusion. This will commonly manifest itself as severe respiratory distress, and is one of the most common causes of transfusion-related morbidity and mortality (105). The pathogenesis of TRALI is not fully understood (106), but it has been suggested that there may be an immune as well as non-immune variant of TRALI. The immune-variant,

or rather antibody-mediated TRALI, is believed to be caused by antibodies that trigger the activation of neutrophils in the pulmonary capillaries (107). These antibodies most likely originate from the transfused blood product (108). Antibodies that can trigger this neutrophil activation include anti-HLA class I and II antibodies, as well as anti-human neutrophil antigen (HNA) antibodies. TRALI induced by leukocyte antibodies is believed to constitute the majority of all TRALI cases (109).

5.9 Anti-HLA Class I Antibody Detection

The following section is a summary of how detection of anti-HLA class I antibodies has evolved, as presented by Bontadini in his article *HLA techniques: Typing and antibody detection in the laboratory of immunogenetics* (110).

Complement-dependent cytotoxicity (CDC) assays with live T cells was the predominant technique for many years. These assays were time-consuming and suffered from a lack of sensitivity, but could provide a direct visualization of the complement-mediated cell damage that resulted from antibodies binding to antigens. Although CDC does not give information of the specificity of the antibodies involved, the assay assesses whether a patient has donor-specific antibodies that potentially could initiate an antibody-mediated graft rejection. A positive CDC is therefore still the "gold standard" for accepting or refusing an organ donor for a transplantation (T. Egeland, personal communication, 16th June 2017).

The next important step was ELISA-based techniques that utilized antigens from individual cells lines. These antigens were attached to wells in microtiter plates, where antibodies could then bind and be detected by anti-human IgG. ELISA assays are significantly less time consuming than CDC assays, can detect both complement and non-complement fixing antibodies, and are much more sensitive. They do however not provide any direct visualization of cell damage.

The last important development is the flow cytometry and Luminex techniques, which rely on soluble or recombinant antigens bound to beads. These techniques generally provide the same benefits as ELISA assays, but at a much higher level of sensitivity. With the latest assays it is possible to get individual fluorescence intensities for binding of antibodies to each of the approximately 100 most common HLA antigens in a Western background population. These

Luminex assays are, however, so sensitive that the clinical relevance of some of the detected antibodies remain controversial.

5.10 Maternal Anti-HLA Class I Antibodies in Connection with Pregnancy

Maternal anti-HLA class I antibodies are commonly found during and after pregnancy, and are detected in 30-60% of all pregnant women, depending on detection method and parity (111-114). It is a consistent finding that anti-HLA class I antibodies are significantly more frequent in multiparous women than in nulliparous women, which is to be expected since the chance of immunization is likely to increase with repeated HLA antigen exposure. It has been stated that the antibodies are unlikely to be detected before week 28 of gestation (112), but this might be dependent on the detection method applied, since more recent studies using modern detection methods have found antibodies in maternal samples from early in the second trimester, even in nulliparous women (115, 116).

Many factors regulate which fetal antigens the mother is exposed to. First and foremost, the maternal and fetal circulations are - to some extent - separated by the placenta. As the fetal trophoblasts invade the maternal decidua and the inner part of the myometrium to generate a fetal-maternal interface that will supply the fetus with nutrients and oxygen (as well as a myriad of other functions), the maternal side is exposed to fetal trophoblasts. These invading fetal trophoblasts do not, however, express polymorphic HLA antigens in the same way as most other nucleated cells. Some of the invading fetal trophoblasts (villous trophoblasts) do not express any HLA class I antigens at all, while the extravillous trophoblasts (which control vascular remodeling) express HLA-C, HLA-E and HLA-G, but not HLA-A or HLA-B (117-122). HLA-E and HLA-G are not particularly polymorphic, with only 23 and 53 reported alleles, respectively (6), and HLA-C is significantly less polymorphic than HLA-A and HLA-B. HLA-C is also expressed at a much lower degree than HLA-A and HLA-B on normal cells (123), and is therefore less of a clinical concern during transplantation and transfusion. In summary, this means that the fetal HLA class I antigens expressed in the fetal-maternal interface are generally less likely to induce an alloreactive response. This is in contrast to the fetal HLA class I antigens expressed on most nucleated cells elsewhere. Still, pregnancy-induced anti-HLA class I antibodies, even in connection with normal pregnancies, are often specific towards fetal HLA-A and HLA-B (124, 125).

Although the maternal and fetal circulations are separated by the placenta, it is becoming more and more clear that there is a rather large passage of fetal material into the maternal circulation during pregnancy. Fetal cells and fetal cell-free DNA are detectable in the maternal circulation even during the first trimester, and increases steadily towards delivery (126-128). So far little is known about how this microchimerism interacts with the maternal immune system, and whether or not this fetal material is sufficient to induce a maternal alloimmune response, although there is an increasing research focus on its role during and after pregnancy (3). It has generally been believed that maternal alloimmunization mostly occurs in connection with fetal-maternal hemorrhage during delivery, or as a result of a fetal-maternal hemorrhage earlier in pregnancy. Although a large fetal-maternal hemorrhage is a rare occurrence, it is probable that nearly all pregnancies experience a limited degree of fetal hemorrhage into the maternal circulation (129).

Since anti-HLA class I antibodies are such a common finding even during otherwise normal pregnancies, they are generally regarded as a normal occurrence that do not require further action during pregnancy. This is in some ways surprising given the harmful effects they can illicit in other settings, and the knowledge that maternal IgG is transported across the placenta by neonatal Fc receptors (4, 5). It is also known that pregnancy-induced anti-HLA antibodies can become a problem in other settings later: Most cases of TRALI are likely caused by anti-HLA class I antibodies (106, 109, 130), and among blood donors these antibodies are most frequently found in samples from multiparous women (131-133). Women that receive transplants from their partner or child have a higher risk of an acute antibody-mediated rejection of the graft compared to other transplants (134), There is also a greater increase in anti-HLA class I antibody levels following an incompatible renal transplantation if a pregnancy was the initial sensitization rather than a previous transplantation (135).

Although maternal anti-HLA class I antibodies in general are regarded as a normal finding during pregnancy, there are many reports of associations between these antibodies and several different pregnancy complications. Many of the studies on this topic are well described in the systematic review and meta-analysis by Lashley et al. (136).

Multiple sources have cited a possible connection with recurrent miscarriage (137-140), while others have contradicted this (141-144), and even argue that a maternal response to paternal alloantigens might be important for a successful pregnancy. In line with this, it has been reported that couples with recurrent miscarriages may actually have an increased number of

shared common HLA antigens (145, 146), and that the lack of a maternal immune response is correlated with an increased risk of recurrent miscarriage (142). A recent study even found that a dissimilar HLA class I type between partners may promote partnership, sexuality and the desire to procreate (147).

One study reported an increased frequency of anti-paternal HLA antibodies in women with preterm placental abruption (148), and it has also been shown that the presence of anti-HLA antibodies in maternal sera, sometimes as early as <16 weeks of gestation, is associated with chronic chorioamnionitis at delivery (116). There has also been suggested an association with preeclampsia (149), but this connection may not be related to fetal antigen-specific IgG antibodies at all, but rather a nonspecific reaction to placental damage (149, 150).

Several of the cited reports hypothesize that the presence of maternal anti-HLA class I antibodies that target paternally-inherited antigens may be a sign of a maternal rejection of the fetus, similar to a transplanted allograft. Still, it is also known that coincidental proinflammatory events, such as infections, surgeries and traumatic injuries, can increase the breadth and strength of these antibodies in other settings (151). Given the generally inconclusive nature of the few systematic studies on anti-HLA class I antibodies during pregnancy (136), and the likelihood that their presence is increased by other proinflammatory events, it remains a possibility that they are simply an epiphenomenon in this setting. Especially since the exact state of inflammation during pregnancy, and its potential effects on maternal and fetal health, remains a challenging topic to grasp (152).

In summary, there are clear indications that the maternal immune system both sees and reacts to fetal alloantigens, although in many cases it remains unclear what, if any, effects this might have on fetal and/or maternal health. The topic of fetal-maternal tolerance is a vast field, and encompasses many suggested mechanisms on how the maternal immune system is modulated to accommodate the fetus during pregnancy. Some of the suggested mechanisms have already been described, such as the unique expression of HLA on trophoblasts, while others include an increased regulation of T-cell responses and complement activation (2). Fetal-maternal microchimerism, which has been a topic of growing interest in recent years, is also likely to contribute to this development of tolerance (3).

5.11 Antigens and Epitopes

The term antigen is "used to describe any substance that can be recognized and responded to by the adaptive immune system" (153). Epitopes, on the other hand, are the exact parts of an antigen that are recognized by an antibody. One antigen usually expresses multiple epitopes, and many of these epitopes will not be unique to that one antigen (shared epitopes). Antibodies that target a shared epitope may therefore react with other antigens than the original immunizing antigen. This is the explanation for why an anti-HLA class I antibody may react with multiple HLA class I antigens, even ones that the immunized individual was never exposed to, and is referred to as cross-reactivity. HLA class I antigens have historically been grouped into cross-reactivity-groups (CREGs) based on observed cross-reactivity patterns in serologic testing (154). CREG groups have historically consisted of approximately ten groups of HLA antigens, where the antigens within each group demonstrate a strong serological cross-reactivity (154-156). But, even though the antigens within a CREG group often have several epitopes in common, giving rise to the strong serological cross-reactivity, there may still be some epitopes expressed on these antigens that they share with antigens in other CREG groups (157). Immunization against one antigen may therefore still yield reactivity both inside and outside the given CREG group.

As sequencing became more common, these cross-reactivity groups were changed to instead reflect the expression of common polymorphic amino acid sequences that constituted epitopes (155, 156). Initially these polymorphic amino acids were only considered as short linear sequences (triplets) that were part of the whole sequence of the HLA antigen. However, more recently it has been shown that many epitopes also are made up of amino acids from discontinuous positions in the sequence (eplets), that due to the ultimate folding of the protein are brought together to form a functional epitope (158). The identification of antibody reactive eplets is an ongoing process (124).

5.12 HLA Class I on Platelets

Platelets express HLA-A, HLA-B and HLA-C (159, 160). It has been suggested that HLA class I antigens on platelets may not have their own de novo synthesis of HLA class I antigens, but rather that they are primarily adsorbed (159, 161) or are vestiges from the megakaryocyte precursor stage that do not directly stimulate cytotoxic T-cells (160). But there

are also studies that contradict this, and suggest that platelets have the capability to synthesize their own HLA proteins (162). Platelets have also been shown to not induce T-cell proliferation on their own *in vitro* (163), suggesting that platelets are not usually the primary immunizing agents, but rather that they are affected by immunizations towards antigens that they share with other cells, such as for example HLA class I. This is supported by the widely accepted notion that leukocyte-depletion of platelet concentrates significantly reduces primary HLA alloimmunization (164). That being said, T-cell mediated cytotoxicity has been suggested as a possible mechanism of platelet destruction in ITP (165).

5.13 Anti-HLA Class I Antibodies and FNAIT

Since platelets express all of the major HLA class I antigens, and anti-HLA class I antibodies are well known to cause refractoriness following platelet transfusion, it has long been hypothesized that anti-HLA class I antibodies may be a cause of FNAIT (166). This association has primarily been suggested by case studies (35-45). The few prospective studies so far have been negative or inconclusive (113, 167-171), and were often limited by small study populations. Whether or not anti-HLA class I antibodies may be a cause of FNAIT remains an open question that is still being investigated (172).

In general, the noted case studies have excluded anti-HPA antibodies as a possible cause of the observed thrombocytopenia either by negative detection assays, and/or by finding compatible expression of HPA in mother and child. Other apparent causes of neonatal thrombocytopenia, primarily congenital infections, are usually excluded by a lack of clinical symptoms and/or by negative blood cultures and other infection parameters. Reactivity of maternal samples with paternal or fetal platelets is usually demonstrated, and in later reports the specificity of the detected anti-HLA class I antibodies is often evaluated and compared with paternal or fetal genotype. Some of the reports also detect the antibody that is suspected to have caused the thrombocytopenia in fetal circulation. There are both nulli- and multiparous mothers among the described cases. This kind of rigorous examination, and similarities with anti-HPA antibody induced FNAIT, is mainly why the hypothesis of anti-HLA class I antibodies as a cause of FNAIT has persisted.

Anti-HLA class I antibodies have been reported to appear alongside anti-HPA antibodies in 37-45% of cases with suspected FNAIT (33, 34), which is not surprising given the frequent

occurrence of anti-HLA class I antibodies during pregnancies (111-114). If maternal anti-HLA class I antibodies can induce fetal/neonatal thrombocytopenia, as suggested by multiple case reports (35-45), it would be reasonable to assume that their added presence alongside anti-HPA-1a antibodies might worsen the fetal/neonatal platelet count compared to cases with only anti-HPA-1a antibodies. There are however no published reports on a systematic investigation into such a hypothesis.

6. Aims of thesis

The main aim of this thesis was to characterize cases with suspected FNAIT due to maternal anti-HLA class I alloantibodies. More specifically, the aims were:

- To examine the maternal and perinatal clinical characteristics of pregnancies with suspected FNAIT, where the only suspected cause of thrombocytopenia was anti-HLA class I antibodies detected in the mother.
- To determine the anti-HLA class I antibody specificities and -levels in pregnancies with suspected FNAIT, and compare these findings with normal controls.
- To determine whether certain fetal-maternal HLA class I mismatches are associated with neonatal thrombocytopenia
- To assess if the additional presence of maternal anti-HLA class I antibodies alongside anti-HPA-1a antibodies in confirmed cases of FNAIT influence severity of FNAIT.
- To generate new hypotheses that can inform the design of future prospective studies on the relationship between maternal anti-HLA class I antibodies and FNAIT.

7. Methods

7.1 Selection of Study Populations

Papers I and II:

The case group and control group presented in papers I and II are identical, except that some sub-groups are described to a differing degree of detail in the two papers.

The case group was selected from all pregnancies referred to the Norwegian National Unit for Platelet Immunology in Tromsø, Norway due to suspected FNAIT during the period 1998-

2009. Pregnancies were included as cases if maternal anti-HLA class I antibodies were detected and neonatal thrombocytopenia confirmed. Pregnancies were excluded if platelet-specific (anti-HPA-) antibodies were detected or if other identifiable causes of neonatal thrombocytopenia were found. Information regarding demographic characteristics, obstetric history, course and outcome of pregnancy was obtained from the medical records. All maternal blood samples were taken postpartum, and stored as plasma.

Of 82 mothers who fulfilled the inclusion criteria, 62 consented to participate. There was one twin pregnancy. Thirteen neonates were further excluded from analysis: Eight due to other possible reasons for neonatal thrombocytopenia (two congenital cytomegalovirus infections, one Jacobsen's syndrome, one maternal immune thrombocytopenic purpura, one neonatal haemochromatosis, one Noonan's syndrome, one Down's syndrome, one case of neonatal death 18 days after birth where autopsy showed underdeveloped bone marrow) and five cases where maternal sera were unavailable for antibody analysis. Thus, data from 50 cases over a period of 11 years were included for further analysis.

The control group consisted of an unselected population of pregnant women originally included in a prospective study investigating maternal-fetal haemodynamics at the University Hospital of North Norway during 2006-2010 (173, 174). Maternal blood samples were taken at 22-24 weeks of gestation, and stored as plasma. Additional maternal blood samples acquired within three days of delivery were available for seven controls. All samples were tested for presence of maternal anti-HLA class I antibodies and categorized as either anti-HLA class I antibody-negative or -positive. Of 250 pregnancies in the control group, 72 (29%) tested positive for maternal anti-HLA class I antibodies. 60 of these 72 antibody positive controls were further analyzed for antibody specificities. Platelet counts were obtained from 45 randomly selected neonates in the control group, of which none were thrombocytopenic.

Paper III:

In the third paper we investigated samples that were originally collected as part of a prospective screening and intervention study aiming to reduce morbidity and mortality of neonatal alloimmune thrombocytopenia (NAIT) through detection and intervention (28). 100 448 pregnant women were recruited consecutively with no applied inclusion criteria during December 1995 until March 2004 from North Norway, and during September 2001 until March 2004 in the health regions South and East in the southern part of Norway. All included

women were HPA-1a typed, and HPA-1a negative women were screened for anti-HPA-1a antibodies and HLA-DRB3 genotyped. Maternal blood samples were collected approximately every fourth week during pregnancy. If development of anti-HPA-1a antibodies were detected, delivery was performed by caesarean section 2-4 weeks prior to term. HPA-1a negative platelets were transfused to the neonate if platelet count was less than 35 x 10^9 /L and/or if petechiae were seen.

Of the 100 448 pregnant women that were recruited, 2111 were typed as HPA-1a negative. 1990 of these HPA-1a negative women were examined for anti-HPA-1a antibodies, with a positive detection in 210 (10.6%). These 210 women underwent 233 pregnancies. Anti-HPA-1a antibodies were detected *during* pregnancy in 194 of these 233 cases. 170 of these 194 pregnancies were managed according to the described program. Further details, and study selection figure, are described by Kjeldsen-Kragh et. al. (28).

166 of the 170 included pregnancies from the original screening study were later rescreened for anti-HLA class I antibodies during the investigation described in the third paper. 111 (67%) of the samples tested positive for anti-HLA class I antibodies.

7.2 Antibody Detection (All Papers)

Identification of anti-HPA antibodies was performed by monoclonal antibody immobilization of platelet antigen (MAIPA) (175), both during the original screening study described in the third paper, and on referrals to the Norwegian National Unit for Platelet Immunology in Tromsø, Norway during the period 1998-2009. Quantification of anti-HPA-1a antibody levels was performed using a modified MAIPA test (56, 176).

Anti-HLA class I antibodies were originally identified by using an in-house MAIPA technique (177) at the Norwegian National Unit for Platelet Immunology during 1998-2009. Samples were tested against paternal platelets when available. If paternal platelets were not available, the study sample was tested against random donor platelets from at least four donors. All samples that tested negative for antibodies in MAIPA panels with random donor platelets, were subsequently also tested with FlowPRA 1 Screening Test (One Lambda, Canoga Park, CA) to uncover false negatives.

All samples included for the current studies were later retested using the FlowPRA 1 Screening Test (One Lambda, Canoga Park, CA) to acquire comparable measurements. The panel of antigens present in the FlowPRA I Screening Test contains all of the most common, and several rare, HLA class I antigens present in a Northern European population. It gives an overall mean fluorescence intensity (MFI) that reflects the presence or absence of anti-HLA class I antibodies targeting any of the HLA antigens present. An anti-human IgG antibody was used to detect the antibodies.

As an estimate of anti-HLA class I antibody level we calculated the ratio of the median fluorescence intensity (MFI) for each sample to the MFI of the respective negative control.

7.3 Anti-HLA Class I Antibody Specificities (Paper II)

After the initial detection, anti-HLA class I antibodies were further analyzed with regards to antibody specificity using the LABScreen Single Antigen HLA Class I assay at the Department of Immunology, Oslo University Hospital, Rikshospitalet, Norway. This assay does not only detect the presence or absence of anti-HLA class I antibodies, like the FlowPRA I Screening Test, but also gives individual MFI responses for the binding of antibodies to each specific HLA class I antigen. Also here, an anti-human IgG antibody was used to detect the antibodies.

Each of the beads in the LABScreen Single Antigen HLA Class I assay are coated with one specific HLA-A, HLA-B or HLA-C antigen, for example HLA-A*01:01. The whole assay includes the 97 most common antigens in the general population, and each of these antigens are comprised of multiple epitopes that are potential antibody targets. One specific epitope can be shared among several antigens, and it is therefore common to see reactivity towards several antigens in the LABScreen Single Antigen HLA Class I assay, even though the investigated antibody was the result of immunization towards one specific antigen.

We also diluted ten maternal samples from the suspected FNAIT cases at 1:10, 1:50 and 1:500, and reanalysed them with LABScreen Single Antigen HLA Class I assay to evaluate the stability of the reactivity patterns.

7.4 HLA Class I Genotyping (Paper II)

To further evaluate antibody specificities we therefore genotyped maternal and neonatal HLA class I. This was done by an in-house sequence-based typing and analyzed using the Assign Software (Conexio Genomics, Fremantle, Australia) at the Department of Immunology, Oslo University Hospital, Rikshospitalet, Norway. In cases where genotyping indicated two or more likely alleles, the most frequent allele according to data from the Norwegian Bone Marrow Donor Registry (178) was chosen to represent the genotype in question for further analyses.

7.5 HLA Class I Epitopes (Paper II)

Maternal and neonatal genotyping data was combined with data on HLA class I epitope expression. Epitope data was retrieved from HLAMatchmaker (http://www.epitopes.net) and the HLA Epitope Registry (http://www.epregistry.com.br/index/databases/database/ABC/) in February 2016. With this data we could determine which epitopes on the paternally-inherited HLA antigens that were the most probable causes of immunization, i.e. neonatal epitopes that were inherited from the father and not shared by the mother. In order to be a likely cause of immunization the epitope also had to be labeled as "antibody reactive" (confirmed or provisional) in the HLA Epitope Registry (179).

By combining maternal and neonatal genotyping with the data from the HLA Epitope Registry and the MFI signals from the LABScreen Single Antigen HLA Class I assay, it was possible to determine which MFI signals that represented a paternal-specific reactivity, and which MFI signals that represented reactivity towards a third-party or self (reactivity towards maternal epitopes).

7.6 Definitions

All pregnancies were dated based on ultrasonography performed in the second trimester. Infants born before 37+0 gestational weeks were defined as premature.

Small for gestational age (SGA) was defined as birth weight less than the 10th percentile for gestational age based on singleton percentile curves (180). A placental weight/birth weight

ratio (PW/BW-ratio) was calculated and included in some analyses, given that this ratio has been considered a significant predictor of long-term fetal health (181, 182).

Preeclampsia was diagnosed according to current ISSHP criteria (183).

Thrombocytopenia was defined as a platelet count < 150×10^9/L, moderate thrombocytopenia was defined as a platelet count between 50 - 149×10^9/L, and severe thrombocytopenia was defined as a platelet count < 50×10^9/L.

7.7 Ethics

The described studies were approved by the Regional Committee for Medical Research Ethics, North Norway (Ref. no. REKNORD 2013/1863: date of approval 15.05.2014, and REKNORD 5.2005.1386). Informed written consent was obtained from all women included.

7.8 Statistics

All papers:

Data were analyzed using different versions of SPSS software (SPSS Inc., Chicago, IL, USA). Figures were created using plot.ly, Microsoft Excel, Adobe Photoshop and SPSS.

Normality of data distribution was tested using Kolmogorov-Smirnov test. An independent samples t-test was used to compare means for continuous variables with a normal distribution, while the Mann-Whitney U test was used to compare means without a normal distribution. The Fisher's exact test was used to compare frequencies for categorical variables.

Variance of continuous variables between groups was tested using One-Way ANOVA with Bonferroni post-hoc test.

When testing correlation between normally and not normally distributed data we report Pearson's correlation coefficient or Spearman's correlation coefficient, respectively.

Missing data was treated by pairwise deletion when comparing all unadjusted data, and by listwise deletion when performing regression analyses.

A *P*-value of < 0.05 was considered significant.

Regression Models Paper I:

All regression models included the following independent variables: Maternal age (continuous), parity (nulli- or multiparity), preeclampsia (yes/no), sex of the newborn (boy/girl) and gestational age at time of delivery (continuous). When looking at associations between maternal antibody level and birth weight, SGA, placental weight or PW/BW within each study group, maternal antibody level was included as an independent variable. We did not adjust for gestational age at delivery when SGA was defined as the dependent variable.

Data on smoking habits were available for 70% of cases. All regression analyses were done both with and without adjusting for smoking habits. Data presented in the results did not take into account smoking habits unless otherwise stated.

Regression Models Paper III:

Measurements of antibody level were log-transformed due to positive skew.

Logistic regression analyses included thrombocytopenia (yes/no) as the dependent variable, with parity (discrete), maternal anti-HLA class I antibody status (positive/negative) and maternal anti-HPA-1a antibody level (continuous) as independent variables. Linear regression analyses included platelet count at birth (continuous) as the dependent variable, with the same independent variables as in the logistic regression analyses. An interaction term between parity and maternal anti-HLA class I antibody status (positive/negative) was included in all models. Both logistic and linear regression analyses were repeated including maternal anti-HLA class I antibody level (continuous) substituted for maternal anti-HLA class I antibody status (dichotomous) as an independent variable.

8. Results

8.1 Paper I

Maternal anti-HLA class I antibodies are associated with reduced birth weight in thrombocytopenic neonates

In this comparative cross-sectional study we investigated the clinical characteristics and antibody levels of pregnancies where the neonate had suspected FNAIT due to presence of maternal anti-HLA class I antibodies (cases, n=50), and compared them to normal pregnancies (controls, n=250) screened for maternal anti-HLA class I antibodies. We found that thrombocytopenic neonates born to mothers with anti-HLA class I antibodies had significantly lower birth weight compared to controls, and that an increasing level of maternal anti-HLA class I antibodies was linearly and inversely associated with birth weight and placental weight among the thrombocytopenic neonates. There was no difference in birth weight between the antibody-positive and antibody-negative controls, and antibody level was not significantly associated with birth weight or placental weight among controls.

8.2 Paper II

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

In this comparative cross-sectional study we investigated the antibody specificities of maternal anti-HLA class I antibodies in neonates with suspected FNAIT (cases, n = 50), and compared them to normal pregnancies with maternal anti-HLA class I antibodies (controls, n = 60). By combining maternal and neonatal genotype with data on HLA class I epitope expression we could show maternal anti-HLA class I antibodies in connection with pregnancies complicated by neonatal thrombocytopenia are fetal/paternal-specific, with very little reactivity towards self or any third-party. We did not find that the observed HLA immunization was tied to any particular HLA antigen, but mothers in the case group had an overall higher antibody level compared to normal antibody-positive controls. Fetal/paternal-specific antibody levels were increased in cases with more severe clinical outcome.

8.3 Paper III

Maternal anti-HLA class I antibodies in addition to anti-HPA-1a antibodies influence severity of fetal and neonatal alloimmune thrombocytopenia (FNAIT) – data from a large prospective screening study

In this study we used data and samples collected as part of a previous large prospective screening study (28) to investigate the potential impact of anti-HLA class I antibodies alongside anti-HPA-1a antibodies on neonatal platelet count. We reanalyzed samples collected from HPA-1a negative women who developed anti-HPA-1a antibodies during pregnancy, and found that 67% of the mothers also had detectable anti-HLA class I antibodies during pregnancy. The presence of anti-HLA class I antibodies was significantly associated with an increased risk of neonatal thrombocytopenia, after adjusting for anti-HPA-1a antibody level and parity. This association was primarily predicated on pregnancies where the mother was nulliparous (25% of the study population).

9. Methodological Considerations

9.1 Study Design

Both papers I and II concern identical case and control groups, with some sub-groups being described with greater detail in each of the two papers. Ultimately, paper I and II were designed as comparative cross-sectional studies. A cross-sectional study is primarily defined by exposure and outcome being measured simultaneously, although the point of measurement does not necessarily have to be the same point in time for all cases. For the case group in paper I and II these points of measurement stretched over an eleven-year period, even though exposure and outcome were measured simultaneously for each individual case. The control group in papers I and II was identified prospectively, and included as a comparative group to the identified cases.

Why paper I and II are described as comparative cross-sectional might not be readily apparent, but there are a couple of crucial factors concerning the case group that determines this. First, as already described, there was no latency period between exposure (detected antibodies) and outcome (neonatal thrombocytopenia), although it can likely be inferred (see later discussion on Sampling Time) that the antibodies were also present during the pregnancy

itself. This lack of observation time between exposure and outcome invalidates any inferrals on causality.

Second, if we were to designate it as a retrospective cohort, we would need to have data on both exposed and non-exposed cases in a complete study population. An alternative study design might then be to look at FNAIT referrals with and without anti-HLA class I antibodies, or all Norwegian pregnancies with and without anti-HLA class I antibodies. The first of these two options would probably provide more answers on how the described cases compare with other cases of FNAIT, but not necessarily how they compare with a normal background population. The second option would likely provide better answers to all our stated research questions, but would require extensive effort and large study populations to achieve sufficient power, especially given the rarity of the cases we describe. We ended up choosing the described approach, since it provides a concise initial description of a rather rare group of cases.

The data in paper III stems from a prospective screening and intervention study that was conducted during 1995-2004, with maternal samples being reanalyzed for the purposes of our investigation. In this study design there is latency between the exposure and the outcome, as well as descriptions of a complete study population. These results can therefore inform arguments regarding causality. However, the initial screening was conducted with the detection of anti-HPA-1a antibodies in mind, not anti-HLA class I antibodies. Also, the neonatal platelet count was not measured in HPA-1bb women without detectable anti-HPA-1a antibodies. This means that the described results are only valid within the context of an HPA-1a negative pregnancy where the mother developed anti-HPA-1a antibodies. We do not know whether anti-HLA class I antibodies in an HPA-1bb pregnant women without anti-HPA-1a antibodies may affect neonatal platelet count. We also do not know whether the effect of anti-HLA class I antibodies during pregnancy would differ between an HPA-1a positive woman compared to an HPA-1a negative woman. A more thorough investigation on the relationship between anti-HLA class I antibodies and neonatal thrombocytopenia during pregnancy, would likely include both pregnancies with and without anti-HPA-1a antibodies, as well as extensive descriptions on other possible causes of neonatal thrombocytopenia.

9.2 Selection Bias

The case group in papers I and II was selected from referrals made to the Norwegian National Unit for Platelet Immunology with suspected FNAIT during 1998-2009. Since this laboratory is the only one conducting FNAIT investigations in Norway, these cases are likely representative of clinically significant FNAIT cases during this period. It is, however, important to remember that FNAIT is an under-diagnosed condition without any implemented screening programs (184). Clinical signs of bleeding are usually only present in cases with severe thrombocytopenia (platelet count of $< 50 \times 10^9$/L), and a neonatal platelet count is not part of any routine perinatal investigation in Norway. Clinical referrals due to suspected FNAIT will, therefore typically consist of the more severe cases of neonatal thrombocytopenia, while moderate cases with less severe thrombocytopenia and lower antibody levels will likely go unnoticed.

The very low median platelet count among the described cases (24×10^9/L), as well as the high frequency of neonates that were SGA (46%), could be a reflection of this selection bias. If this is the case, it would also mean that correlation analyses between antibody presence and platelet count become inconclusive, since we are probably only seeing a small part of the scale. This kind of selection bias is a common problem for most, if not all, retrospective studies. However, given the apparent rarity of cases with suspected FNAIT due to anti-HLA class I antibodies, this kind of study design becomes a necessity. The described case population is the largest of its kind reported in the literature, and with few missing data.

The included control group for papers I and II does not suffer from this kind of selection bias, since it consists of pregnancies included prospectively without any inclusion criteria. These controls were, however, only recruited at the University Hospital of North Norway, while the included cases were included nationwide. To evaluate the external validity of the controls, we compared data for the controls with data from the Medical Birth Registry of Norway (MBRN) for 2010. Except for a lower frequency of smokers (6.4% versus 18.5% in the MBRN), all maternal and neonatal characteristics were similar, and we therefore concluded that the controls are representative of a general population of Norwegian pregnancies during the given time-period.

For all papers it is also important to note that the results are not necessarily valid outside of the geographical region in which the data was collected. This kind of bias is likely also present in the prospectively selected population in paper III. For maternal alloimmunization in

general, this kind of geographical bias will often be related to differing allele frequencies between populations. For example, incompatibility for HPA-4, which is not a likely cause of FNAIT in Caucasians, is the most common cause of FNAIT in Japan (185). Also, HLA-B*27, which is a quite common allele among the Ugro-Finnish and Northern Scandinavian sub-groups of Caucasians, remains rather rare in other parts of Europe (186).

9.3 Confounders

We excluded cases with other identifiable reasons for neonatal thrombocytopenia, such as congenital infections, genetic syndromes and maternal ITP, after going through medical records from the local hospitals where the delivery had taken place. A few of these causes, such as Jacobsen's syndrome, are very rare conditions in the background population. One would perhaps expect more common causes of neonatal thrombocytopenia, such as neonatal infections, to constitute the vast majority of causes for exclusion, but this was not the case. On the other hand, if the diagnosis of infection was made, it is highly unlikely that these cases would be referred for FNAIT investigation, since FNAIT is still in many ways considered a diagnosis of exclusion. It is easier to see how a genetic syndrome, often diagnosed later in life, would be part of the referrals. This is also consistent with a similar study from Sweden (187), which found several rare conditions when going through the medical records of cases with suspected FNAIT due to maternal anti-HLA class I antibodies.

There were some commonly cited causes of thrombocytopenia that we did not have sufficient information to evaluate. One of these causes were fetal hypoxia/asphyxia, which is linked to neonatal thrombocytopenia (85). Given the high frequency of emergency caesarian sections in the case population (20/24), it is possible that some neonates may have suffered from this. However, even asphyxia rarely leads to platelets counts $< 50 \times 10^9$/L (188, 189), and it is therefore unlikely that this would explain the consistently low platelet counts among most all neonates in the case group, although it could of course be a contributing factor. IUGR is also a cited cause of thrombocytopenia that we could not properly evaluate, since we did not have access to repeated growth measurements during pregnancy. The increased frequency of SGA (46%) could indicate that IUGR was present in several cases, but it is important to remember that some children are SGA but not IUGR. Furthermore, as we will discuss later, it may impair an investigation to exclude all conditions that have been linked to thrombocytopenia, when a sequence of causality has not been established. Were the neonates thrombocytopenic

due to a low birth weight, or could perhaps the antibodies and/or fetal thrombocytopenia have influenced the birth weight?

As is discussed under *Discussion of Results - Inflammation*, it is also important to note that the presence of inflammation in general may have impacted both antibody presence (151) as well as platelet count (190). We did not have access to information on inflammatory markers during pregnancy.

9.4 Measurements of Antibody Level

In all three papers we used MFI values from the FlowPRA I Screening Test and the LABScreen Single Antigen HLA Class I assay as measurements of anti-HLA class I antibody levels. None of these assays are validated as quantitative tests by the producer, although it is not uncommon to use these or similar assays as indications, or semi-quantitative measurements, of antibody level/strength (191, 192). Different epitopes will be expressed at different frequencies in the assay, but this should not affect the MFI levels in a significant way as long as the antibodies do not completely saturate the binding sites. We also found that the MFI levels correlated with clinical variables, such as parity, in a manner that was in accordance with what has been reported for anti-HLA class I antibody levels in the literature (112, 114). In conclusion, the given MFI levels are likely to be a good indicator of actual antibody levels, but should be interpreted with these limitations kept in mind.

9.5 Sampling Time

Maternal samples in the described study populations were not collected at the same time points. Maternal samples from the case group in papers I and II were collected postpartum, with a median sample time of six days after delivery. Only four of the cases were collected at > 15 days postpartum. We only detected IgG antibodies with the described antibody analysis methods. It is unlikely that the maternal antibodies had underwent an isotype switch from IgM to IgG in only 6 days. The detected antibodies are therefore likely to have originated during the pregnancy in question or during an earlier pregnancy, rather than during delivery.

The maternal samples in the control group were collected at week 22-24 of gestation. IgG detected in the fetal circulation has been shown to rapidly increase following week 20 of

gestation (193, 194), indicating an increase in placental transport. Furthermore, as maternal anti-HLA class I antibodies can be detected well before week 22-24 of gestation with modern detection methods (115, 116), samples taken at week 22-24 should reflect either immunization during the pregnancy in question or a previous one. However, if samples had been obtained at a later time point, it is likely that more pregnancies would have tested positive, since it could have given the maternal immune system a more prolonged exposure to fetal cells/antigens.

Little is known with regards to how anti-HLA class I antibody levels develop during pregnancy. In seven of the antibody positive control pregnancies we had samples available from both week 22-24 and postpartum. We found that samples taken at week 22-24 had a MFI that was on average 66% higher than MFI of the postpartum samples (Mean 1.66, 95% CI 0.94-2.38), indicating that anti-HLA class 1 antibody level might decline after delivery (195). If these findings are representative, the described difference in antibody level should be even larger if the samples had been obtained postpartum for both case and control group. It should be further investigated whether this potential difference in antibody levels is due to an increased maternal production, or an increased placental transfer.

In paper III we chose to use the latest available sample taken before delivery, with a median sample time of 35 weeks of gestation. This may have impacted the rather high frequency of anti-HLA class I antibody positive samples (67%), but should give an accurate reflection of the total number of pregnancies that had an anti-HLA class I antibody presence. This sampling time is likely the more optimal of the three described, since it removes any uncertainty regarding immunization during delivery, as well as reflect the steadily increasing transport of maternal IgG into maternal circulation towards the third trimester (193, 194).

9.6 Antibody Isotypes and Subclasses

As mentioned, we only tested for IgG antibodies, since this is the only isotype known to cross the placenta (4, 5). Obtaining information on particularly IgM could have given valuable information on whether the detected antibodies were indeed due to an immunization during the pregnancy in question, and also give indications on the timing of isotype switches. Since it remains to be investigated whether a potential effect of anti-HLA class I antibodies on the fetus is due to passage of antibodies across the placenta, or if there also are affected targets at

the fetal-maternal interface, it may be interesting to also consider IgM antibodies. Future studies would likely also benefit from considering the different IgG subclasses, since it is known that the subclasses have a varying capacity for transport across the placenta (193). Still, there are no clear indications that different isotype compositions, at least for anti-HPA-1a antibodies, predict clinical outcome (196).

10. Discussion of Results

10.1 Antibodies and Fetal Growth

In paper I we found that there was an inverse association between maternal anti-HLA class I antibody level and birth weight, as well as placental weight. These findings are consistent with a published report that found a similar association between maternal antibodies against HPA-1a and reduced birth weight in boys (62), and another study that found an increased frequency of low birth weight among neonates with FNAIT (29).

Chronic villitis of unknown etiology is associated with IUGR (197), and has also been reported in cases of FNAIT(198). The integrin αVβ3 is used as an adhesion receptor by invading trophoblasts (21) and plays an important role in angiogenesis (19). Since the HPA-1a antigen is expressed on integrin αVβ3, it was suggested that the binding of anti-HPA-1a antibodies to αVβ3 could disrupt placentation, and this hypothesis is currently being investigated (64). Although there have been similar reports of a connection between maternal anti-HLA class I antibodies and chronic chorioamnioitis at delivery (116), the connection between anti-HLA class I antibodies and birth weight is not readily apparent.

As was discussed in the cited report (116), most of the detected anti-HLA class I antibodies was directed against HLA-A and -B, which are not expressed on trophoblasts. The reported associations between anti-HLA-A and -B antibodies and miscarriage (137-139), as well as placental abruption (148), supports the idea that anti-HLA class I antibodies may affect placental function, but the mechanism remains to be elucidated. With anti-HLA-C antibodies, the connection with adverse neonatal outcomes seems more obvious, as HLA-C is expressed on trophoblasts (119). But as noted by several of the cited sources, as well as our data, anti-HLA-C antibodies are not as strongly associated with the reported complications as are anti-HLA-A and -B antibodies.

Another suggested mechanism on how anti-HPA-1a antibodies could influence birth weight, is through fetal thrombocytopenia itself. Platelets play an important role in angiogenesis (199) and have been shown to be crucial in stabilization of tumor vessels (200). If similar mechanisms are at work during pregnancy, the reported complications may be due to the thrombocytopenia itself. This would be consistent with the reported association of neonatal thrombocytopenia with a multitude of adverse neonatal outcomes (70, 84), as well as the similarities between our case group and other populations with anti-HPA-1a antibody induced thrombocytopenia.

10.2 Intracranial Hemorrhage

Five (10%) of the described children in the case group of papers I and II were reported to have an intracranial hemorrhage, with one dying the day after delivery and the remaining four developing significant neurological sequelae. This frequency of ICH is similar to what has been reported for retrospectively identified cases of FNAIT due to anti-HPA antibodies (29, 67-69), as is the frequency of neurological sequelae (63). Thrombocytopenia in itself was long thought to be the primary mechanism behind fetal/neonatal ICH. However, recent studies have shown that ICH in FNAIT may also be due to anti-HPA-1a antibodies impairing angiogenesis (201), by induction of apoptosis and disruption of proliferation in endothelial cells (202). There are reports that anti-HLA class I antibodies can cause vasculopathy in transplants through multiple pathways (203), and that anti-HLA class I antibodies can cause endothelial cell death at high concentration in in-vitro studies (204). Still, no studies have been conducted on possible links between anti-HLA class I antibodies and fetal/neonatal ICH, particularly during pregnancy. Given the available literature, it appears more likely that a possible relation between maternal anti-HLA class I antibodies and fetal/neonatal ICH should be mediated through the thrombocytopenia itself, although this also remains a tenuous connection.

10.3 Antibodies and Platelet Count

None of our investigations were designed to directly address the central question of causality between anti-HLA class I antibodies and FNAIT, as it has been suggested by multiple case reports (35-45). The findings in Paper III indicate that anti-HLA class I antibodies also have

an effect on neonatal platelet count in a population of prospectively screened pregnancies, but it is important to remember that these results pertain to a very narrow group of the general population, namely HPA-1a negative women who have developed anti-HPA-1a antibodies. It is not inherently clear why anti-HLA class I antibodies should have a different effect on the pregnancy and/or fetus when the mother is HPA-1a negative as opposed to positive, but it is certainly a possibility that the alloimmunization and production of one type of antibody might stimulate the presence of another, without there being a causal relationship between both antibodies and the outcome. However, what we found was that there was a significant inverse unadjusted correlation between an increasing anti-HLA class I antibody level and neonatal platelet count, but only in pregnancies where the mother was nulliparous. Furthermore, this is in line with the high frequency of nulliparous mothers also found in the retrospectively selected case population in paper I and II (45%).

We did not find a significant correlation between anti-HLA class I antibody level and neonatal platelet count among the case population in papers I and II, but this was likely influenced by the narrow distribution of reported neonatal platelet counts: 89% had severe thrombocytopenia, with a median platelet count of 24 x 10^9/L (SD 19 x 10^9/L), while in paper III (among the nulliparous pregnancies) 38% had a severe thrombocytopenia, with a median neonatal platelet count of 135 x 10^9/L (SD 113 x 10^9/L). This narrow distribution was, of course, also influenced by the *nadir* neonatal platelet count being chosen as the reported platelet count in paper I and II.

10.4 Nulliparity

The link between between nulliparity and an adverse neonatal outcome is well-known, with nulliparity being strongly associated with an increased risk of SGA neonates and neonatal mortality (205). Nulliparity is also associated with an increased rate of preeclampsia (206, 207) as well as several other maternal complications (208). With this is mind, it is not surprising that a high number of mothers included in the case population for papers I and II were nulliparous. However, these pregnancies were not only selected due to the occurrence of neonatal thrombocytopenia, but also due to the presence of anti-HLA class I antibodies, which are consistently reported to be more frequent in multiparous women than nulliparous (111-114). We therefore expected there to be fewer nulliparous pregnancies in the case group. On the other hand, there is an increasing focus on the passage of fetal cells into the maternal

circulation, even during normal pregnancies. A recent extensive review highlights how this process is believed to promote maternal tolerance towards paternal antigens in both the pregnancy in question, as well as future pregnancies (3). This provides an explanation as to why preeclampsia is generally considered a disease of the first pregnancy (209), and why changing partners in subsequent pregnancies impacts the risk of preeclampsia (210, 211) and preterm delivery (212). The same paternal antigen may be the source of a much more adverse immunological reaction during a first pregnancy than during a subsequent pregnancy, when some form of maternal tolerance has been established. In this context, it is perhaps to be expected that a retrospectively selected population of adverse events would include several nulliparous pregnancies.

The high number of nulliparous pregnancies also meant that there were fewer events likely to have caused a previous maternal alloimmunization. The main sources of alloimmunization are transfusion, transplantation and pregnancy. Transfusion and transplantation are rare in women of fertile age. The close match between expected immunizing epitopes based on fetal-maternal genotype and the observed antibody specificities reflects this. Most of the cases were specific towards fetal/paternal epitopes, with little reactivity against any third-party or self. If more cases had been multiparous we would likely have required paternal genotype, in addition to the maternal and neonatal genotype, to map out the maternal antibody reactivity patterns. This was further underlined by all of the cases (n = 7) with unresolved antibody reactivity patterns being from cases where the mother was multiparous (data missing for one case).

Even though maternal antibody level was more closely associated with clinical outcome among the nulliparous pregnancies than among the multiparous, the antibody level was consistently higher among the multiparous pregnancies. This was to be expected, since anti-HLA class I antibodies are consistently reported to be more frequent among multiparous women (111-114). It is nonetheless interesting, since it further emphasizes that immunization in a first/nulliparous pregnancy may be qualitatively different from that in a multiparous one. Still, it also needs to be considered that the weaker association between antibodies and clinical outcome among multiparous pregnancies could also be the result of the detected antibodies being directed towards antigens expressed in a previous pregnancy, rather than in the current one.

The frequency of anti-HLA class I antibodies are reported to increase with the number of pregnancies (111-114), and are also reported to be associated with an increased risk of miscarriage (137-140). We had data available on reported gravida status, but we have for the most part chosen not to include gravida status in our analyses, due to the unreliable nature of these data. Early miscarriage is very common (213, 214) and is typically considered a normal event, and the very early cases may even go unnoticed (213). In the 16 nulliparous cases in paper I and II where data on gravidity was available, nine were primigravida and seven were multigravida. In the 40 nulliparous pregnancies in paper III where data on gravidity was available, 13 were primigravida while 27 were multigravida. Most of the multiparous women in both groups were gravida 2 (5/7 and 17/27, respectively). There were a few women in both groups that had multiple abortions behind them, but they constituted a small minority. These pregnancies also did not seem to significantly influence the antibody presence, since antibody levels were consistently higher among multiparous women, and the detected antibodies for the most part were specific towards the child in question.

10.5 Antibody Characteristics

During our initial investigations into the antibody specificities we were still only considering antigens as antibody targets rather than epitopes. This meant that the only part of the reactivity patterns that we could address with any certainty were the MFI signals that belonged to either the fetal or maternal antigens, since these were the only antigens that we could be certain had been present during pregnancy. We expected most of the maternal antibodies to be directed against paternal antigens, since they were likely the only alloantigens the mother had ever encountered. However, for most cases there were also strong MFI signals against multiple other, often rare, antigens, which it was highly unlikely that the mother had ever been exposed to.

The reactions against these third-party antigens were most probably the result of cross-reactivity between HLA class I antigens, but the initial analysis with conventional CREG groups failed to demonstrate this, with all but four of the 33 genotyped cases having beads with MFI > 10 000 in four or more CREGs. It was only when we applied the much higher resolution data on epitope expression from the HLA Epitope Registry that it became evident that the broad reactivity in most cases was due to the same mismatched paternal epitopes being present on multiple beads. We could therefore conclude that most, if not all, of the

maternal anti-HLA class I antibodies in our study population were fetal/paternal-specific, as initially suspected.

Although detection and verification of antibody-defined epitopes is an ongoing process (124), there are already benefits to including this data in anti-HLA class I antibody analysis. In particular, it may help unravel reactivity patterns that are not fully explained by conventional CREG groups. These kinds of data may also provide a good basis and documentation for using epitope mapping when designing algorithms to search for platelet donors in multi-immunized patients with platelet transfusion refractoriness.

Paper II is the first publication on epitope-focused analysis of anti-HLA class I antibodies in relation to FNAIT, but similar analyses of pregnancy-induced anti-HLA class I antibodies have been published by others (124, 140, 215-221). These studies generally demonstrate that maternal anti-HLA class I antibodies are fetal-specific also in normal pregnancies. We could not find that the presence of anti-HLA class I antibodies was tied to any particular maternal allele or fetal-maternal mismatch, although this has been reported by others (221-223). This was likely affected by our relatively low sample size, with regards to allele frequency analysis. Still, it should be noted that cases where HLA-B*27 was the most likely immunizing allele had a significantly lower platelet count compared to the rest of the case group. This is noteworthy, given the known association between HLA-B*27 and inflammatory diseases (224, 225), as well as an earlier case report where anti-HLA-B*27 antibodies were suggested as a cause of FNAIT (37).

Since we did not have access to maternal and neonatal genotype for the complete control group in papers I and II, there are limitations as to what conclusions can be drawn from the described antibody specificity data. The fetal/paternal-specific pattern is likely not exclusive for the case group, but we did not have the opportunity to demonstrate this. For the five controls where maternal and fetal genotype was available, we found that one had a reactivity pattern similar to that described for most of the cases, one appeared strongly reactive but not against antigens expressed by the present child, and the remaining three controls appeared to be only borderline positive, or even negative. These findings seem representative for the control group as a whole if you only look at MFI levels: Among the antibody positive controls there were some that had antibody levels comparable to the cases, but for the most part they were at a significantly lower level.

One interpretation of this is that the antibodies in both groups were specific toward the child in question, but that cases had a significantly higher antibody level compared to controls. Alternatively, the detected antibodies in many of the controls may have been remnants from a previous pregnancy/pregnancies. A third possibility is that the assays might be too sensitive, and that some of the controls were false-positives. Whatever the case may be, any future investigations into the role of anti-HLA class I antibodies during pregnancy need to obtain maternal and fetal genotype (and likely also paternal genotype) to properly evaluate whether the observed antibodies are consistent with a maternal alloimmunization during the pregnancy in question.

10.6 Preeclampsia

There was a high frequency of preeclampsia (13%) among mothers in the case group described in papers I and II. Since maternal preeclampsia is associated with neonatal thrombocytopenia (226), we reanalyzed all data with these cases excluded, but this did not significantly change any of the described results. A link between anti-HLA class I antibodies and preeclampsia has been reported (149), but this connection may just be the result of a nonspecific reaction to placental damage rather than fetal antigen-specific IgG (149, 150). Anti-HLA class I antibodies are, as mentioned, also associated with chronic chorioamnionitis at delivery (116). In line with this, we found that an increasing anti-HLA class I antibody level was significantly associated with a decreasing placental weight among the described cases in paper I and II. However, the relationship between anti-HLA class I antibodies and preeclampsia may not be a causal one, but rather one predicated on inflammation in general.

10.7 Inflammation

It has been reported that the level and number of specificities of anti-HLA antibodies can increase with inflammation (151), even following non-infectious events such as a traumatic injury. This could potentially mean that any inflammation, regardless of cause, could impact the presence of anti-HLA class I antibodies during pregnancy, such as for example preeclampsia, placental villitis or fetal hypoxia. Pregnancy in itself is often referred to as an increased inflammatory state for women, although this is more complex than increased/decreased (152). Furthermore, antibody-mediated platelet destruction by human

phagocytes has also been shown to be enhanced by the inflammatory marker CRP (190). Still, even though the presence of antibodies may be secondary, it does not mean that they were not harmful.

Even though we excluded or adjusted for multiple complicating factors that could affect neonatal thrombocytopenia, preeclampsia or birth weight, maternal anti-HLA class I antibodies may still not have been the only influencing factor present. This is also consistent with the findings of a report on similar cases with suspected FNAIT due to anti-HLA class I antibodies in Sweden (187), that found other plausible causes for the observed thrombocytopenia in many of their cases.

10.8 Anti-HLA Class I Antibodies as a Possible Cause of FNAIT

It is difficult to ensure that all confounders were accounted for, but the fact remains that the retrospectively selected cases we have described in papers I and II were initially referred due to a suspicion of FNAIT - a suspicion that in most cases remained after careful assessment of full medical records. Most of these children had severe thrombocytopenia detected shortly after delivery, without any other clear diagnosis. Since FNAIT is the most common cause of severe thrombocytopenia in an otherwise healthy newborn (57, 58), the suspicion seemed warranted in most cases. This suspicion was further emphasized by the observed frequency of ICH at 10%, which is similar to the frequency of ICH due to severe FNAIT reported in both retrospective studies (7-26%) (29, 67-69) as well as prospective studies (10%) (46). The antibody reactivity patterns were also consistent with an immunization towards a fetal/maternal incompatibility, with very little reactivity towards any third-party. These findings indicate that the reported cases have clinical features that are consistent with the diagnosis of FNAIT as we know it, and could be an indication that anti-HLA class I antibody-induced FNAIT may be as severe as anti-HPA-1a induced FNAIT. However, a causal relationship remains to be established, and such an investigation will require large prospectively collected data.

Paper III does, however, give a clear indication that there is some relationship between anti-HLA class I antibodies and platelet count that remains consistent when a retrospective selection bias is removed. This association remained significant after adjusting for anti-HPA-1a antibody level, and there were no significant co-linearity/correlation between anti-HPA-1a

antibody level and anti-HLA class I antibody level. There were, however, a significant influence by parity status, with most of the effect of anti-HLA class I antibodies on platelet count being predicated on the mother being nulliparous. Any future studies on this topic should keep this in mind.

11. Implications

The data we have presented emphasizes the severe clinical outcome of pregnancies with suspected FNAIT due to maternal anti-HLA class I antibodies, although the sequence of causality remains uncertain. Further studies are required before any conclusions on clinical practice can be drawn.

Still, there is a clear trend in all three papers that the group that should likely receive extra attention is nulliparous women who develop antibodies. This may be particularly relevant for HPA-1a negative women where the fetus is HPA-1a positive, although identification of any such women will remain difficult without an implemented screening program. This hypothesis concerning an increased risk in nulliparous pregnancies are also in line with many well-established risk-assessments of fetal and maternal complications during pregnancy, as well as the increasing focus on how the exchange of fetal and maternal cell material during pregnancy likely promotes fetal-maternal tolerance in subsequent pregnancies. If the first pregnancy goes well, the next is more likely to follow suit.

Obtaining maternal and neonatal HLA class I genotype proved essential to our investigation when trying to evaluate maternal anti-HLA class I antibody specificities, and it is our recommendation that any future studies or clinical investigations into these antibodies should include these data, as well as paternal genotype. If an antibody reactivity pattern remains unresolved after initial analysis, further analysis using HLA class I epitope data should be considered. This kind of analysis might prove even more beneficial in other settings, such as evaluation of potential donors for multi-transfused patient, and should likely be considered for clinical application in the near future.

As always, it is also vital to any future advances in this field that clinicians consider the diagnosis of FNAIT when presented with neonatal thrombocytopenia with no apparent cause such as a congenital infection or maternal ITP. Unless such cases are referred, most of the potential lessons that can be learned will be lost.

12. Suggestions for Future Research

The physicians working with FNAIT in Tromsø are commonly approached and asked what to do with pregnant women with detectable anti-HLA class I antibodies – women who often have previously delivered a thrombocytopenic newborn. This question is still not easily answered.

Given the frequently adverse clinical outcome of newborns with suspected FNAIT due to maternal anti-HLA class I antibodies, and the persisting uncertainty concerning causality, it seems apparent that a large collaborative prospective study on maternal anti-HLA class I antibodies during pregnancy should be undertaken. Such a study would need access to clinical data on mother and child both during the pregnancy, and for the immediate neonatal period following delivery. Maternal peripheral blood samples should be checked for anti-HLA class I antibodies at least once during pregnancy, preferably in the third trimester, neonatal platelet counts should be taken at delivery, and maternal as well as neonatal DNA (even paternal, if possible) should be collected for HLA class I genotyping. Detected maternal anti-HLA class I antibodies should also be quantified, using appropriate assays. It might not be necessary to detect a high number of thrombocytopenic neonates during such a study to draw any worthwhile conclusions, since information on how antibody levels correlate with platelet counts also in the normal range could prove highly informative.

We would also be very interested in learning if similar cases to the ones we have described are referred to reference laboratories in other countries as well, and if there are similar findings among these to what we have described. This might also give an indication as to whether there are trends within different countries that influence what kind of cases that are ultimately referred due to suspected FNAIT, and if reported data based on referrals are actually comparable between different countries.

While we wait for a large prospective study on maternal anti-HLA class I antibodies during pregnancy, it would be interesting to do a follow-up study of all women included in the case group for papers I and II. We know that the risk of recurrence for HPA-1a alloimmunization, including risk of ICH, is high. We know nothing about recurrence risk for neonatal thrombocytopenia, with or without ICH, when maternal anti-HLA class I antibodies were the suspected cause. Further, IVIg is commonly used to prevent severe FNAIT, and it is an open question whether IVIg may be beneficial to prevent HLA-class I antibody-induced FNAIT - if such a condition actually exists.

13. Concluding Remarks

The presence of anti-HLA class I antibodies was consistently associated with a more severe clinical outcome in the data we have presented, particularly among nulliparous pregnancies, but the central question of whether anti-HLA class I antibodies can be a cause of FNAIT remains unanswered. It is our recommendation that future studies of this potential causality emphasize proper quantification of antibody levels and genotyping of both parents as well as the neonate. Any causal clinical investigation would also need access to complete medical records.

Given the frequency of anti-HLA class I antibodies during pregnancy, and their close association with severe clinical outcomes in other settings, there are surprisingly few reports of prospective studies on this topic. With precise analytical tools, such as epitope mapping, becoming readily available, it might be high time that the many unanswered questions surrounding this phenomenon is reconsidered in a larger prospective setting.

14. References

1. Valenzuela, N. M., and E. F. Reed. 2013. Antibodies in transplantation: the effects of HLA and non-HLA antibody binding and mechanisms of injury. *Methods Mol Biol* 1034: 41-70.
2. Guleria, I., and M. H. Sayegh. 2007. Maternal acceptance of the fetus: true human tolerance. *J Immunol* 178: 3345-3351.
3. Kinder, J. M., I. A. Stelzer, P. C. Arck, and S. S. Way. 2017. Immunological implications of pregnancy-induced microchimerism. *Nature reviews. Immunology*.
4. Chen, P., C. Li, S. Lang, G. Zhu, A. Reheman, C. M. Spring, J. Freedman, and H. Ni. 2010. Animal model of fetal and neonatal immune thrombocytopenia: role of neonatal Fc receptor in the pathogenesis and therapy. *Blood* 116: 3660-3668.
5. Simister, N. E., and C. M. Story. 1997. Human placental Fc receptors and the transmission of antibodies from mother to fetus. *Journal of reproductive immunology* 37: 1-23.
6. Robinson, J., J. A. Halliwell, J. D. Hayhurst, P. Flicek, P. Parham, and S. G. Marsh. 2015. The IPD and IMGT/HLA database: allele variant databases. *Nucleic acids research* 43: D423-431.
7. Schuh, A. C., N. A. Watkins, Q. Nguyen, N. J. Harmer, M. Lin, J. Y. Prosper, K. Campbell, D. R. Sutherland, P. Metcalfe, W. Horsfall, and W. H. Ouwehand. 2002. A tyrosine703serine polymorphism of CD109 defines the Gov platelet alloantigens. *Blood* 99: 1692-1698.
8. Landau, M., and N. Rosenberg. 2011. Molecular insight into human platelet antigens: structural and evolutionary conservation analyses offer new perspective to immunogenic disorders. *Transfusion* 51: 558-569.
9. Rozman, P. 2002. Platelet antigens. The role of human platelet alloantigens (HPA) in blood transfusion and transplantation. *Transplant immunology* 10: 165-181.
10. Davoren, A., B. R. Curtis, R. H. Aster, and J. G. McFarland. 2004. Human platelet antigen-specific alloantibodies implicated in 1162 cases of neonatal alloimmune thrombocytopenia. *Transfusion* 44: 1220-1225.
11. Porcelijn, L., E. S. Van den Akker, and D. Oepkes. 2008. Fetal thrombocytopenia. *Seminars in fetal & neonatal medicine* 13: 223-230.
12. Newman, P. J., R. S. Derbes, and R. H. Aster. 1989. The human platelet alloantigens, PlA1 and PlA2, are associated with a leucine33/proline33 amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *The Journal of clinical investigation* 83: 1778-1781.
13. Bennett, J. S., B. W. Berger, and P. C. Billings. 2009. The structure and function of platelet integrins. *Journal of thrombosis and haemostasis : JTH* 7 Suppl 1: 200-205.
14. Lawler, J., and R. O. Hynes. 1989. An integrin receptor on normal and thrombasthenic platelets that binds thrombospondin. *Blood* 74: 2022-2027.
15. Coller, B. S., D. A. Cheresh, E. Asch, and U. Seligsohn. 1991. Platelet vitronectin receptor expression differentiates Iraqi-Jewish from Arab patients with Glanzmann thrombasthenia in Israel. *Blood* 77: 75-83.
16. Bennett, J. S. 2005. Structure and function of the platelet integrin alphaIIbbeta3. *The Journal of clinical investigation* 115: 3363-3369.
17. Cheresh, D. A., J. W. Smith, H. M. Cooper, and V. Quaranta. 1989. A novel vitronectin receptor integrin (alpha v beta x) is responsible for distinct adhesive properties of carcinoma cells. *Cell* 57: 59-69.
18. Drake, C. J., D. A. Cheresh, and C. D. Little. 1995. An antagonist of integrin alpha v beta 3 prevents maturation of blood vessels during embryonic neovascularization. *Journal of cell science* 108 (Pt 7): 2655-2661.
19. Brooks, P. C., R. A. Clark, and D. A. Cheresh. 1994. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* 264: 569-571.
20. Vanderpuye, O. A., C. A. Labarrere, and J. A. McIntyre. 1991. A vitronectin-receptor-related molecule in human placental brush border membranes. *The Biochemical journal* 280 (Pt 1): 9-17.
21. Kumpel, B. M., K. Sibley, D. J. Jackson, G. White, and P. W. Soothill. 2008. Ultrastructural localization of glycoprotein IIIa (GPIIIa, beta 3 integrin) on placental syncytiotrophoblast

microvilli: implications for platelet alloimmunization during pregnancy. *Transfusion* 48: 2077-2086.
22. Kumpel, B., M. J. King, S. Sooranna, D. Jackson, J. Eastlake, R. Cheng, and M. Johnson. 2008. Phenotype and mRNA expression of syncytiotrophoblast microparticles isolated from human placenta. *Annals of the New York Academy of Sciences* 1137: 144-147.
23. Shulman, N. R., R. H. Aster, H. A. Pearson, and M. C. Hiller. 1962. Immunoreactions involving platelet. VI. Reactions of maternal isoantibodies responsible for neonatal purpura. Differentiation of a second platelet antigen system. *The Journal of clinical investigation* 41: 1059-1069.
24. Blanchette, V. S., L. Chen, Z. S. de Friedberg, V. A. Hogan, E. Trudel, and F. Decary. 1990. Alloimmunization to the PlA1 platelet antigen: results of a prospective study. *British journal of haematology* 74: 209-215.
25. Maslanka, K., K. Guz, and B. Zupanska. 2003. Antenatal screening of unselected pregnant women for HPA-1a antigen, antibody and alloimmune thrombocytopenia. *Vox sanguinis* 85: 326-327.
26. Williamson, L. M., G. Hackett, J. Rennie, C. R. Palmer, C. Maciver, R. Hadfield, D. Hughes, S. Jobson, and W. H. Ouwehand. 1998. The natural history of fetomaternal alloimmunization to the platelet-specific antigen HPA-1a (PlA1, Zwa) as determined by antenatal screening. *Blood* 92: 2280-2287.
27. Turner, M. L., H. Bessos, T. Fagge, M. Harkness, F. Rentoul, J. Seymour, D. Wilson, I. Gray, R. Ahya, J. Cairns, and S. Urbaniak. 2005. Prospective epidemiologic study of the outcome and cost-effectiveness of antenatal screening to detect neonatal alloimmune thrombocytopenia due to anti-HPA-1a. *Transfusion* 45: 1945-1956.
28. Kjeldsen-Kragh, J., M. K. Killie, G. Tomter, E. Golebiowska, I. Randen, R. Hauge, B. Aune, P. Oian, L. B. Dahl, J. Pirhonen, R. Lindeman, H. Husby, G. Haugen, M. Gronn, B. Skogen, and A. Husebekk. 2007. A screening and intervention program aimed to reduce mortality and serious morbidity associated with severe neonatal alloimmune thrombocytopenia. *Blood* 110: 833-839.
29. Mueller-Eckhardt, C., V. Kiefel, A. Grubert, H. Kroll, M. Weisheit, S. Schmidt, G. Mueller-Eckhardt, and S. Santoso. 1989. 348 cases of suspected neonatal alloimmune thrombocytopenia. *Lancet* 1: 363-366.
30. Murphy, M. F., and J. B. Bussel. 2007. Advances in the management of alloimmune thrombocytopenia. *British journal of haematology* 136: 366-378.
31. Kroll, H., V. Kiefel, G. Mueller-Eckhardt, S. Santoso, and C. Mueller-Eckhardt. 1990. [219 Zw(a) positive mothers of children with clinically suspected neonatal alloimmune thrombocytopenia]. *Beitr Infusionsther* 26: 397-400.
32. Panzer, S., W. R. Mayr, and B. Eichelberger. 2005. Light chain phenotypes of HLA antibodies in cases with suspected neonatal alloimmune thrombocytopenia. *Vox sanguinis* 89: 261-264.
33. Uhrynowska, M., K. Maslanka, and B. Zupanska. 1997. Neonatal thrombocytopenia: incidence, serological and clinical observations. *American journal of perinatology* 14: 415-418.
34. Taaning, E., S. Petersen, J. Reinholdt, J. Bock, and A. Svejgaard. 1994. Neonatal Immune Thrombocytopenia Due to Allo-or Autoantibodies: Clinical and Immunological Analysis of 83 Cases. *Platelets* 5: 53-58.
35. Saito, S., M. Ota, Y. Komatsu, S. Ota, S. Aoki, K. Koike, I. Tokunaga, T. Tsuno, G. Tsuruta, T. Kubo, and H. Fukushima. 2003. Serologic analysis of three cases of neonatal alloimmune thrombocytopenia associated with HLA antibodies. *Transfusion* 43: 908-917.
36. Moncharmont, P., V. Dubois, C. Obegi, M. Vignal, Y. Merieux, L. Gebuhrer, and D. Rigal. 2004. HLA antibodies and neonatal alloimmune thrombocytopenia. *Acta haematologica* 111: 215-220.
37. Thude, H., U. Schorner, C. Helfricht, M. Loth, B. Maak, and D. Barz. 2006. Neonatal alloimmune thrombocytopenia caused by human leucocyte antigen-B27 antibody. *Transfus Med* 16: 143-149.

38. Gramatges, M. M., P. Fani, K. Nadeau, S. Pereira, and M. R. Jeng. 2009. Neonatal alloimmune thrombocytopenia and neutropenia associated with maternal human leukocyte antigen antibodies. *Pediatric blood & cancer* 53: 97-99.
39. Starcevic, M., M. Tomicic, M. Malenica, and V. Zah-Matakovic. 2010. Neonatal alloimmune thrombocytopenia caused by anti-HLA-A24 alloantibodies. *Acta Paediatr* 99: 630-632.
40. Meler, E., R. Porta, C. Canals, B. Serra, and M. Lozano. 2016. Fatal alloimmune thrombocytopenia due to anti-HLA alloimmunization in a twin pregnancy: A very infrequent complication of assisted reproduction. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis*.
41. Chow, M. P., K. J. Sun, C. H. Yung, H. Y. Hu, J. L. Tzeng, and T. D. Lee. 1992. Neonatal alloimmune thrombocytopenia due to HLA-A2 antibody. *Acta haematologica* 87: 153-155.
42. Sternbach, M. S., M. Malette, F. Nadon, and R. M. Guevin. 1986. Severe alloimmune neonatal thrombocytopenia due to specific HLA antibodies. *Current studies in hematology and blood transfusion*: 97-103.
43. del Rosario, M. L., E. R. Fox, T. S. Kickler, and K. J. Kao. 1998. Neonatal alloimmune thrombocytopenia associated with maternal anti-HLA antibody: a case report. *Journal of pediatric hematology/oncology* 20: 252-256.
44. Sasaki, M., A. Yagihashi, D. Kobayashi, N. Watanabe, T. Fujikawa, S. Chiba, S. Sato, K. Morishita, T. Sekimoto, and H. Ikeda. 2001. Neonatal alloimmune thrombocytopenia due to anti-human leukocyte antigen antibody: a case report. *Pediatric hematology and oncology* 18: 519-524.
45. Hutchinson, A. L., P. M. Dennington, R. Holdsworth, and L. Downe. 2015. Recurrent HLA-B56 mediated neonatal alloimmune thrombocytopenia with fatal outcomes. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis* 52: 311-313.
46. Kamphuis, M. M., N. Paridaans, L. Porcelijn, M. De Haas, C. E. Van Der Schoot, A. Brand, G. J. Bonsel, and D. Oepkes. 2010. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *BJOG : an international journal of obstetrics and gynaecology* 117: 1335-1343.
47. Valentin, N., A. Vergracht, J. D. Bignon, M. L. Cheneau, D. Blanchard, C. Kaplan, M. F. Reznikoff-Etievant, and J. Y. Muller. 1990. HLA-DRw52a is involved in alloimmunization against PL-A1 antigen. *Human immunology* 27: 73-79.
48. L'Abbe, D., L. Tremblay, M. Filion, L. Busque, M. Goldman, F. Decary, and P. Chartrand. 1992. Alloimmunization to platelet antigen HPA-1a (PIA1) is strongly associated with both HLA-DRB3*0101 and HLA-DQB1*0201. *Human immunology* 34: 107-114.
49. Parry, C. S., J. Gorski, and L. J. Stern. 2007. Crystallographic structure of the human leukocyte antigen DRA, DRB3*0101: models of a directional alloimmune response and autoimmunity. *Journal of molecular biology* 371: 435-446.
50. Wu, S., K. Maslanka, and J. Gorski. 1997. An integrin polymorphism that defines reactivity with alloantibodies generates an anchor for MHC class II peptide binding: a model for unidirectional alloimmune responses. *J Immunol* 158: 3221-3226.
51. Stuge, T. B., B. Skogen, M. T. Ahlen, A. Husebekk, S. J. Urbaniak, and H. Bessos. 2011. The cellular immunobiology associated with fetal and neonatal alloimmune thrombocytopenia. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis* 45: 53-59.
52. Ahlen, M. T., A. Husebekk, I. L. Killie, B. Skogen, and T. B. Stuge. 2016. T cell responses to human platelet antigen-1a involve a unique form of indirect allorecognition. *JCI insight* 1: e86558.
53. Ahlen, M. T., A. Husebekk, M. K. Killie, B. Skogen, and T. B. Stuge. 2009. T-cell responses associated with neonatal alloimmune thrombocytopenia: isolation of HPA-1a-specific, HLA-DRB3*0101-restricted CD4+ T cells. *Blood* 113: 3838-3844.
54. Killie, M. K., A. Husebekk, J. Kjeldsen-Kragh, and B. Skogen. 2008. A prospective study of maternal anti-HPA 1a antibody level as a potential predictor of alloimmune thrombocytopenia in the newborn. *Haematologica* 93: 870-877.

55. Eksteen, M., H. Tiller, M. Averina, G. Heide, M. Kjaer, C. Ghevaert, T. E. Michaelsen, O. Ihle, A. Husebekk, B. Skogen, and T. B. Stuge. 2015. Characterization of a human platelet antigen-1a-specific monoclonal antibody derived from a B cell from a woman alloimmunized in pregnancy. *J Immunol* 194: 5751-5760.
56. Jaegtvik, S., A. Husebekk, B. Aune, P. Oian, L. B. Dahl, and B. Skogen. 2000. Neonatal alloimmune thrombocytopenia due to anti-HPA 1a antibodies; the level of maternal antibodies predicts the severity of thrombocytopenia in the newborn. *BJOG : an international journal of obstetrics and gynaecology* 107: 691-694.
57. Sainio, S., A. L. Jarvenpaa, M. Renlund, S. Riikonen, K. Teramo, and R. Kekomaki. 2000. Thrombocytopenia in term infants: a population-based study. *Obstetrics and gynecology* 95: 441-446.
58. Burrows, R. F., and J. G. Kelton. 1993. Fetal thrombocytopenia and its relation to maternal thrombocytopenia. *The New England journal of medicine* 329: 1463-1466.
59. Murphy, M. F., H. Hambley, K. Nicolaides, and A. H. Waters. 1996. Severe fetomaternal alloimmune thrombocytopenia presenting with fetal hydrocephalus. *Prenatal diagnosis* 16: 1152-1155.
60. Li, C., S. Piran, P. Chen, S. Lang, A. Zarpellon, J. W. Jin, G. Zhu, A. Reheman, D. E. van der Wal, E. K. Simpson, R. Ni, P. L. Gross, J. Ware, Z. M. Ruggeri, J. Freedman, and H. Ni. 2011. The maternal immune response to fetal platelet GPIbalpha causes frequent miscarriage in mice that can be prevented by intravenous IgG and anti-FcRn therapies. *The Journal of clinical investigation* 121: 4537-4547.
61. Ni, H., P. Chen, C. M. Spring, E. Sayeh, J. W. Semple, A. H. Lazarus, R. O. Hynes, and J. Freedman. 2006. A novel murine model of fetal and neonatal alloimmune thrombocytopenia: response to intravenous IgG therapy. *Blood* 107: 2976-2983.
62. Tiller, H., M. K. Killie, A. Husebekk, B. Skogen, H. Ni, J. Kjeldsen-Kragh, and P. Oian. 2012. Platelet antibodies and fetal growth: maternal antibodies against fetal platelet antigen 1a are strongly associated with reduced birthweight in boys. *Acta obstetricia et gynecologica Scandinavica* 91: 79-86.
63. Tiller, H., M. M. Kamphuis, O. Flodmark, N. Papadogiannakis, A. L. David, S. Sainio, S. Koskinen, K. Javela, A. T. Wikman, R. Kekomaki, H. H. Kanhai, D. Oepkes, A. Husebekk, and M. Westgren. 2013. Fetal intracranial haemorrhages caused by fetal and neonatal alloimmune thrombocytopenia: an observational cohort study of 43 cases from an international multicentre registry. *BMJ open* 3.
64. Eksteen, M., G. Heide, H. Tiller, Y. Zhou, N. H. Nedberg, I. Martinez-Zubiaurre, A. Husebekk, B. R. Skogen, T. B. Stuge, and M. Kjaer. 2017. Anti-human platelet antigen (HPA)-1a antibodies may affect trophoblast functions crucial for placental development: a laboratory study using an in vitro model. *Reproductive biology and endocrinology : RB&E* 15: 28.
65. Dubruc, E., F. Lebreton, C. Giannoli, M. Rabilloud, C. Huissoud, M. Devouassoux-Shisheboran, and F. Allias. 2016. Placental histological lesions in fetal and neonatal alloimmune thrombocytopenia: A retrospective cohort study of 21 cases. *Placenta* 48: 104-109.
66. Looney, C. B., J. K. Smith, L. H. Merck, H. M. Wolfe, N. C. Chescheir, R. M. Hamer, and J. H. Gilmore. 2007. Intracranial hemorrhage in asymptomatic neonates: prevalence on MR images and relationship to obstetric and neonatal risk factors. *Radiology* 242: 535-541.
67. Ghevaert, C., K. Campbell, J. Walton, G. A. Smith, D. Allen, L. M. Williamson, W. H. Ouwehand, and E. Ranasinghe. 2007. Management and outcome of 200 cases of fetomaternal alloimmune thrombocytopenia. *Transfusion* 47: 901-910.
68. Spencer, J. A., and R. F. Burrows. 2001. Feto-maternal alloimmune thrombocytopenia: a literature review and statistical analysis. *The Australian & New Zealand journal of obstetrics & gynaecology* 41: 45-55.
69. Muller, J. Y., M. F. Reznikoff-Etievant, C. Patereau, C. Dangu, and N. Chesnel. 1985. [Neonatal alloimmune thrombopenia. Clinical and biological study of 84 cases]. *Presse Med* 14: 83-86.

70. Roberts, I., S. Stanworth, and N. A. Murray. 2008. Thrombocytopenia in the neonate. *Blood reviews* 22: 173-186.
71. Kamphuis, M. M., and D. Oepkes. 2011. Fetal and neonatal alloimmune thrombocytopenia: prenatal interventions. *Prenatal diagnosis* 31: 712-719.
72. Bussel, J. 2009. Diagnosis and management of the fetus and neonate with alloimmune thrombocytopenia. *Journal of thrombosis and haemostasis : JTH* 7 Suppl 1: 253-257.
73. Rayment, R., S. J. Brunskill, P. W. Soothill, D. J. Roberts, J. B. Bussel, and M. F. Murphy. 2011. Antenatal interventions for fetomaternal alloimmune thrombocytopenia. *The Cochrane database of systematic reviews*: CD004226.
74. Paidas, M. J., R. L. Berkowitz, L. Lynch, C. J. Lockwood, R. Lapinski, J. G. McFarland, and J. B. Bussel. 1995. Alloimmune thrombocytopenia: fetal and neonatal losses related to cordocentesis. *American journal of obstetrics and gynecology* 172: 475-479.
75. Overton, T. G., K. R. Duncan, M. Jolly, E. Letsky, and N. M. Fisk. 2002. Serial aggressive platelet transfusion for fetal alloimmune thrombocytopenia: platelet dynamics and perinatal outcome. *American journal of obstetrics and gynecology* 186: 826-831.
76. Kamphuis, M. M., H. Tiller, E. S. van den Akker, M. Westgren, E. Tiblad, and D. Oepkes. 2017. Fetal and Neonatal Alloimmune Thrombocytopenia: Management and Outcome of a Large International Retrospective Cohort. *Fetal diagnosis and therapy* 41: 251-257.
77. Altarescu, G., T. Eldar-Geva, S. Grisaru-Granovsky, L. Bonstein, H. Miskin, I. Varshver, E. J. Margalioth, E. Levy-Lahad, and P. Renbaum. 2012. Preimplantation genetic diagnosis for fetal neonatal alloimmune thrombocytopenia due to antihuman platelet antigen maternal antibodies. *Obstetrics and gynecology* 119: 338-343.
78. Tiller, H., P. Fedorcsak, and B. R. Skogen. 2016. Old tools revisited give hope - new treatment option for families with a history of severe FNAIT complications. *Acta obstetricia et gynecologica Scandinavica* 95: 486-487.
79. Tiller, H., M. K. Killie, P. Chen, M. Eksteen, A. Husebekk, B. Skogen, J. Kjeldsen-Kragh, and H. Ni. 2012. Toward a prophylaxis against fetal and neonatal alloimmune thrombocytopenia: induction of antibody-mediated immune suppression and prevention of severe clinical complications in a murine model. *Transfusion* 52: 1446-1457.
80. Kjeldsen-Kragh, J., H. Ni, and B. Skogen. 2012. Towards a prophylactic treatment of HPA-related foetal and neonatal alloimmune thrombocytopenia. *Current opinion in hematology* 19: 469-474.
81. Hohlfeld, P., F. Forestier, C. Kaplan, J. D. Tissot, and F. Daffos. 1994. Fetal thrombocytopenia: a retrospective survey of 5,194 fetal blood samplings. *Blood* 84: 1851-1856.
82. Dreyfus, M., C. Kaplan, E. Verdy, N. Schlegel, I. Durand-Zaleski, and G. Tchernia. 1997. Frequency of immune thrombocytopenia in newborns: a prospective study. Immune Thrombocytopenia Working Group. *Blood* 89: 4402-4406.
83. Castle, V., M. Andrew, J. Kelton, D. Giron, M. Johnston, and C. Carter. 1986. Frequency and mechanism of neonatal thrombocytopenia. *The Journal of pediatrics* 108: 749-755.
84. Mehta, P., R. Vasa, L. Neumann, and M. Karpatkin. 1980. Thrombocytopenia in the high-risk infant. *The Journal of pediatrics* 97: 791-794.
85. Bauman, M. E., P. Y. Cheung, and M. P. Massicotte. 2011. Hemostasis and platelet dysfunction in asphyxiated neonates. *The Journal of pediatrics* 158: e35-39.
86. H-G. K. Blaas, A. H., G. Haugen, S. M. Skulstad, H. Tiller. 2014. Veileder i fødselshjelp 2014 -Trombocytt alloimmunisering.
87. Villarreal, L. P. 2009. *Origin of group identity : viruses, addiction, and cooperation*. Springer, New York.
88. Gorer, P. A., S. Lyman, and G. D. Snell. 1948. Studies on the genetic and antigenic basis of tumour transplantation. Linkage between a histocompatibility gene and'fused'in mice. *Proceedings of the Royal Society of London B: Biological Sciences* 135: 499-505.
89. Thorsby, E. 2009. A short history of HLA. *Tissue antigens* 74: 101-116.
90. Klein, J., and A. Sato. 2000. The HLA system. First of two parts. *The New England journal of medicine* 343: 702-709.

91. Klein, J., and A. Sato. 2000. The HLA system. Second of two parts. *The New England journal of medicine* 343: 782-786.
92. Hurley, C. K., M. Fernandez Vina, and M. Setterholm. 2003. Maximizing optimal hematopoietic stem cell donor selection from registries of unrelated adult volunteers. *Tissue antigens* 61: 415-424.
93. Morales-Buenrostro, L. E., P. I. Terasaki, L. A. Marino-Vazquez, J. H. Lee, N. El-Awar, and J. Alberu. 2008. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. *Transplantation* 86: 1111-1115.
94. Morris, P. J., M. R. Mickey, D. P. Singal, and P. I. Terasaki. 1969. Serotyping for homotransplantation. XXII. Specificity of cytotoxic antibodies developing after renal transplantation. *British medical journal* 1: 758-759.
95. Harmer, A. W., C. G. Koffman, A. J. Heads, and R. W. Vaughan. 1995. Sensitization to HLA antigens occurs in 95% of primary renal transplant rejections. *Transplantation proceedings* 27: 666-667.
96. Lee, P. C., P. I. Terasaki, S. K. Takemoto, P. H. Lee, C. J. Hung, Y. L. Chen, A. Tsai, and H. Y. Lei. 2002. All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. *Transplantation* 74: 1192-1194.
97. Terasaki, P. I., and M. Ozawa. 2004. Predicting kidney graft failure by HLA antibodies: a prospective trial. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 4: 438-443.
98. Tait, B. D., C. Susal, H. M. Gebel, P. W. Nickerson, A. A. Zachary, F. H. Claas, E. F. Reed, R. A. Bray, P. Campbell, J. R. Chapman, P. T. Coates, R. B. Colvin, E. Cozzi, Doxiadis, II, S. V. Fuggle, J. Gill, D. Glotz, N. Lachmann, T. Mohanakumar, N. Suciu-Foca, S. Sumitran-Holgersson, K. Tanabe, C. J. Taylor, D. B. Tyan, A. Webster, A. Zeevi, and G. Opelz. 2013. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. *Transplantation* 95: 19-47.
99. Pavenski, K., J. Freedman, and J. W. Semple. 2012. HLA alloimmunization against platelet transfusions: pathophysiology, significance, prevention and management. *Tissue antigens* 79: 237-245.
100. Stanworth, S. J., C. Navarrete, L. Estcourt, and J. Marsh. 2015. Platelet refractoriness--practical approaches and ongoing dilemmas in patient management. *British journal of haematology* 171: 297-305.
101. Doughty, H. A., M. F. Murphy, P. Metcalfe, A. Z. Rohatiner, T. A. Lister, and A. H. Waters. 1994. Relative importance of immune and non-immune causes of platelet refractoriness. *Vox sanguinis* 66: 200-205.
102. Legler, T. J., I. Fischer, J. Dittmann, G. Simson, R. Lynen, A. Humpe, J. Riggert, E. Schleyer, W. Kern, W. Hiddemann, and M. Kohler. 1997. Frequency and causes of refractoriness in multiply transfused patients. *Annals of hematology* 74: 185-189.
103. Laundy, G. J., B. A. Bradley, B. M. Rees, M. Younie, and J. M. Hows. 2004. Incidence and specificity of HLA antibodies in multitransfused patients with acquired aplastic anemia. *Transfusion* 44: 814-825.
104. Kickler, T., S. D. Kennedy, and H. G. Braine. 1990. Alloimmunization to platelet-specific antigens on glycoproteins IIb-IIIa and Ib/IX in multiply transfused thrombocytopenic patients. *Transfusion* 30: 622-625.
105. Williamson, L. M., S. Lowe, E. M. Love, H. Cohen, K. Soldan, D. B. McClelland, P. Skacel, and J. A. Barbara. 1999. Serious hazards of transfusion (SHOT) initiative: analysis of the first two annual reports. *BMJ* 319: 16-19.
106. Bux, J., and U. J. Sachs. 2007. The pathogenesis of transfusion-related acute lung injury (TRALI). *British journal of haematology* 136: 788-799.
107. Bux, J. 2011. Antibody-mediated (immune) transfusion-related acute lung injury. *Vox sanguinis* 100: 122-128.
108. Popovsky, M. A., and S. B. Moore. 1985. Diagnostic and pathogenetic considerations in transfusion-related acute lung injury. *Transfusion* 25: 573-577.

109. Middelburg, R. A., D. van Stein, E. Briet, and J. G. van der Bom. 2008. The role of donor antibodies in the pathogenesis of transfusion-related acute lung injury: a systematic review. *Transfusion* 48: 2167-2176.
110. Bontadini, A. 2012. HLA techniques: typing and antibody detection in the laboratory of immunogenetics. *Methods* 56: 471-476.
111. Morin-Papunen, L., A. Tiilikainen, and A. L. Hartikainen-Sorri. 1984. Maternal HLA immunization during pregnancy: presence of anti HLA antibodies in half of multigravidous women. *Medical biology* 62: 323-325.
112. Regan, L., P. R. Braude, and D. P. Hill. 1991. A prospective study of the incidence, time of appearance and significance of anti-paternal lymphocytotoxic antibodies in human pregnancy. *Hum Reprod* 6: 294-298.
113. King, K. E., K. J. Kao, P. F. Bray, J. F. Casella, K. Blakemore, N. A. Callan, S. D. Kennedy, and T. S. Kickler. 1996. The role of HLA antibodies in neonatal thrombocytopenia: a prospective study. *Tissue antigens* 47: 206-211.
114. Masson, E., C. Vidal, M. Deschamps, S. Bongain, C. Thevenin, I. Dupont, D. Rietmulher, F. Pouthier, G. Mongaillard, J. Chabod, C. Ferrand, P. Tiberghien, and J. M. Rebibou. 2013. Incidence and risk factors of anti-HLA immunization after pregnancy. *Human immunology* 74: 946-951.
115. Lee, J., R. Romero, Y. Xu, J. Miranda, W. Yoo, P. Chaemsaithong, J. P. Kusanovic, T. Chaiworapongsa, A. L. Tarca, S. J. Korzeniewski, S. S. Hassan, N. G. Than, B. H. Yoon, and C. J. Kim. 2013. Detection of anti-HLA antibodies in maternal blood in the second trimester to identify patients at risk of antibody-mediated maternal anti-fetal rejection and spontaneous preterm delivery. *Am J Reprod Immunol* 70: 162-175.
116. Lee, J., R. Romero, Y. Xu, J. S. Kim, J. Y. Park, J. P. Kusanovic, T. Chaiworapongsa, S. S. Hassan, and C. J. Kim. 2011. Maternal HLA panel-reactive antibodies in early gestation positively correlate with chronic chorioamnionitis: evidence in support of the chronic nature of maternal anti-fetal rejection. *Am J Reprod Immunol* 66: 510-526.
117. Kovats, S., E. K. Main, C. Librach, M. Stubblebine, S. J. Fisher, and R. DeMars. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. *Science* 248: 220-223.
118. King, A., C. Boocock, A. M. Sharkey, L. Gardner, A. Beretta, A. G. Siccardi, and Y. W. Loke. 1996. Evidence for the expression of HLAA-C class I mRNA and protein by human first trimester trophoblast. *J Immunol* 156: 2068-2076.
119. Apps, R., S. P. Murphy, R. Fernando, L. Gardner, T. Ahad, and A. Moffett. 2009. Human leucocyte antigen (HLA) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies. *Immunology* 127: 26-39.
120. King, A., T. D. Burrows, S. E. Hiby, J. M. Bowen, S. Joseph, S. Verma, P. B. Lim, L. Gardner, P. Le Bouteiller, A. Ziegler, B. Uchanska-Ziegler, and Y. W. Loke. 2000. Surface expression of HLA-C antigen by human extravillous trophoblast. *Placenta* 21: 376-387.
121. Apps, R., L. Gardner, S. E. Hiby, A. M. Sharkey, and A. Moffett. 2008. Conformation of human leucocyte antigen-C molecules at the surface of human trophoblast cells. *Immunology* 124: 322-328.
122. Ellis, S. A., M. S. Palmer, and A. J. McMichael. 1990. Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule. *J Immunol* 144: 731-735.
123. Apps, R., Z. Meng, G. Q. Del Prete, J. D. Lifson, M. Zhou, and M. Carrington. 2015. Relative expression levels of the HLA class-I proteins in normal and HIV-infected cells. *J Immunol* 194: 3594-3600.
124. Duquesnoy, R. J., G. Honger, I. Hosli, M. Marrari, and S. Schaub. 2016. Detection of newly antibody-defined epitopes on HLA class I alleles reacting with antibodies induced during pregnancy. *International journal of immunogenetics* 43: 200-208.
125. Vilches, M., and A. Nieto. 2015. Analysis of Pregnancy-Induced Anti-HLA Antibodies Using Luminex Platform. *Transplantation proceedings* 47: 2608-2610.

126. Lo, Y. M., E. S. Lo, N. Watson, L. Noakes, I. L. Sargent, B. Thilaganathan, and J. S. Wainscoat. 1996. Two-way cell traffic between mother and fetus: biologic and clinical implications. *Blood* 88: 4390-4395.
127. Herzenberg, L. A., D. W. Bianchi, J. Schroder, H. M. Cann, and G. M. Iverson. 1979. Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell sorting. *Proceedings of the National Academy of Sciences of the United States of America* 76: 1453-1455.
128. Ariga, H., H. Ohto, M. P. Busch, S. Imamura, R. Watson, W. Reed, and T. H. Lee. 2001. Kinetics of fetal cellular and cell-free DNA in the maternal circulation during and after pregnancy: implications for noninvasive prenatal diagnosis. *Transfusion* 41: 1524-1530.
129. Solomonia, N., K. Playforth, and E. W. Reynolds. 2012. Fetal-maternal hemorrhage: a case and literature review. *AJP reports* 2: 7-14.
130. Curtis, B. R., and J. G. McFarland. 2006. Mechanisms of transfusion-related acute lung injury (TRALI): anti-leukocyte antibodies. *Critical care medicine* 34: S118-123.
131. Densmore, T. L., L. T. Goodnough, S. Ali, M. Dynis, and H. Chaplin. 1999. Prevalence of HLA sensitization in female apheresis donors. *Transfusion* 39: 103-106.
132. Powers, A., C. P. Stowell, W. H. Dzik, S. L. Saidman, H. Lee, and R. S. Makar. 2008. Testing only donors with a prior history of pregnancy or transfusion is a logical and cost-effective transfusion-related acute lung injury prevention strategy. *Transfusion* 48: 2549-2558.
133. Triulzi, D. J., S. Kleinman, R. M. Kakaiya, M. P. Busch, P. J. Norris, W. R. Steele, S. A. Glynn, C. D. Hillyer, P. Carey, J. L. Gottschall, E. L. Murphy, J. A. Rios, P. M. Ness, D. J. Wright, D. Carrick, and G. B. Schreiber. 2009. The effect of previous pregnancy and transfusion on HLA alloimmunization in blood donors: implications for a transfusion-related acute lung injury risk reduction strategy. *Transfusion* 49: 1825-1835.
134. Higgins, R., D. Lowe, M. Hathaway, C. Williams, F. T. Lam, H. Kashi, L. C. Tan, C. Imray, S. Fletcher, K. Chen, N. Krishnan, R. Hamer, S. Daga, M. Edey, D. Zehnder, and D. Briggs. 2011. Human leukocyte antigen antibody-incompatible renal transplantation: excellent medium-term outcomes with negative cytotoxic crossmatch. *Transplantation* 92: 900-906.
135. Higgins, R., D. Lowe, S. Daga, M. Hathaway, C. Williams, F. T. Lam, H. Kashi, L. C. Tan, C. Imray, S. Fletcher, N. Krishnan, P. Hart, D. Zehnder, and D. Briggs. 2015. Pregnancy-induced HLA antibodies respond more vigorously after renal transplantation than antibodies induced by prior transplantation. *Human immunology* 76: 546-552.
136. Lashley, E. E., T. Meuleman, and F. H. Claas. 2013. Beneficial or harmful effect of antipaternal human leukocyte antibodies on pregnancy outcome? A systematic review and meta-analysis. *Am J Reprod Immunol* 70: 87-103.
137. Nielsen, H. S., M. D. Witvliet, R. Steffensen, G. W. Haasnoot, E. Goulmy, O. B. Christiansen, and F. Claas. 2010. The presence of HLA-antibodies in recurrent miscarriage patients is associated with a reduced chance of a live birth. *Journal of reproductive immunology* 87: 67-73.
138. Biddle, P. K., C. I. Friedman, and P. M. Johnson. 1987. Lymphocyte-reactive antibodies and recurrent early pregnancy failure. *American journal of obstetrics and gynecology* 157: 785-786.
139. Umapathy, S., A. Shankarkumar, V. Ramrakhiyani, and K. Ghosh. 2011. Role of anti-human lymphocyte culture cytotoxic antibodies in recurrent spontaneous pregnancy loss women. *Journal of human reproductive sciences* 4: 17-19.
140. Meuleman, T., E. van Beelen, R. J. Kaaja, J. M. van Lith, F. H. Claas, and K. W. Bloemenkamp. 2016. HLA-C antibodies in women with recurrent miscarriage suggests that antibody mediated rejection is one of the mechanisms leading to recurrent miscarriage. *Journal of reproductive immunology* 116: 28-34.
141. Bartel, G., K. Walch, M. Wahrmann, S. Pils, L. Kussel, S. Polterauer, C. Tempfer, and G. A. Bohmig. 2011. Prevalence and qualitative properties of circulating anti-human leukocyte antigen alloantibodies after pregnancy: no association with unexplained recurrent miscarriage. *Human immunology* 72: 187-192.

142. Kishore, R., S. Agarwal, A. Halder, V. Das, B. R. Shukla, and S. S. Agarwal. 1996. HLA sharing, anti-paternal cytotoxic antibodies and MLR blocking factors in women with recurrent spontaneous abortion. *The journal of obstetrics and gynaecology research* 22: 177-183.
143. Coulam, C. B. 1992. Immunologic tests in the evaluation of reproductive disorders: a critical review. *American journal of obstetrics and gynecology* 167: 1844-1851.
144. Agrawal, S., M. K. Pandey, S. Mandal, L. Mishra, and S. Agarwal. 2002. Humoral immune response to an allogenic foetus in normal fertile women and recurrent aborters. *BMC pregnancy and childbirth* 2: 6.
145. Bolis, P. F., V. Soro, M. Martinetti Bianchi, and M. Belvedere. 1985. HLA compatibility and human reproduction. *Clinical and experimental obstetrics & gynecology* 12: 9-12.
146. Ho, H. N., T. J. Gill, 3rd, R. P. Nsieh, H. J. Hsieh, and T. Y. Lee. 1990. Sharing of human leukocyte antigens in primary and secondary recurrent spontaneous abortions. *American journal of obstetrics and gynecology* 163: 178-188.
147. Kromer, J., T. Hummel, D. Pietrowski, A. S. Giani, J. Sauter, G. Ehninger, A. H. Schmidt, and I. Croy. 2016. Influence of HLA on human partnership and sexual satisfaction. *Scientific reports* 6: 32550.
148. Steinborn, A., C. Seidl, C. Sayehli, C. Sohn, E. Seifried, M. Kaufmann, and E. Schmitt. 2004. Anti-fetal immune response mechanisms may be involved in the pathogenesis of placental abruption. *Clin Immunol* 110: 45-54.
149. Buurma, A., D. Cohen, K. Veraar, D. Schonkeren, F. H. Claas, J. A. Bruijn, K. W. Bloemenkamp, and H. J. Baelde. 2012. Preeclampsia is characterized by placental complement dysregulation. *Hypertension* 60: 1332-1337.
150. Kim, E. N., B. H. Yoon, J. Y. Lee, D. Hwang, K. C. Kim, J. Lee, J. Y. Shim, and C. J. Kim. 2015. Placental C4d deposition is a feature of defective placentation: observations in cases of preeclampsia and miscarriage. *Virchows Archiv : an international journal of pathology* 466: 717-725.
151. Locke, J. E., A. A. Zachary, D. S. Warren, D. L. Segev, J. A. Houp, R. A. Montgomery, and M. S. Leffell. 2009. Proinflammatory events are associated with significant increases in breadth and strength of HLA-specific antibody. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 9: 2136-2139.
152. Mor, G., I. Cardenas, V. Abrahams, and S. Guller. 2011. Inflammation and pregnancy: the role of the immune system at the implantation site. *Annals of the New York Academy of Sciences* 1221: 80-87.
153. Murphy, K. 2012. *Janeway's Immunobiology 8th Edition*.
154. Rodey, G. E., and T. C. Fuller. 1987. Public epitopes and the antigenic structure of the HLA molecules. *Critical reviews in immunology* 7: 229-267.
155. McKenna, R. M., and S. K. Takemoto. 2000. Improving HLA matching for kidney transplantation by use of CREGs. *Lancet* 355: 1842-1843.
156. Takemoto, S. K. 1996. HLA amino acid residue matching. *Clinical transplants*: 397-425.
157. Duquesnoy, R. J., M. Marrari, A. Mulder, L. C. Sousa, A. S. da Silva, and S. J. do Monte. 2014. First report on the antibody verification of HLA-ABC epitopes recorded in the website-based HLA Epitope Registry. *Tissue antigens* 83: 391-400.
158. Duquesnoy, R. J. 2006. A structurally based approach to determine HLA compatibility at the humoral immune level. *Human immunology* 67: 847-862.
159. Kao, K. J., D. J. Cook, and J. C. Scornik. 1986. Quantitative analysis of platelet surface HLA by W6/32 anti-HLA monoclonal antibody. *Blood* 68: 627-632.
160. Gouttefangeas, C., M. Diehl, W. Keilholz, R. F. Hornlein, S. Stevanovic, and H. G. Rammensee. 2000. Thrombocyte HLA molecules retain nonrenewable endogenous peptides of megakaryocyte lineage and do not stimulate direct allocytotoxicity in vitro. *Blood* 95: 3168-3175.
161. Lalezari, P., and A. M. Driscoll. 1982. Ability of thrombocytes to acquire HLA specificity from plasma. *Blood* 59: 167-170.

162. Santoso, S., R. Kalb, V. Kiefel, and C. Mueller-Eckhardt. 1993. The presence of messenger RNA for HLA class I in human platelets and its capability for protein biosynthesis. *British journal of haematology* 84: 451-456.
163. Goeken, N. E. 1984. Human suppressor cell induction in vitro: preferential activation by class I MHC antigen. *J Immunol* 132: 2291-2299.
164. van Marwijk Kooy, M., H. C. van Prooijen, M. Moes, I. Bosma-Stants, and J. W. Akkerman. 1991. Use of leukocyte-depleted platelet concentrates for the prevention of refractoriness and primary HLA alloimmunization: a prospective, randomized trial. *Blood* 77: 201-205.
165. Olsson, B., P. O. Andersson, M. Jernas, S. Jacobsson, B. Carlsson, L. M. Carlsson, and H. Wadenvik. 2003. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. *Nature medicine* 9: 1123-1124.
166. Taaning, E. 2000. HLA antibodies and fetomaternal alloimmune thrombocytopenia: myth or meaningful? *Transfusion medicine reviews* 14: 275-280.
167. Sharon, R., and A. Amar. 1981. Maternal anit-HLA antibodies and neonatal thrombocytopenia. *Lancet* 1: 1313.
168. Koyama, N., Y. Ohama, K. Kaneko, Y. Itakura, T. Nakamura, J. Takasaki, T. Tanaka, H. Eguchi, A. Kawase, K. Kamiya, and et al. 1991. Association of neonatal thrombocytopenia and maternal anti-HLA antibodies. *Acta paediatrica Japonica; Overseas edition* 33: 71-76.
169. Panzer, S., L. Auerbach, E. Cechova, G. Fischer, A. Holensteiner, E. M. Kitl, W. R. Mayr, M. Putz, P. Wagenbichler, and S. Walchshofer. 1995. Maternal alloimmunization against fetal platelet antigens: a prospective study. *British journal of haematology* 90: 655-660.
170. Skacel, P. O., T. E. Stacey, C. E. Tidmarsh, and M. Contreras. 1989. Maternal alloimmunization to HLA, platelet and granulocyte-specific antigens during pregnancy: its influence on cord blood granulocyte and platelet counts. *British journal of haematology* 71: 119-123.
171. Marshall, L. R., F. E. Brogden, T. S. Roper, and A. L. Barr. 1994. Antenatal platelet antibody testing by flow cytometry--results of a pilot study. *Transfusion* 34: 961-965.
172. Bonstein, L., N. Atweh, N. Haddad, and Y. Fruchtman. 2015. Anti-HLA Antibodies in Neonatal Alloimmune Thrombocytopenia-Is There Any Clinical Significance? (Abstract). *Blood* 126: 4647-4647.
173. Flo, K., T. Wilsgaard, A. Vartun, and G. Acharya. 2010. A longitudinal study of the relationship between maternal cardiac output measured by impedance cardiography and uterine artery blood flow in the second half of pregnancy. *BJOG : an international journal of obstetrics and gynaecology* 117: 837-844.
174. Flo, K., C. Widnes, A. Vartun, and G. Acharya. 2014. Blood flow to the scarred gravid uterus at 22-24 weeks of gestation. *BJOG : an international journal of obstetrics and gynaecology* 121: 210-215.
175. Kiefel, V., S. Santoso, M. Weisheit, and C. Mueller-Eckhardt. 1987. Monoclonal antibody--specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. *Blood* 70: 1722-1726.
176. Bertrand, G., V. Jallu, M. Gouet, K. M. Kjaer, P. Lambin, A. Husebekk, and C. Kaplan. 2005. Quantification of human platelet antigen-1a antibodies with the monoclonal antibody immobilization of platelet antigens procedure. *Transfusion* 45: 1319-1323.
177. Killie, M. K., W. Salma, E. Bertelsen, B. Skogen, and A. Husebekk. 2010. Quantitative MAIPA: Comparison of different MAIPA protocols. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis* 43: 149-154.
178. Nordang, G. B., S. T. Flam, M. T. Maehlen, T. K. Kvien, M. K. Viken, and B. A. Lie. 2013. HLA-C alleles confer risk for anti-citrullinated peptide antibody-positive rheumatoid arthritis independent of HLA-DRB1 alleles. *Rheumatology (Oxford)* 52: 1973-1982.
179. Duquesnoy, R. J., M. Marrari, M. S. L. C. da, M. B. J. R. de, S. U. A. K. M. de, A. S. da Silva, and S. J. do Monte. 2013. 16th IHIW: a website for antibody-defined HLA epitope Registry. *International journal of immunogenetics* 40: 54-59.
180. Skjaerven, R., H. K. Gjessing, and L. S. Bakketeig. 2000. Birthweight by gestational age in Norway. *Acta obstetricia et gynecologica Scandinavica* 79: 440-449.

181. Hemachandra, A. H., M. A. Klebanoff, A. K. Duggan, J. B. Hardy, and S. L. Furth. 2006. The association between intrauterine growth restriction in the full-term infant and high blood pressure at age 7 years: results from the Collaborative Perinatal Project. *International journal of epidemiology* 35: 871-877.
182. Shehata, F., I. Levin, A. Shrim, B. Ata, B. Weisz, R. Gamzu, and B. Almog. 2011. Placenta/birthweight ratio and perinatal outcome: a retrospective cohort analysis. *BJOG : an international journal of obstetrics and gynaecology* 118: 741-747.
183. Tranquilli, A. L., G. Dekker, L. Magee, J. Roberts, B. M. Sibai, W. Steyn, G. G. Zeeman, and M. A. Brown. 2014. The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health* 4: 97-104.
184. Tiller, H., M. K. Killie, B. Skogen, P. Oian, and A. Husebekk. 2009. Neonatal alloimmune thrombocytopenia in Norway: poor detection rate with nonscreening versus a general screening programme. *BJOG : an international journal of obstetrics and gynaecology* 116: 594-598.
185. Ohto, H. 1997. [Neonatal alloimmune thrombocytopenia]. *Nihon rinsho. Japanese journal of clinical medicine* 55: 2310-2314.
186. Khan, M. A. 1995. HLA-B27 and its subtypes in world populations. *Current opinion in rheumatology* 7: 263-269.
187. Refsum, E., A. Mortberg, J. Dahl, S. Meinke, M. K. Auvinen, M. Westgren, M. Reilly, P. Hoglund, and A. Wikman. 2016. Characterisation of maternal human leukocyte antigen class I antibodies in suspected foetal and neonatal alloimmune thrombocytopenia. *Transfus Med*.
188. Nako, Y., T. Tomomasa, and A. Morikawa. 1997. Plasma thrombomodulin level in newborn infants with and without perinatal asphyxia. *Acta Paediatr* 86: 91-95.
189. Phelan, J. P., C. Kirkendall, L. M. Korst, and G. I. Martin. 2007. Nucleated red blood cell and platelet counts in asphyxiated neonates sufficient to result in permanent neurologic impairment. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet* 20: 377-380.
190. Kapur, R., K. M. Heitink-Polle, L. Porcelijn, A. E. Bentlage, M. C. Bruin, R. Visser, D. Roos, R. B. Schasfoort, M. de Haas, C. E. van der Schoot, and G. Vidarsson. 2015. C-reactive protein enhances IgG-mediated phagocyte responses and thrombocytopenia. *Blood* 125: 1793-1802.
191. Akalin, E., R. Dinavahi, R. Friedlander, S. Ames, G. de Boccardo, V. Sehgal, B. Schroppel, M. Bhaskaran, S. Lerner, M. Fotino, B. Murphy, and J. S. Bromberg. 2008. Addition of plasmapheresis decreases the incidence of acute antibody-mediated rejection in sensitized patients with strong donor-specific antibodies. *Clinical journal of the American Society of Nephrology : CJASN* 3: 1160-1167.
192. Lefaucheur, C., A. Loupy, G. S. Hill, J. Andrade, D. Nochy, C. Antoine, C. Gautreau, D. Charron, D. Glotz, and C. Suberbielle-Boissel. 2010. Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. *Journal of the American Society of Nephrology : JASN* 21: 1398-1406.
193. Malek, A., R. Sager, P. Kuhn, K. H. Nicolaides, and H. Schneider. 1996. Evolution of maternofetal transport of immunoglobulins during human pregnancy. *Am J Reprod Immunol* 36: 248-255.
194. Gitlin, D., and A. Biasucci. 1969. Development of gamma G, gamma A, gamma M, beta IC-beta IA, C 1 esterase inhibitor, ceruloplasmin, transferrin, hemopexin, haptoglobin, fibrinogen, plasminogen, alpha 1-antitrypsin, orosomucoid, beta-lipoprotein, alpha 2-macroglobulin, and prealbumin in the human conceptus. *The Journal of clinical investigation* 48: 1433-1446.
195. Dahl, J., A. Husebekk, G. Acharya, K. Flo, T. B. Stuge, B. Skogen, B. Straume, and H. Tiller. 2016. Maternal anti-HLA class I antibodies are associated with reduced birth weight in thrombocytopenic neonates. *Journal of reproductive immunology* 113: 27-34.
196. Proulx, C., M. Filion, M. Goldman, A. Bradley, D. Devine, F. Decary, and P. Chartrand. 1994. Analysis of immunoglobulin class, IgG subclass and titre of HPA-1a antibodies in

alloimmunized mothers giving birth to babies with or without neonatal alloimmune thrombocytopenia. *British journal of haematology* 87: 813-817.
197. Labarrere, C. A., and O. H. Althabe. 1986. Intrauterine growth retardation of unknown etiology: II. Serum complement and circulating immune complexes in maternal sera and their relationship with parity and chronic villitis. *American journal of reproductive immunology and microbiology : AJRIM* 12: 4-6.
198. Althaus, J., E. G. Weir, F. Askin, T. S. Kickler, and K. Blakemore. 2005. Chronic villitis in untreated neonatal alloimmune thrombocytopenia: an etiology for severe early intrauterine growth restriction and the effect of intravenous immunoglobulin therapy. *American journal of obstetrics and gynecology* 193: 1100-1104.
199. Italiano, J. E., Jr., J. L. Richardson, S. Patel-Hett, E. Battinelli, A. Zaslavsky, S. Short, S. Ryeom, J. Folkman, and G. L. Klement. 2008. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood* 111: 1227-1233.
200. Ho-Tin-Noe, B., T. Goerge, S. M. Cifuni, D. Duerschmied, and D. D. Wagner. 2008. Platelet granule secretion continuously prevents intratumor hemorrhage. *Cancer research* 68: 6851-6858.
201. Yougbare, I., S. Lang, H. Yang, P. Chen, X. Zhao, W. S. Tai, D. Zdravic, B. Vadasz, C. Li, S. Piran, A. Marshall, G. Zhu, H. Tiller, M. K. Killie, S. Boyd, H. Leong-Poi, X. Y. Wen, B. Skogen, S. L. Adamson, J. Freedman, and H. Ni. 2015. Maternal anti-platelet beta3 integrins impair angiogenesis and cause intracranial hemorrhage. *The Journal of clinical investigation* 125: 1545-1556.
202. Santoso, S., H. Wihadmadyatami, T. Bakchoul, S. Werth, N. Al-Fakhri, G. Bein, V. Kiefel, J. Zhu, P. J. Newman, B. Bayat, and U. J. Sachs. 2016. Antiendothelial alphavbeta3 Antibodies Are a Major Cause of Intracranial Bleeding in Fetal/Neonatal Alloimmune Thrombocytopenia. *Arteriosclerosis, thrombosis, and vascular biology* 36: 1517-1524.
203. Zhang, X., N. M. Valenzuela, and E. F. Reed. 2012. HLA class I antibody-mediated endothelial and smooth muscle cell activation. *Current opinion in organ transplantation* 17: 446-451.
204. Narayanan, K., A. Jaramillo, D. L. Phelan, and T. Mohanakumar. 2004. Pre-exposure to sub-saturating concentrations of HLA class I antibodies confers resistance to endothelial cells against antibody complement-mediated lysis by regulating Bad through the phosphatidylinositol 3-kinase/Akt pathway. *European journal of immunology* 34: 2303-2312.
205. Kozuki, N., A. C. Lee, M. F. Silveira, A. Sania, J. P. Vogel, L. Adair, F. Barros, L. E. Caulfield, P. Christian, W. Fawzi, J. Humphrey, L. Huybregts, A. Mongkolchati, R. Ntozini, D. Osrin, D. Roberfroid, J. Tielsch, A. Vaidya, R. E. Black, and J. Katz. 2013. The associations of parity and maternal age with small-for-gestational-age, preterm, and neonatal and infant mortality: a meta-analysis. *BMC public health* 13 Suppl 3: S2.
206. Saftlas, A. F., R. J. Levine, M. A. Klebanoff, K. L. Martz, M. G. Ewell, C. D. Morris, and B. M. Sibai. 2003. Abortion, changed paternity, and risk of preeclampsia in nulliparous women. *American journal of epidemiology* 157: 1108-1114.
207. Klungsoyr, K., N. H. Morken, L. Irgens, S. E. Vollset, and R. Skjaerven. 2012. Secular trends in the epidemiology of pre-eclampsia throughout 40 years in Norway: prevalence, risk factors and perinatal survival. *Paediatric and perinatal epidemiology* 26: 190-198.
208. Bai, J., F. W. Wong, A. Bauman, and M. Mohsin. 2002. Parity and pregnancy outcomes. *American journal of obstetrics and gynecology* 186: 274-278.
209. Sibai, B., G. Dekker, and M. Kupferminc. 2005. Pre-eclampsia. *Lancet* 365: 785-799.
210. Li, D. K., and S. Wi. 2000. Changing paternity and the risk of preeclampsia/eclampsia in the subsequent pregnancy. *American journal of epidemiology* 151: 57-62.
211. Campbell, D. M., I. MacGillivray, and R. Carr-Hill. 1985. Pre-eclampsia in second pregnancy. *British journal of obstetrics and gynaecology* 92: 131-140.
212. Li, D. K. 1999. Changing paternity and the risk of preterm delivery in the subsequent pregnancy. *Epidemiology* 10: 148-152.

213. Wilcox, A. J., C. R. Weinberg, J. F. O'Connor, D. D. Baird, J. P. Schlatterer, R. E. Canfield, E. G. Armstrong, and B. C. Nisula. 1988. Incidence of early loss of pregnancy. *The New England journal of medicine* 319: 189-194.
214. Wang, X., C. Chen, L. Wang, D. Chen, W. Guang, and J. French. 2003. Conception, early pregnancy loss, and time to clinical pregnancy: a population-based prospective study. *Fertility and sterility* 79: 577-584.
215. Duquesnoy, R. J., A. Mulder, M. Askar, M. Fernandez-Vina, and F. H. Claas. 2005. HLAMatchmaker-based analysis of human monoclonal antibody reactivity demonstrates the importance of an additional contact site for specific recognition of triplet-defined epitopes. *Human immunology* 66: 749-761.
216. Dankers, M. K., M. D. Witvliet, D. L. Roelen, P. de Lange, N. Korfage, G. G. Persijn, R. Duquesnoy, Doxiadis, II, and F. H. Claas. 2004. The number of amino acid triplet differences between patient and donor is predictive for the antibody reactivity against mismatched human leukocyte antigens. *Transplantation* 77: 1236-1239.
217. Duquesnoy, R. J., M. Marrari, and A. Mulder. 2015. Usefulness of the Nonself-Self Algorithm of HLA Epitope Immunogenicity in the Specificity Analysis of Monospecific Antibodies Induced during Pregnancy. *Frontiers in immunology* 6: 180.
218. Geneugelijk, K., G. Honger, H. W. van Deutekom, K. A. Thus, C. Kesmir, I. Hosli, S. Schaub, and E. Spierings. 2015. Predicted Indirectly Recognizable HLA Epitopes Presented by HLA-DRB1 Are Related to HLA Antibody Formation During Pregnancy. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 15: 3112-3122.
219. Resse, M., R. Paolillo, B. P. Minucci, F. Cavalca, A. Casamassimi, and C. Napoli. 2015. Epitope-specificities of HLA antibodies: the effect of epitope structure on Luminex technique-dependent antibody reactivity. *Human immunology* 76: 297-300.
220. Lashley, L. E., M. L. van der Hoorn, G. W. Haasnoot, D. L. Roelen, and F. H. Claas. 2014. Uncomplicated oocyte donation pregnancies are associated with a higher incidence of human leukocyte antigen alloantibodies. *Human immunology* 75: 555-560.
221. Honger, G., I. Fornaro, C. Granado, J. M. Tiercy, I. Hosli, and S. Schaub. 2013. Frequency and determinants of pregnancy-induced child-specific sensitization. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 13: 746-753.
222. Dankers, M. K., D. L. Roelen, N. Korfage, P. de Lange, M. Witvliet, L. Sandkuijl, Doxiadis, II, and F. H. Claas. 2003. Differential immunogenicity of paternal HLA Class I antigens in pregnant women. *Human immunology* 64: 600-606.
223. Picascia, A., V. Grimaldi, C. Sabia, and C. Napoli. 2016. Comprehensive assessment of sensitizing events and anti-HLA antibody development in women awaiting kidney transplantation. *Transplant immunology* 36: 14-19.
224. Braun, J., M. Bollow, G. Remlinger, U. Eggens, M. Rudwaleit, A. Distler, and J. Sieper. 1998. Prevalence of spondylarthropathies in HLA-B27 positive and negative blood donors. *Arthritis and rheumatism* 41: 58-67.
225. Hammer, R. E., S. D. Maika, J. A. Richardson, J. P. Tang, and J. D. Taurog. 1990. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human beta 2m: an animal model of HLA-B27-associated human disorders. *Cell* 63: 1099-1112.
226. Burrows, R. F., and M. Andrew. 1990. Neonatal thrombocytopenia in the hypertensive disorders of pregnancy. *Obstetrics and gynecology* 76: 234-238.