Significance of ABO Blood Group Incompatibility between Mother and Child Regarding Incidence and Severity of Fetal and Neonatal Alloimmune Thrombocytopenia

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1 Introduction

1.1 FNAIT

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) involves the mother generating alloantibodies against fetal platelet antigens inherited from the father (Pearson et al. 1964). After transplacental transfer of IgG antibodies, this results in the opsonization of fetal platelets and subsequent destruction of platelets by different mechanisms; thrombocytopenia and signs of bleeding are present in the newborn or remain unapparent. Most commonly, bleeding signs such as petechiae (90%), hematomas (66%) and melena (30%) occur. Other less frequent bleeding signs are hemoptysis (8%), retinal bleeding (7%) and hematuria (3%) (Mueller-Eckhardt et al. 1989). The incidence of FNAIT in Caucasians is 1 in 1000 pregnancies and the incidence of severe bleeding complications due to FNAIT is 1 in 10,000 pregnancies (Kamphuis et al. 2014). Approximately 30% of FNAIT cases result in severe disease progression (Kamphuis et al. 2010). Intracranial hemorrhage (ICH) occurs in 10% of severely thrombocytopenic cases (Kamphuis et al. 2010) and leads to perinatal mortality in almost 50% of these cases, with severe neurodevelopmental impairment occurring in 60% of surviving children (Winkelhorst et al. 2019). Alloimmunization occurs when the father is positive for a target antigen which the mother does not inherit. In FNAIT, target antigens are human platelet antigens (HPA), expressed as molecular variants of glycoproteins (GP), located primarily on the surface of thrombocytes and megakaryocytes. Several different human platelet antigen-systems are known so far, and each system comprises different alleles, of which the frequent allele is labelled with the letter a and the less common allele with the letter b (Sachs 2013).

In Caucasians, the most frequent alloantibody responsible for approximately 75% of all cases of FNAIT is directed against HPA-1a (Ghevaert et al. 2007b), which is expressed on the integrin β3-chain (GP IIIa) and present in various complexes on different cells, for example on thrombocytes (see also chapter Pathogenesis). 75% of all Caucasians are homozygous HPA-1aa, 23% heterozygous HPA-1ab and 2% are HPA-1bb homozygous (Ahlen et al. 2012; Décary 1982).

The risk of HPA-1a alloimmunization in HPA-1bb women after delivery of an HPA-1a-positive child is strongly associated with the HLA class II allele HLA-DRB3*01:01

(Wienzek-Lischka et al. 2017). 12.7% of HPA-1bb mothers who are HLA-DRB3*01:01 positive undergo alloimmunization during pregnancy if they encounter the fetal HPA-1a peptide, as opposed to only 0.5% who lack this allele (Kjeldsen-Kragh and Olsen 2019).

HPA-1a and HPA-1b differ in a single leucine/proline substitution at position 33 (leu33/pro33) of the integrin β3 chain. In HPA-1a, leu33 acts as anchor residue for the peptide binding groove of HLA-DRB3*01:01. Its hydrophobic side chain is predicted to fit to the hydrophobic P9 pocket of DRA/DRB3*01:01 (Wu et al. 1997) and is buried in the peptide binding groove, which enables the stable MHC binding necessary for T-cell activation. In contrast, the pro33 in HPA-1b associates poorly to the hydrophobic P9 pocket of DRA/DRB3*01:01; only non-physiologically high concentrations of autologous HPA-1b peptides enable HPA-1a-specific T-cell activation (Ahlen et al. 2016).

Another frequently targeted antigen (approximately 15% of FNAIT cases) in Caucasians is HPA-5b on GPIIb, whereas in Asian regions the most frequent antibody is directed against HPA-4b (Mueller-Eckhardt et al. 1989; Shibata et al. 1986).

1.2 Pathogenesis

Regarding hemolytic disease of the fetus and newborn (HDFN), the red blood cell (RBC) counterpart of FNAIT, the alloimmunization of pregnant women against RBC antigens primarily occurs during parturition, when fetomaternal hemorrhage is at its strongest (Woodrow and Finn 1966). Consequently, HDFN does not regularly occur in the first pregnancy of mothers not immunized before by pregnancy or blood transfusion. In contrast, immunization against HPA-1a does already occur early during the first pregnancy (Skogen et al. 2009). However, it is doubtful whether the amount of fetal platelets present in antenatal FMH could sufficiently cause immunization against HPA-1a, taking into account that platelets seldomly stimulate anti-HPA-1a immunization after allogeneic transfusions (Kiefel et al. 2001). Therefore, Kumpel et al. propose that particles of the syncytiotrophoblast may trigger alloimmunization in early pregnancy. These particles expressing the β3 integrin (carrier of the HPA-1a/1b polymorphism) are shed into the maternal bloodstream (Kumpel et al. 2008) as early as 4-6 weeks p.c. and are phagocytized by maternal dendritic cells, whereupon HPA-peptides are presented on

MHC molecules and matching maternal T-cells are activated (Kumpel and Manoussaka 2012). However, this route of maternal immunization could account for alloimmunization against HPA located on the β3 integrin only (Curtis 2015).

Once fetal antigens enter the maternal circulation, the alloantigens are processed and presented by professional antigen presenting cells, e.g., dendritic cells, to T-cells. Ahlen et al. were able to demonstrate the existence of HPA-1a– specific HLA-DRB3*01:01–restricted $CD4^+$ T cells in alloimmunized women (Ahlen et al. 2009). Activated, antigen-specific $CD4^+$ T helper cells in turn activate antigen-specific B cells in undergoing a class switch from IgM to IgG and secreting anti-HPA-1a antibodies of IgG class. The level of antibody synthesis depends on the specificity of cognate T-B cell interaction and the strength of the co-stimulatory signal (Kumpel and Manoussaka 2012).

The maternal IgG-antibodies are transferred into the fetal circulation by fetal Fragment crystallizable Receptor neonatal (FcRn) receptors on the syncytiotrophoblast (Chen et al. 2010) and opsonize fetal platelets, which are afterwards degraded in the reticuloendothelial system of the fetus. Already in week 16 of gestational age, fetal platelets express HPAs (Gruel et al. 1986). Since low platelet counts were already detected in some cases prior to gestational week 20, the transplacental IgG transfer must be active before gestational week 20 (Bussel et al. 1997).

The β3 integrin is expressed as a heterodimer with αIIb (GP IIbIIIa, fibrinogen receptor), mainly on platelets, or as a heterodimer with αv (αvβ3 integrin, vitronectin receptor), mainly on endothelial cells (Bennett 1996; Bennett et al. 1997; Bennett et al. 1999; Paul et al. 2003). The expression of αvβ3 integrin on endothelial cells and interaction with extracellular matrix plays a major role in the formation of new vessels, e.g., angiogenesis (Bennett et al. 1999; Brooks et al. 1994). Santoso et al. demonstrated that three different types of anti-HPA-1a antibodies exist: anti-αIIbβ3, anti-β3 and anti-αvβ3.Whereas anti-αIIbβ3 is only reactive with the fibrinogen receptor (GP IIbIIIa) on platelets, anti-β3 and anti-αvβ3 (anti-αvβ3 predominantly) react with integrin αvβ3 (receptor for vitronectin) on endothelial cells (as well as smooth muscle cells and platelets). Only anti-αvβ3 impairs angiogenesis and induces apoptosis by blocking the attachment to vitronectin and increasing the synthesis of reactive oxygen species (ROS) (Santoso et al. 2016). In support of these findings, van Gils et al. described the

interference of HPA-1a antibodies with the formation of a stable endothelial monolayer by reallocation of junctional proteins (van Gils et al. 2009).

Regarding the pathogenesis of ICH, Yougbaré et al. showed that mouse fetuses without platelets survived in utero without developing ICH, while in a mouse FNAIT model of isoimmunization in β3 -/- mice, ICH only occurred in the presence of anti-β3 integrin-antibodies (Yougbaré et al. 2015). Santoso et al. detected anti-αvβ3 antibodies in sera from mothers with children suffering from ICH (Santoso et al. 2016). Therefore, it can be assumed that ICH is more likely caused by an impairment of angiogenesis by anti-αvβ3 antibodies rather than because of low platelet counts.

Since the αvβ3-integrin is expressed on infiltrating trophoblasts, anti-β3 antibodies could possibly be responsible for causing intrauterine growth retardation (IUGR) and miscarriage in FNAIT cases (Eksteen et al. 2017; Yougbaré et al. 2015). Additionally, FNAIT is associated with chronic placental inflammation mostly affecting the fetomaternal interface, e.g., chronic chorioamnionitis and villitis (Althaus et al. 2005; Dubruc et al. 2016). Killer cells naturally occurring in the uterus contribute to this inflammatory environment and tend to impair trophoblast function in the presence of anti-β3 antibodies and therefore impair placental development (Yougbaré et al. 2017).

1.3 Diagnosis

FNAIT is usually discovered when the first obviously affected child is born and other causes for thrombocytopenia (e.g., septicemia) have been ruled out. Early onset thrombocytopenia and very low platelet counts in otherwise healthy newborns are found in FNAIT cases; other maternal clinical features (e.g. immune thrombocytopenia, ITP) and thrombocytopenia among sick, preterm children usually do not cause a neonatal platelet count this low and this early (Burrows and Kelton 1993). Laboratory diagnosis of FNAIT is based on a positive crossmatch between maternal serum and paternal platelets, discrepancy between maternal and paternal or maternal and neonatal HPA-genotypes, and the detection of maternal platelet-specific alloantibodies.

Commonly, the detection of anti-HPA is performed serologically via monoclonal antibody immobilization of platelet antigens (MAIPA). MAIPA was also used in this

study to detect maternal antibodies and is therefore described in detail in chapter 2, Material and Methods (Kiefel et al. 1987).

Another possibility for detecting anti-HPA antibodies is the platelet immunofluorescence test (PIFT), where test platelets are incubated with maternal serum. If anti-HPA antibodies exist, they are made visible by adding a fluorescence labelled "secondary antibody" and measurement of fluorescent signals with flow cytometry afterwards (Borne et al. 1978). However, since pregnant mothers develop anti-HLA antibodies in approximately 50% of pregnancies at term, it is not possible to distinguish between HPA and HLA antibodies using platelets as targets (Kiefel et al. 1987). Thus, glycoprotein-specific capture tests are the gold-standard for the detection of HPA antibodies.

If initial platelet serology results are negative, but FNAIT is nonetheless strongly suspected, serology should be repeated some weeks later (Socher et al. 2009). Anti-HPA antibodies are not always detectable directly after delivery (Killie et al. 2008) and detection of low-avidity antibodies, which can demonstrably induce platelet destruction in a NOD-SCID mouse model, may be missed by MAIPA due to its steps involving washing (Bakchoul et al. 2011). After affinity maturation, these antibodies can be detected some weeks after delivery.

1.4 Therapy

Neonatal treatment depends on clinical presentation and platelet count. Neonates without bleeding signs and no family history of ICH should receive platelet (PLT) transfusions if the PLT count is $< 25 \times 10^9$/l, in the case of bleeding signs and family history if the PLT count is $< 50 \times 10^9$/l, and in the case of ICH if the PLT count is $< 100 \times 10^9$/l (Lieberman et al. 2019; New et al. 2016). If antigen-negative platelets or washed platelets of the mother are not available, random buffy coat platelets can be used, although their time of survival might be shortened (Kiefel et al. 2006).

The subsequent pregnancy is designated as risk pregnancy and antenatal fetal bleeding prophylaxis depends on the outcome of the previously affected child. Standard care remains the administration of intravenous immunoglobulin (IVIG) for the expectant mother (Winkelhorst et al. 2017). IVIG might block the neonatal Fc receptor on the

syncytiotrophoblast and on fetal macrophages, inhibiting the transplacental transfer and the binding of anti-HPA on opsonized platelets to fetal macrophages (Ueda et al. 2015). Mechanisms of the downregulation of maternal antibodies independent of the FcRn receptor pathway and how IVIG acts against ICH cannot yet be explained. Direct effects on endothelial cells or systemic platelet-mediated cytotoxicity are conceivable (Yougbaré et al. 2015), and this assumption is supported by the prevention of chronic villitis through IVIG (Althaus et al. 2005). IVIG administration can reduce the recurrence risk of ICH from 79% to 11% (Tiller et al. 2013). In order to do so, the onset of administration should be tailored to the approximate gestational age in which ICH occurred in the previous sibling (Bussel et al. 2010), mostly starting before 20 weeks of gestation because ICH mainly occurs by the end of the second trimester (Tiller et al. 2013), (Giovangrandi et al. 1990).

In the 1990s, fetal blood sampling (FBS) was offered to all pregnant women with HPA-1a antibodies, to assess the fetal platelet count (Brojer et al. 2016). FBS comes with a high risk of transplacental hemorrhage (Denomme and Fernandes 2007), boosting of alloimmunization, 1.6% fetal loss and 2.4% other complications per procedure. FBS was combined with intrauterine platelet transfusion (IUT) to avoid prolonged bleeding from the umbilical vein. Nowadays IUTs are mostly used as a rescue strategy only, since IUT alone is associated with premature birth or abortion in 1-2% per intervention and non-invasive approaches i.e. empiric IVIG gave adequate results in several studies (Winkelhorst et al. 2017).

Nevertheless, IVIG administration contains disadvantages. Its isolation from a large donor pool involves the (albeit small) risk of transmission of blood associated diseases. The standard dose of 1g/kg maternal weight weekly until delivery is based on a recommendation for ITP and has not been investigated in randomized studies. Dose-related maternal side effects occur: light discomfort and headaches are common, and aseptic meningitis, renal and cardiovascular dysfunctions are rare (Paridaans et al. 2015; Winkelhorst et al. 2017). IVIG still contains isohemagglutinins, which can cause intravascular hemolysis, reticuloendothelial degradation of erythrocytes and complement-activation. Blood group A women therefore have a higher risk for anemia than blood group O women, especially reported for treatment with 2g IVIG per kg maternal body weight weekly. A reduction of the IVIG dose to 0.5 g/kg body weight

was reported in a case series with pregnant women with standard risk (no ICH in history) (Lakkaraja et al. 2016). Data on the value of adding steroids to IVIG therapy is insufficient (Winkelhorst et al. 2017).

A prospective screening study from Norway has recommended performing a Caesarean section two to four weeks before term (Kjeldsen-Kragh et al. 2007). Practice guidelines advise induction of labor in week 37 and vaginal delivery in cases without history of ICH and a history of vaginal delivery in the previous pregnancy (Lieberman et al. 2019; van den Akker et al. 2006). In general, potentially traumatic delivery assistance which increase the neonatal bleeding risk, such as forceps delivery or vacuum delivery and scalp electrodes, should be avoided (Lieberman et al. 2019). However, trials assessing the safest delivery mode for pregnancies affected by FNAIT are needed.

New experimental treatment methods are investigated, mostly focusing on administration of non-destructive antibodies which block pathogenic maternal antibodies and are unable to activate complement, phagocytosis or antibody-dependent cytotoxicity (Bakchoul et al. 2013; Ghevaert et al. 2013; Mathiesen et al. 2013). Further approaches relate to blocking FcRn to prevent the transplacental IgG-transfer (Chen et al. 2010), targeting uterine natural killer cells and/or their receptors (Yougbaré et al. 2017), targeting T-cell response in order to inhibit alloimmunization (Ahlen et al. 2009) and oral administration of peptides in a tolerogenic formulation to induce tolerance and prevent anti-HPA-1a formation (Ahlen et al. 2016). However, these new treatment methods are not yet established and cannot replace IVIG, making further research necessary.

1.5 Predictors of Incidence and Severity

Many predictors for the incidence and severity of FNAIT have been proposed, but only a few appear reliable and can be used to decide on a therapy regime.

One predictor for the appearance of HPA-1a alloimmunization is the HLA-DRB3*01:01 status of the mother (Delbos et al. 2016; Wienzek-Lischka et al. 2017). 90% of the immunized HPA-1bb women are carriers of HLA-DRB3*01:01, whereas only 27% of the general population carry HLA-DRB3*01:01 (Ahlen et al. 2009). Recently, a dose-dependent effect of maternal HLA-DRB3*01:01 status (negative,

hemi- or heterozygous) on the neonatal platelet count was described, depicting an inversely proportional relationship between maternal HLA-DRB3*01:01 allele dose and neonatal platelet counts (Kjeldsen-Kragh et al. 2019).

Another positive correlation with the incidence of FNAIT might be the HLA-DQB1*02:01 status (Ahlen et al. 2009). Whether HLA-DRB4*01:01 might play a role in FNAIT is still discussed. While Delbos et al. stated that therapy response in the presence of HLA-DRB4*01:01 was significantly better and no ICH in mothers with low-avidity antibodies occurred (Delbos et al. 2016), Loewenthal et al. observed an aggravation of FNAIT progression and impaired therapy response in presence of a combination of HLA-DRB3*01:01 and HLA-DRB4*01:01. In contrast to both, Wienzek-Lischka et al. in turn found no association between FNAIT and HLA-DRB4*01:01 (Wienzek-Lischka et al. 2017).

Study results on maternal antibody levels as severity predictors are inconsistent. Killie et al. demonstrated an association between antibody-levels in gestational weeks 22 and 34 and FNAIT severity for both primiparous and multiparous women (Killie et al. 2008). Tiller et al. also confirmed an association between maternal antibody levels and neonatal platelet counts (Tiller et al. 2016). In contrast, Bertrand et al. came to the conclusion that the antibody level did not correlate with the severity in index cases, but in subsequent pregnancies (Bertrand et al. 2011), while Ghevaert et al. observed that neither the potency nor the bioactivity of the maternal antibody permitted conclusions about the severity of FNAIT (Ghevaert et al. 2007a).

The commonly accepted assumption that the severity of FNAIT increases with subsequent pregnancies, for example illustrated by the data of Kamphuis et al. (Kamphuis et al. 2010) and Delbos et al. (Delbos et al. 2016), has recently been challenged by Tiller et al., whose study on the natural course of FNAIT in subsequent pregnancies revealed unchanged or higher platelet counts in about 66% of subsequent siblings at the time of delivery (Tiller et al. 2016).

Concerning severity, the only reliable predictor thus far seems to be the outcome of the previous child regarding ICH (Bussel et al. 1997; Kamphuis et al. 2010; Kjeldsen-Kragh et al. 2007; Porcelijn et al. 2008). If ICH has once occurred, the reoccurrence rate in subsequent pregnancies is very high (Birchall et al. 2003; Radder et al. 2003),

ranging between 72% and 79% including fetal death. If the index child did not present with ICH, the risk of ICH for the subsequent child without IVIG prophylaxis is estimated at about 7% (Radder et al. 2003).

The mode of delivery has no influence on the risk of ICH, not even in fetuses with platelet counts below 50 x 10^9/l (van den Akker et al. 2006).

Anti-HLA class I antibodies cannot be excluded as possible predictors of incidence or severity of FNAIT so far, since their detection in association with FNAIT can be pure coincidence and independent from the pathogenesis of FNAIT. Since anti-HLA-antibodies are detectable in 30-50% of all pregnant woman at term and their frequency rises with the number of pregnancies, Marin et al. and Delbos et al. hypothesized that anti-HLA class I antibodies work synergistically with anti-HPA and may aggravate the severity of FNAIT (Delbos et al. 2016; Marin et al. 2005). Marin speculates that anti-HLA class I antibodies, which enter the fetal circulation, are mainly neutralized by thrombocytes and thus cause fetal thrombocytopenia (Marin et al. 2005). Fetal HLA-antigens -C, -E, -G are expressed on extravillous trophoblasts and HLA-antigens -A, -B, -C can also become accessible during fetomaternal hemorrhaging, when fetal thrombocytes enter maternal circulation. There are several FNAIT case reports in which anti-HLA are the only detectable antibodies (Thude et al. 2006), though admittedly in the majority of cases the sera were not tested for antibodies against rare HPA. However, in all of these cases other particular circumstances applied, e.g. infections, asphyxia, maternal ITP (Refsum et al. 2017). A recent large study in 817 cases of suspected FNAIT demonstrated no association of maternal anti-HLA class I antibodies with FNAIT incidence and severity (Sachs et al. 2020).

Recently, a correlation between maternal ABO blood groups and the severity of FNAIT has been reported. Ahlen et al. reported that women with non-O-blood groups had a higher risk of having a child with severe FNAIT than blood group O women (Ahlen et al. 2012), while alloimmunization occurred independent from ABO blood groups.

1.6 General Screening in Pregnancy

The majority of ICH occurs in utero (Ghevaert et al. 2007b; Jin et al. 2019; Kamphuis et al. 2014; Winkelhorst et al. 2019) and often the platelet count of affected fetuses is below 20 x 10^9/l (Ghevaert et al. 2007b). An early general screening in pregnancy and prophylactic interventions in women where anti-HPA-1a antibodies have been detected could possibly prevent ICH. Although costs would be high, the treatment costs of FNAIT sequelae are higher (Kamphuis et al. 2010).

A prophylactic screening could proceed as follows (Kamphuis et al. 2010):

1. Detection of HPA-1bb women in the first trimester through HPA genotyping or antigen detection by ELISA.

2. Fetal HPA genotyping from cell-free DNA in maternal plasma in HPA-1bb pregnant women. This replaces paternal genotyping and excludes HPA-1bb children from a heterozygous father, where no alloimmunization will occur.

3. The group of HPA-1bb women could be reduced by testing for HLA-DRB3*01:01. Note that in 10% of cases alloimmunization occurs independent from HLA-DRB3*01:01.

4. Screening HPA-1bb pregnant women at risk for immunization for anti-HPA-1a antibodies.

Reconsidering from a preclinical standpoint, it seems wiser to narrow the group of women at risk for FNAIT as much as possible before the application of fetal HPA genotyping and thus change the proposed order and reverse step 3 before step 2.

Currently, a general screening program is being evaluated in the Netherlands (Kjeldsen-Kragh et al. 2007; Tiller et al. 2017). In Germany, however, no screening measures have yet been implemented.

1.7 Prophylaxis of Maternal Immunization in HDFN and FNAIT

The concept of prophylaxis of maternal immunization in FNAIT has been proposed in analogy to the efficacy of anti-RhD prophylaxis in RhD-negative women to prevent hemolytic disease of the fetus and newborn (HDFN). In HDFN, anti-RhD and anti-ABO antibodies are frequently involved in prenatal and postnatal disease in the fetus or newborn, respectively.

Albeit ABO incompatibility between mother and fetus is present in between 14% to 20% of pregnancies (depending on ethnicities) (Akanmu et al. 2015; Cariani et al. 1995; Clarke 1973), only approximately 1% of children is affected by ABO hemolytic disease (ABO-HD) (Peevy and Wiseman 1978; Voak and Bowley 1969). The course of the disease is mainly benign and includes jaundice, likely because most of the transferred antibodies are neutralized by ABO antigens on other cells tissues and plasma proteins (Ottenberg 1911). The quantitative expression of ABO antigens varies widely among infants' RBCs (Grundbacher 1980) and thus, red blood cells of the fetus with low expression of ABO antigens survive in the presence of maternal ABO antibodies and disease is almost always observed only postnatally.

In HDFN due to anti-RhD antibodies, severe manifestations with fetal anemia and hydrops, severe hyperbilirubinemia and kernicterus in the newborn used to occur more often. Since immunization of the mother occurs mainly during delivery by fetomaternal (micro-) hemorrhage, the subsequent child is at risk (Costumbrado and Ghassemzadeh 2019).

By introduction of RhD phenotyping for all primiparous women and anti-D-immunoprophylaxis in RhD-negative pregnant women more than 50 years ago, the incidence of HDFN due to anti-RhD decreased from 16% (Bowman 1997) to less than 0.1% (Hendrickson and Delaney 2016). By default, possibly after non-invasive detection of fetal RhD, RhD-negative women in Germany receive 300 µg anti-D-immunoglobulin between 28th and 30th week of gestation as well as within 72 hours after birth in case the newborn is RhD-positive (and in case of invasive interventions or fetomaternal hemorrhage during pregnancy) (Legler 2018). Interestingly, anti-D-immunoprophylaxis not only reduces the risk for anti-D-alloimmunizaton, but also for non-D-alloimmunization against other antigens located on RBCs (Zwiers et al. 2018). One possibility for explaining this non-antigen specific immunosuppression is the

“rapid clearance”, i.e., the destruction of the RBC by splenic macrophages before the maternal immune system is triggered for alloimmunization (Woodrow et al. 1975).

Although the rate of FNAIT in newborns of primiparous women is already high (at least 25%), alloimmunization may occur in association with delivery in a major proportion of anti-HPA-1 immunized women (Killie et al. 2008; Kjeldsen-Kragh et al. 2007; Kjeldsen-Kragh et al. 2012). Therefore, FNAIT may resemble anti-RhD HDFN more than previously assumed (Skogen et al. 2009).

Profnait, a project set up by eleven European project partners and promoted by the European Union since 2012, works to establish a safe and effective FNAIT immunoprophylaxis (Geisen 2013; Kjeldsen-Kragh et al. 2012). In HPA-1bb women who gave birth to an HPA-1a positive child without the occurrence of alloimmunization, it is planned to administer anti-HPA-1a immunoprophylaxis immediately after delivery (similar to anti-D-immunoprophylaxis), in order to degrade fetal HPA-1a positive platelets before they are able to trigger the maternal immune system (Kamphuis et al. 2010). It is still uncertain whether a postpartal prophylaxis for FNAIT would be as successful as anti-D-immunoprophylaxis, if antigens on particles of the syncytiotrophoblast alternative to fetal platelets trigger for already early immunization in FNAIT.

However, in cases of severe FNAIT complicated by intracranial hemorrhage, maternal immunization nonetheless occurs mostly before delivery (Jin et al. 2019). To prevent all severe FNAIT cases, antenatal prophylaxis is required in addition to postnatal prophylaxis (Kjær et al. 2020). Assuming that fetal platelet antigen expression is initiated by 12 weeks of gestation and since the transplacental IgG transfer must be active before gestational week 20 (in order to cause intracranial hemorrhage before gestational week 18), available prophylaxis for pregnant women positively screened for FNAIT would need to be administered early in pregnancy (Bussel et al. 2010; Jin et al. 2019).

1.8 ABO Incompatibility

In ABO-incompatible pregnancies, isohemagglutinins are directed against the fetal blood group antigens. As early as 1943, Levine noticed that the prevalence of anti-RhD alloimmunization was higher in ABO-compatible than in ABO-incompatible pregnancies (Levine 1943, 1959) pregnancies. This observation was confirmed by several authors and led to the development of anti-RhD immunoprophylaxis, trying to mimic the described protection through ABO incompatibility with a suitable antibody.

In 1997 Bowman depicted the risk of anti-RhD alloimmunization to be 16% in ABO-compatible pregnancies, but only 2% in ABO-incompatible pregnancies (Bowman 1997). Recently, Zwiers confirmed that ABO incompatibility has a preventive effect on anti-D and non-D alloimmunization (Zwiers et al. 2018). Furthermore, Zizka et al. hypothesize that ABO incompatibility significantly reduces the risk of severe fetomaternal hemorrhage (FMH). They suspect that in case of fetomaternal blood-contact, agglutination almost immediately closes the pathological leaks of placental vessels and the fetal blood loss is smaller (Zizka et al. 2008).

Regarding FNAIT, in 1977 Gratwohl and Shulman described 25 cases of “isoimmune neonatal thrombopenia” (FNAIT), all resulting from ABO-compatible pregnancies and assumed that not only fetomaternal incompatibility regarding platelet antigens but also fetomaternal compatibility regarding ABO blood groups are requirements for the development of FNAIT. Consequently, they speculated ABO incompatibility might prevent alloimmunization, probably because fewer fetal platelets enter maternal circulation or their time of survival is shortened (Gratwohl and Shulman 1977).

1.9 ABO Blood Group System

In 1900 Karl Landsteiner described three blood groups: A, B and C (later called O), after observing different agglutinating effects in mixing sera and RBC of different individuals together (Schwarz and Dorner 2003). Based on Landsteiner’s discoveries, the ABO blood group antigens on erythrocytes, small carbohydrate determinants on glycoproteins and glycolipids, were identified. Products of the encoding genes are glycosyltransferase enzymes, which transfer the immunodominant terminal monosaccharide to the membranous acceptor substrates.

The ABO system is now structured in four major groups A, B, O, AB (Yamamoto et al. 1990b) and various subgroups and variants. Since ABO antigens are not only expressed on erythrocytes but on other cells and tissues as well, e.g., leukocytes and epithelial cells, they are often referred to as "histo-blood group antigens." Several genes collaborate for their synthesis. The glycosyltransferases' genes are located on chromosome 9 q34.1-q34.2 and include 7 exons which span over 18kb. 77% of the coding sequences and 91% of the catalytically active soluble transferase proteins are comprised in exon 6 and 7 (Bennett et al. 1995; Ferguson-Smith et al. 1976).

All ABO phenotypes are closely related to DNA sequence variants of the glycosyltransferases. A sequence encoding for the frequent phenotype A_1, a 1062 bp long coding region (Yamamoto et al. 1990a) is used as reference to describe the differences between ABO alleles, which are found mainly in exons 6 and 7, where substrate specificity and activity of the glycosyltransferase is coded (Bennett et al. 1995). Two deletions, seven sense mutations and ten missense mutations recurring in various alleles depict the evolutionary history of the ABO gene (Blumenfeld and Patnaik 2004).

Blood group A individuals express the α1→3 N-acetylgalactosaminyltransferase (GTA), which transfers N-acetylgalactosamine (GalNAc), while B individuals express α1→3 galactosyltransferase (GTB), which transfers galactose (Gal) to the acceptor substrate called H substance. Uridine diphosphate-GalNAc (UDP-GalNAc) operates as donor substrate for GTA and UDP-Gal for GTB. The DNA sequence of A- and B-glycosyltransferases differs in 7 base substitutions, which results in the exchange of 4 amino acids of altogether 353 amino acids (Yamamoto et al. 1990b). The amino acids in position 266 and 268 communicate with the donor- and acceptor-substrate and therefore distinguish the glycosyltransferase enzymes substrate specificity and activity (Yamamoto and McNeill 1996; Yip 2002). Leu/Met266 is most important for the selection of donor carbohydrates; the smaller Leu266 in GTA interacts with the larger acetamido group of UDP-GalNAc, while the larger Met266 in GTB interacts with the smaller UDP-Gal (Yamamoto and McNeill 1996). However, GTA and GTB share a small degree of overlap in their substrate specificity, i.e. GTA can transfer Gal if GalNAc is absent or barely there and vice versa (Yates and Watkins 1982). AB individuals express both the A- and B-glycosyltransferases.

Alleles *ABO*A1.01* and *ABO*A2.01* differ from each other in a base substitution (nucleotide position 297, exon 6) and a deletion (nucleotide position 1059, exon7), which leads to the loss of a stop codon and consecutive shift of the reading frame and results in an *ABO*A2.01* glycosyltransferase prolonged by 21 amino acids at the C-terminus (Yamamoto et al. 1992). Its enzymatic activity is weakened 5 to 10 times, thus the antigenic density of *ABO*A2.01* RBC is only 20% of the *ABO*A1.01* antigenic density on RBC (Schachter and Michaels 1971).

The *ABO*O.01.02* allele (= $O1^v$) arises through a deletion at position 261 in the *ABO*A1.01* DNA sequence, resulting in the generation of a premature stop codon. The *ABO*O.01.01* allele (= O1) is formed subsequently through interallelic exchange between *ABO*A1.01* and *ABO*O.01.02* (Roubinet et al. 2004). These alleles encode truncated proteins with no catalytic domain.

In contrast to the nonsense mutations generating *ABO*O.01.02* and *ABO*O.01.01*, a single missense mutation (base substitution in position 802, exon 7) generating allele *ABO*O.02* (= O2) has an inactivating effect on the *ABO*A1.01* glycosyltransferase albeit lacking 261delG (Yamamoto et al. 1993). In *ABO*O.02*, arginine in position 268 blocks the donor-GalNAc binding site of the A-glycosyltransferase (Lee et al. 2005). The eventuality that non-deletional O alleles can likely produce small amounts of the A antigen cannot yet be ruled out (Seltsam et al. 2005; Yazer et al. 2008). If *ABO*O.02* is not considered in genotyping, one might mistake genotype *ABO*O.02*/*O.01* for *ABO*A1.01*/*O.01*.

The H acceptor substance is synthetized by a α1→2 fucosyltransferase encoded through the FUT1-gene on chromosome 19. The dominant H allele is necessary for the expression of the α1→2 fucosyltransferase, however, in rare cases the FUT1-gene is homozygous h and no fucosyltransferase, and accordingly, no acceptor substrate for A- and B- glycosyltransferases will be synthesized, which results in the Bombay phenotype without any H-substance and hence no ABO antigens (Morgan and Watkins 1969).

Approximately 80% of the Caucasian population also has ABO antigens in soluble form in plasma and other body fluids (Kelly et al. 1995). Indispensable presupposition is the secretion of H-substance in body fluids, which is controlled through FUT2, a gene

closely related to FUT1. Individuals who inherit the dominant allele FUT2 Se are called secretors; Se is dominant over non-secretor Se (Morgan and Watkins 1969).

The ABO system meets the criteria for definition as a blood group because natural antibodies, isohemagglutinins, are built as a result of immunization against A-and B-substances on bacteria (Ahlstedt et al. 1977; Springer et al. 1959) during the settlement and construction of intestinal microbiota. Therefore, neonatal IgM isohemagglutinins are first detected at the age of three months and reach adult titers within the age of 5 to 10 years (Maur et al. 1993). This immunization occurs only against the ABO phenotypes that the neonate itself lacks. AB individuals therefore lack isohemagglutinins in their sera completely, while O individuals express antibodies against RBC phenotypes A and B, and individuals with Bombay phenotype even generate antibodies against the H-substance, reacting with all red blood cells regardless of ABO group.

1.10 ABO Antigens on Platelets

On platelets, the blood group A and B determinants are expressed on various glycoproteins (e.g. GPIb, GPIIa, GPIIb, GPIIIa, GPIV, GPV, CD109), PECAM and glycosphingolipids (maintained from the time as megakaryocyte, newly synthetized, passively adsorbed from plasma to a minor extent). The *ABO*A1.01* antigen expression on adult platelets varies widely, while *ABO*A2.01* and B determinants almost always demonstrate a minimal level of expression. (Cooling et al. 2005; Curtis et al. 2000; Farias et al. 2016; Hou et al. 1996; Kelton et al. 1998; Moureau and Andre 1954; Santoso et al. 1991; Skogen et al. 1988; Stockelberg et al. 1996).

In most cases, the platelet *ABO*A1.01* antigenic density is so low that ABO-incompatible platelet transfusions are thought to not have much impact on the duration of platelet survival (Curtis et al. 2000).

However, approximately 7% of adults with the *ABO*A1.01* phenotype and 4% of adults with the B phenotype are "high expressers" (HXP) for their ABO determinants on platelets (Curtis et al. 2000; Ogasawara et al. 1993), defined through an increase of the antigenic density beyond two standard deviations. Respectively, approximately 5% of A and B phenotype adults are “low expressors” (O'Donghaile et al. 2020).

In the case of HXP type 1, the antigenic density is three times higher than average, and for HPX type 2, seven times higher than average. In HXP, *ABO*A1.01* antigens on platelets are mainly expressed on GPIIb and PECAM; as a consequence, incompatible transfusions can also affect the duration of platelet survival (Curtis et al. 2000).

The *ABO*A1.01* antigenic density on HXP RBC increases the H antigen expression drops to the same effect, but on platelets, the H antigen seems much more strongly expressed than the A antigen. Cooling et al. speculated that the ABO antigen expression on platelets depends more on the activity of the fucosyltransferase FUT1, proposing that the H antigen on platelets is expressed on sterically hidden, complex carbohydrates. The ABO glycosyltransferase may not modify these residues to *ABO*A1.01* antigens (Cooling et al. 2005). The H-to-A antigen expression ratio on platelets appears to be directly related to the genotype of the individual, with *ABO*A1.01*/*A1.01* individuals showing a lower H-to-A ratio than for example *ABO*A1.01*/*O.01* individuals (DeLelys et al. 2013; Xu et al. 2019). O'Donghaile et al. concluded that the *ABO*A1.01*/*A1.01* genotype must be one of the major determinants of ABO high-expresser trait (O'Donghaile et al. 2020), while factors such as epigenetic effects, ABO gene transcription rates and messenger RNA stability may also contribute to the expression traits and explain the variety of antigen expression among individuals of the same genotypes (DeLelys et al. 2013).

The HXP phenotype, among others, is of importance, with Curtis et al. reporting a case of FNAIT with an RBC phenotype O mother and a Type 2 HPX child with RBC phenotype B. No other antibodies than anti-B were detectable. They may have induced FNAIT despite the competition of antibody binding through erythrocytes and other tissues (Curtis et al. 2008). Likewise, Kato et al. presented a case where FNAIT was possibly induced by maternal high-titer anti-A antibodies. Anti-HPA or anti-HLA were excluded serologically. Unfortunately, these authors were not able to examine the A antigen expression on the infant´s platelets because of its death due to severe ICH, but maternal anti-A reacted strongly with paternal platelets. (Kato et al. 2013).

The ISBT (International Society of Blood Transfusion) terminology (Table 1) is used throughout this paper. Since further discrimination between *ABO*O.01.01* and *ABO*O.01.02* was not implemented, these alleles were summarized as *ABO*O.01*.

Table 1: ABO terminology

Phenotype	ISBT 001 (version v1.1 170123) [1]	dbRBC/ BGMUT[2]	Traditional designation
A1	*ABO*A1.01*	A101	A^1
A2	*ABO*A2.01*	A201	A^2
B	*ABO*B.01*	B101	B
O	*ABO*O.01.01*	O01	O^1
	*ABO*O.01.02*	O02	O^{1v}
	*ABO*O.02.01*	O03	O^2

1.11 Hypothesis and Aim of the Study

FNAIT is a disorder which, in severe cases, can proceed to fetal/neonatal death or neurologic sequelae. Non-invasive monitoring of the fetal platelet count is not possible. Prophylaxis of fetal bleeding during pregnancy itself is not harmless either and cannot be applied to all pregnant women at risk. Thus far, a predictor for FNAIT severity, which may be relied on in order to find the necessary intensity of therapy with the least significant side-effects, does not yet exist, especially not for antenatal management in pregnant women with a history of FNAIT in a previous pregnancy. An FNAIT screening program in the general population of pregnant women would also require reliable prediction of the fetal bleeding risk to avoid overtreatment.

In hemolytic disease of the fetus and newborn (HDFN), ABO incompatibility between mother and fetus has a profound effect on maternal immunization to fetal red blood cell antigens (Bowman 1997; Levine 1959; Zwiers et al. 2018).

It is hypothesized that the ABO group of the mother and ABO incompatibility between pregnant mother and fetus may be associated with the incidence of anti-HPA-1a immunization and/or may represent a disease-modifying factor (Ahlen et al. 2012; Gratwohl and Shulman 1977).

The aim of this study is to verify whether there is a genetic association between ABO incompatibility between mother and fetus, and the incidence and severity of FNAIT. To this purpose, maternal and fetal/neonatal ABO blood groups are determined.

[1] International Society of Blood Transfusion

[2] Blood Group Antigen Gene Mutation Database, Patnaik et al. (2012).

In the course of blood group examination, the proposed correlation between maternal ABO blood groups and FNAIT severity (Ahlen et al. 2012) is investigated and a possible association between fetal/neonatal ABO blood groups and FNAIT severity is tested.

The results of this study may enable refined prediction of maternal anti-HPA-1a immunization risk as well as prediction of fetal bleeding risk in already immunized mothers. Furthermore, results may be informative for the development of prepartal and/or postpartal anti-HPA-1a immunoprophylaxis strategies in HPA-1bb pregnant women.

2 Material and Methods

2.1 Subjects

165 blood samples from mother-child pairs with a history of FNAIT living in Germany were collected by the Center for Transfusion Medicine and Hemotherapy of the Justus Liebig University Giessen between 2000 and 2015. The FNAIT diagnosis was based on maternal anti-HPA antibodies or HPA-1a antigen incompatibility in association with typical clinical data entries, including the neonatal platelet count (nadir) and the presence or absence of intracranial hemorrhage. Thrombocytopenia was defined as a platelet count $\leq 150 \times 10^9$/l and severe thrombocytopenia less than 50×10^9/l. The common denominator was the circumstance that neither the mother nor the unborn child received prenatal therapy in any form.

In average, maternal age upon childbirth was 30½ years and the affected child arose from the second pregnancy as well as second delivery.

Of the 165 affected children, 113 were male, 51 female and one intersex (at a later record classified as male). 15 children suffered from intracranial hemorrhage; data presented in table 2.

Table 2: Data of children suffering from ICH.

Maternal antibody	Maternal ABO genotype		Neonatal ABO genotype		ABO-incompatible	Neonatal PLT count nadir (/µl)	Birth weight (g)
	Allele 1	Allele 2	Allele 1	Allele 2			
Anti-HPA-1a	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	no	8000	2810
Anti-HPA-1a	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	no	3000	unknown
Anti-HPA-1a	*ABO*O.01*	*ABO*O.01*	*ABO*A1.01*	*ABO*O.01*	yes	21000	2275
Anti-HPA-1a	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	no	13000	unknown
Anti-HPA-1a	*ABO*O.01*	*ABO*O.01*	*ABO*A1.01*	*ABO*O.01*	yes	13000	unknown
Anti-HPA-1a	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	no	30000	2275
Anti-HPA-1a	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	no	4000	3205
Anti-HPA-1a	*ABO*A1.01*	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	no	5000	3680
Anti-HPA-1a	*ABO*A1.01*	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	no	45000	3000
Anti-HPA-1a	*ABO*A1.01*	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	no	11000	3270
Anti-HPA-1a	*ABO*A1.01*	*ABO*O.01*	*ABO*O.01*	*ABO*O.01*	no	16000	3320
Anti-HPA-1a	*ABO*A1.01*	*ABO*B.01*	*ABO*B.01*	*ABO*O.01*	no	6000	2230
Anti-HPA-1a	*ABO*B.01*	*ABO*O.01*	*ABO*B.01*	*ABO*O.01*	no	9000	2720
Anti-HPA-1a	*ABO*B.01*	*ABO*O.01*	*ABO*A1.01*	*ABO*B.01*	yes	26000	unknown
Anti-HPA-1a	*ABO*B.01*	*ABO*O.02*	*ABO*O.01*	*ABO*O.02*	no	5000	3420

All ICH-kids arose from HPA-1a incompatible pregnancies (the most common cause) and had severe thrombocytopenia. Most pregnancies were ABO-compatible.

In 125 cases, FNAIT was caused by maternal anti-HPA-1a (anti-PL^{A1}), including 4 cases caused by a combination with anti-HPA-5b (anti-Br^a) and one case in combination with anti-HPA-2b (anti-Ko^a), as well as one case combined with anti-A. 24 cases were caused by anti-HPA-5b (anti-Br^a) alone, 4 cases by anti-HPA-3a (anti-Bak^a) and 2 cases by anti-HPA-15a (anti-Gov^b). Anti-HPA-15b (anti-Gov^a), anti-HPA-2b (anti-Ko^a) and anti-HPA-8bw (anti-Sr^a) were responsible for one case each. The anti-HPA antibodies were detected using MAIPA (monoclonal antibody immobilization of platelet antigen assay).

In 7 cases, FNAIT was clinically diagnosed but no antibodies were detectable. In these cases, however, fetomaternal HPA-1a incompatibility was confirmed through HPA-genotyping of mother and child.

The Ethics Committee of the Medical Faculty at the Justus Liebig University in Giessen, Germany, officially approved the use of all human material (vote 21.07.2009, docket file nr. 82/09).

2.2 Materials and Manufacturer

2.2.1 Reagents and buffer

Table 3: Reagents, in alphabetical order.

Affinity Pure Goat Anti-Mouse IgG, Fc8 Fragment Specific	Jackson ImmunoResearch Laboratories, INK, Pennsylvania, USA (ordered via: DIANOVA GmbH, Hamburg, GER)
Aqua destillata	Baxter Deutschland GmbH, Unter-Schleißheim, GER
BSA (Bovine Serum Albumine 22%)	Ortho Clinical Diagnostics GmbH, Neckargemünd, GER
C$_6H_8O_7$ x H_2O (citric acid monohydrate)	Merck KGaA, Darmstadt, GER

$CaCl_2$ (calcium chloride dihydrate)	Sigma-Aldrich Chemie GmbH, München, GER
EZ1® DNA Blood 350 µl Kit	Qiagen GmbH, Hilden, GER, REF
H$_2O_2$ (hydrogen peroxide)	Merck KGaA, Darmstadt, GER
H_2SO_4 (sulphuric acid (2.5 N))	Merck KGaA, Darmstadt, GER
Isotonic saline solution 0.9% Braun injection solution	B. Braun Melsungen AG, Melsungen, GER
NABO*A2.01CO_3 (sodium carbonate)	Merck KGaA, Darmstadt, GER
NABO*A2.01HPO_4 x 12 H2O (sodium hydrogen phosphate)	Merck KGaA, Darmstadt, GER
$NaHCO_3$ (sodium hydrogen carbonate)	Merck KGaA, Darmstadt, GER
NaN_3 (sodium acid)	Merck KGaA, Darmstadt, GER
Nuclease-free water	Promega, REF: P119E, USA
OPD-tablets (2 mg Orthophenylenediamine)	Kem-En-Tec Diagnostics A/S, Taastrup, DK
PBS (Phosphate Buffered Saline (1x))	PAA Laboratories GmbH, Cölbe, GER
Peroxidase-conjugates Affinity Pure Goat Anti-Human IgG Fc8 Fragment Specific	Jackson ImmunoResearch Laboratories, INK, Pennsylvania, USA (ordered via: DIANOVA GmbH, Hamburg, GER)
Probes for ABO genotyping	Applied Biosystems by Thermo Fisher Scientific, LSG Strategic Oligo Solutions, California, USA
TaqMan® Universal 2× PCR Master Mix	Applied Biosystems, Life Technologies LTD, Warrington, UK
TRIS PUFFERAN® ≥99.3%, Buffer Grade (Tris-(hydroxymethyl)-aminomethan)	Carl Roth GmbH + Co. KG, Karlsruhe, GER
Triton™ X-100	Sigma-Aldrich Chemie GmbH, München, GER
Tween^{20} ®	Sigma-Aldrich Chemie GmbH, München, GER

Table 4: Buffer, in alphabetical order.

Buffer	Components
Coating buffer (durability: 14 d)	1.59 g NABO*A2.01CO_3 2.93 g $NaHCO_3$ 0.2 g NaN_3 dissolve in 1000 ml distilled water (target pH 9.6)
PBS/BSA 2%	10 ml Phosphate Buffered Saline (PBS) 1 ml 22% Bovine Serum Albumine (BSA)
Substrate buffer peroxidase (durability: 3 months)	3.65 g $C_6H_8O_7$ x H_2O 11.94 g NABO*A2.01HPO_4 x 12 H_2O bring volume up to 500 ml with distilled water (target pH 5.0), take 7.5 ml of this solution and add 3 OPD tablets and 7.5 µl H_2O_2
Trisbuffer / washing buffer (durability: 3 months)	3.63 g Tris, dissolve in 3 l isotonic saline solution (target pH 7.4) 15 ml Triton x 100 15 ml $Tween^{20}$ 1.5 ml 1m$CaCl_2$

2.2.2 Consumables

Table 5: Consumables, in alphabetical order.

MicroAmp® Fast 96-Well Reaction Plate	Applied Biosystems, Life Technologies, Beijing, China
MicroAmp™ Optical Adhesive Film	Life Technologies Corporation, Carlsbad, California, USA
Microtiter tray (high binding capacity, F-form)	Greiner Bio One International GmbH, Kremsmünster, GER
Pipettes tips: 10 µl, achromatic 200 µl, yellow 1000 µl, blue Biozym tips, achromatic, 10 µl	 Sarstedt, Nürnbrecht, GER Sarstedt, Nürnbrecht, GER Sarstedt, Nürnbrecht, GER Biozym Scientific GmbH,

	Hess. Oldendorf, GER
Filter Mikro reach low binding, 10 µl	Biozym Scientific GmbH,
	Hess. Oldendorf, GER
Filter Tip XL Low Binding, 200 µl	Biozym Scientific GmbH,
	Hess. Oldendorf, GER
Filter Tip, 1250 µl	Biozym Scientific GmbH,
	Hess. Oldendorf, GER
Reaction tubes:	
0.5 ml	Sarstedt, Nürnbrecht, GER
1.5 ml	Sarstedt, Nürnbrecht, GER
1.5 ml DNA LoBind Tubes	Eppendorf AG, Hamburg, GER

2.2.3 Hardware

Table 6: Hardware, in alphabetical order.

Centrifuges:	
Biofuge pico	Heraeus Instruments GmbH,
	Hanau, GER
Rotina 380	Hettich Zentrifugen, Mühlheim a.d.
	Ruhr, GER
DNA extraction:	Qiagen Instruments AG,
Bio Robot EZ1 Advanced XL	Hombrechtikon, CH
pH measurement:	Wissenschaftlich-Technische
inoLab pH Level 1	Werkstätten GmbH, Weilheim, GER
Photometer:	
NanoDrop® ND-1000	NanoDrop Technologies, Wilmington,
	USA
Tecan Sunrise	Tecan Austria GmbH, Grödig, Austria
Pipettes:	
Proline® Plus 0.5-10 µl	Sartorius AG, Göttingen, GER
Eppendorf Reference (0.5-10 µl)	Eppendorf AG, Hamburg, GER
Eppendorf Reference (10-100 µl)	Eppendorf AG, Hamburg, GER

Eppendorf Research (0.5-10 µl) Eppendorf Research (10-100 µl) Eppendorf Research (100-1000 µl) Starlab MicroOne® Eppendorf Multipette Eppendorf Titerman 4908	Eppendorf AG, Hamburg, GER Eppendorf AG, Hamburg, GER Eppendorf AG, Hamburg, GER Starlab GmbH, Ahrensburg, GER Eppendorf AG, Hamburg, GER Eppendorf AG, Hamburg, GER
StepOnePlus™ Real-Time PCR System	Applied Biosystems, Life Technologies GmbH, Darmstadt, GER
Vortexer: MS2 Minishaker IKA Reax 2000 Sea Star Vortexer All-in-one	 Heidolph Instruments GmbH & Co.KG, Schwabach, GER Biozym Scientific GmbH, Hessisch Oldendorf, GER

2.2.4 Software

Table 7: Software, in alphabetical order.

IBM SPSS Statistics Version 25 for Windows	IBM Deutschland GmbH, Ehningen, GER
MAGELLAN V7.2 TRA. 2PC PAC SUNRISE	Tecan Austria GmbH, Grödig, Austria
Microsoft® Excel 2010	Microsoft Corporation, Redmond, USA
Microsoft® Office 365 Word	Microsoft Corporation, Redmond, USA
NanoDrop-1000 V. 3.8.1	NanoDrop Technologies, Wilmington, USA
Prism 8, GraphPad Inc.	GraphPad Software, La Jolla, California, USA
StepOnePlus™Software v2.3	Thermo Fisher Scientific, Life Technologies GmbH®2012, Darmstadt, GER

2.3 MAIPA

The verification of anti-HPA was performed serologically via indirect monoclonal antibody immobilization of platelet antigens (MAIPA); after incubation of test platelets with maternal serum, the relevant glycoproteins were able to be immobilized by adding monoclonal murine antibodies. Maternal and murine antibodies and HPA form a stable trimolecular complex, which was liberated during platelet solubilization and afterwards fixated via a murine antibody on a microtiter tray coated with polyspecific anti-murine-antibodies. By adding enzyme-labelled anti-human-IgG, the antibody complex with the maternal antibody was visualized through substrate reaction. This cross-match procedure allowed the detection of fetal-maternal incompatibilities beyond the frequent HPA-1 and -5 and of HLA antibodies. Prerequisite was the existence of monoclonal antibodies for all the relevant glycoproteins (Table 8).

Table 8: Monoclonal murine antibodies used for MAIPA.

Specificity	Clone	Isotype	Form	Sales
GP IIb IIIa	Ü Gi 5		Liquid	Dr. Santoso
GP Ia IIa	Ü Gi 9		Liquid	Dr. Santoso
GP Ib IX CD42a	FMC25	IgG1	Liquid	Serotec
GP V CD 42d	SW16	IgG1	Liquid	CLB
HLA Cl. I	ABO* B.01G6	IgG2a	Purified	Immunotech

The MAIPA was conducted by employees of the laboratory from the Center for Transfusion Medicine and Hemotherapy.

Test platelets were isolated from EDTA-coagulated blood through cell-fractioning, washed three times and stored at 4 °C for at least 12 hours in isotonic saline solution. Samples, not stored on ice, were examined within 2 days after blood drawing. Examination of hemolytic samples was not possible.

A microtiter tray was coated with 100 µl buffer per well (consisting of coatingbuffer and anti-Mouse-IgG in a ratio of 1:500) and incubated for at least 120 minutes at 37 °C or allowed to rest for 24 hours at room temperature.

20 million test platelets (in one reaction tube) were centrifuged (1 min, 10000 rpm) and resuspended in 30 µl PBS 2% BSA immediately after the supernatant was discarded. Serum/platelet-free plasma was also centrifuged (1 min, 10000 rpm), then 20 – 50 µl were added to the platelets and the mixture was incubated for 30 minutes at 37 °C. After a wash step with 100 µl isotonic saline solution and centrifugation (1min, 10000 rpm), the supernatant was again discarded and the platelets were again resuspended in 30 µl PBS 2% BSA. After adding 10 µl of monoclonal mouse antibodies, the mixture was incubated for 30 minutes at 37 °C and then washed three times in 100 µl isotonic saline solution, then centrifugation was repeated (1 min, 10000 rpm) and the supernatant discarded. For platelet lysis, the remaining platelet pellet was resuspended in 100 µl solubilisation buffer (containing Tris-buffered saline and Triton x 100 in the same ratio as the washing buffer), incubated for 30 minutes at 4 °C and then centrifuged for 30 minutes at 4 °C (13000 rpm). Afterwards, 50 µl supernatant was carefully taken (without stirring up insoluble platelet residues) and diluted with 200 µl washing buffer.

During solubilization time, the microtiter tray was washed four times (with 200 µl washing buffer / well) and blocked for 15 minutes at 4 °C (with 100 µl washing buffer / well). Subsequently, the microtiter tray was beaten and 100 µl of the diluted supernatant was added in each well, then the tray was incubated at 4 °C for 90 minutes. After this, the tray was washed again four times with 200 µl washing buffer / well. 100 µl peroxidase labelled Affinity Pure Goat Anti-Human IgG was added per well. After incubation for 120 minutes at 4 °C the tray was washed again in the usual manner (4x, 200 µl washing buffer / well) to remove excess antibodies. Then 100 µl substrate puffer per well was added and the tray was incubated in the dark for 10 minutes at room temperature. Finally, the color reaction was stopped with 50 µl 2.5 N H_2SO_4.

Using a Photometer (Sunrise™, Tecan), the extinction was measured at 450 and 620 nm. Each extinction and a blank value (distilled water) was measured twice. The single extinction values should not differ by more than 20%. The patients mean value of extinction was subtracted from the mean blank value, and the resulting value of optical density (OD-value) was interpreted according to table 9. A positive control for each monoclonal murine antibody was also included.

Table 9: Interpretation of OD-Values.

Assessment of OD-Values	Altitude OD-Value
Negative	< 0.15
Borderline (+)	0.15-0.20
Weakly positive +	0.21-0.40
Moderately positive ++	0.41-0.80
Explicitly positive +++	0.81-1.20
Strongly positive ++++	> 1.21

HPA-genotyping was performed by employees of the laboratory at the Center for Transfusion Medicine and Hemotherapy using TaqMan® real time PCR.

2.4 DNA Extraction

DNA was isolated from whole blood and buffy coats using the "Bio Robot EZ1 Advanced XL" and its proven EZ1® DNA Blood 350 µl Kit. The DNA extraction was carried out as specified by the manufacturer, using the preprogramed protocol on EZ1 Advanced XL Cards. After arranging the disposable filter-tips and tip-holders, elution tubes and the sample tubes (filled with 350 µl EDTA-coagulated blood) and loading the Bio Robot with the prefilled cartridges (Geno Prep Cartridge B 350, Qiagen) containing all required reagents, the instrument prepared the DNA extraction automatically. After the cell lysis and in the presence of a chaotropic salt, which keeps the proteins denatured and soluble, the DNA bound to the silica surface of magnetic particles. Using a magnet, these particles were separated from the lysates and the DNA was washed and eluted in a 200 µl elution volume in the elution tubes. With this set-up, 14 samples can be processed in approximately 20 minutes (EZ1 DNA Blood Handbook 04/2010).

2.5 Measurement of DNA Concentration

The DNA concentration was measured using the NanoDrop®ND-1000 Spectrophotometer. After 2 µl samples were placed between two fiber optic cable ends, a ray of light from a pulsed xenon flash lamp was analyzed by the spectrometer after passing through the sample. The sample absorbance at a given wavelength was calculated relative to the initial absorbance of distilled water set as a baseline value. If the ratio of sample absorbance at 260 and 280 nm was lower than 1.8, the DNA sample was considered pure. The calculated absorbance was then correlated with the concentration using a modified Beer-Lambert equation with an extinction coefficient for double stranded DNA of 50 ng/ml. The ND-1000 software V.3.8.1 run from a PC was used to control the instrument. A template volume of 2 µl contained enough DNA for a TaqMan® real time PCR.

2.6 ABO Genotyping

Maternal and fetal ABO genotyping was performed with a TaqMan® real time PCR assay to detect the major ABO alleles *ABO*A1.01*, *ABO*A2.01*, *ABO*B.01*, *ABO*O.01* (O^1/O^{1v}) and *ABO*O.02* (O^2). Further discrimination between *ABO*O.01.01* and *ABO*O.01.02* was not implemented.

To identify the mentioned ABO alleles, 4 SNP sites were selected (Table 10) and detected by customized primers and probes.

Table 10: The selected SNP sites in the mentioned ABO alleles.

SNP site	*ABO*O.01.01, ABO*O.01.02*	*ABO*B.01*	*ABO*O.02*	*ABO*A2.01*
rs-number	77641731	8176743	41302905	56392308
Exon	6	7	7	7
Chr:bp	9:133257521	9:133256028	9:133255929	9:133255670
Codon	Val87/Thr88=fs	235	268	354
Nucleotide position	c.260/262insG	703	802	1061
*ABO**A1.01 (consensus)	G	G	G	C
*ABO**A2.01	G	G	G	**deletion**
*ABO**B.01	G	**A**	G	C
*ABO**O.01.01, *ABO**O.01.02	**deletion**	G	G	C
*ABO**O.02	G	G	**A**	C

Allele *ABO*A1.01* served as consensus sequence. It was only detected by exclusion of the other alleles.

Probes were stored at -15 to -25 °C. Samples were stored at -25 to -80 °C.

The TaqMan® probes were labelled with a fluorescent reporter dye at the 5′ end as well as a fluorescent quencher dye at the 3′ end (Table 11). Until the DNA-polymerase separated the 5′ reporter dye from the physical proximity to the quencher dye due to its 5′- 3′ exonuclease activity, the fluorescence of the reporter dye would not be emitted. Depending on which probe hybridized (the wildtype or the variation specific probe), different fluorescence signals were able to be detected and monitored in real time.

Table 11: Sequences, quencher and reporter molecules of TaqMan® probes used for the detection of the selected SNPs.

SNP	**Sequence of probes (P), quencher and reporter molecules**
*ABO*O.01.01, ABO*O.01.02*	P1: 5'-VIC-GCCTCGTGGTGCCCCTTGG-3'-MGB P2: 5'-FAM-GCCTCGTGGTACCCCTTGG-3'-MGB
*ABO*B.01*	P1: 5'-VIC-CCGTAGAAGCTGGGGTGCAGG-3'-MGB P2: 5'-FAM-CCGTAGAAGCCGGGGTGCAGG-3'-MGB
*ABO*O.02*	P1: 5'-VIC-CCGAAGAACCCCCCCAGGT-3'-MGB P2: 5'-FAM-CCGAAGAACCTCCCCAGGT-3'-MGB
*ABO*A2.01*	P1: 5'-VIC-AGCCGCTCACGGGTTCCGGAC-3'-TAMRA P2: 5'-FAM-AGCCGCTCAC*GGTTCCGGAC-3'-TAMRA

The target in each allele is underlined. Deletions are indicated with an asterisk. For alleles *ABO*O.01*, *ABO*O.02* and *ABO*A2.01* probe 1 always detected the allele-specific target and probe 2 detected *ABO*A1.01* consensus sequence and vice versa for allele *ABO*B.01*.

For each probe, a reaction mix was set up in 1,5 ml DNA LoBind Tubes. This reaction mix consisted of 10 µl TaqMan® Universal 2× PCR Master Mix, containing AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, Uracil-DNA Glycosylase, buffer components and a passive internal reference ROX™ dye, as well as 1µl assay mix (made up of primers and probes) and 7 µl nuclease-free water. After preparation, the reaction mix was vortexed for a few seconds.

The 2 µl DNA templates were pipetted in the according wells of a 96-well-plate following the plate layout in the StepOne™ software v2.3. By adding the reaction mix, the final reaction volume was brought up to 20 µl per well. Negative (NTC = no template control) and positive controls for each target were prepared in the same manner. The 96-well-plate was then covered with a MicroAmp™ Optical Adhesive Film.

Finally, thermal cycling was undertaken in a StepOne Plus™ Real-time PCR System using the following cycling procedure: 60 °C for 30 seconds, initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C annealing and elongation for 1 minute, concluding with 60 °C for 30 seconds.

Analysis of the results was again undertaken with the StepOne™ software v2.3 by examining the allelic discrimination plots (Figure 1) and following the evaluation scheme (Table 4).

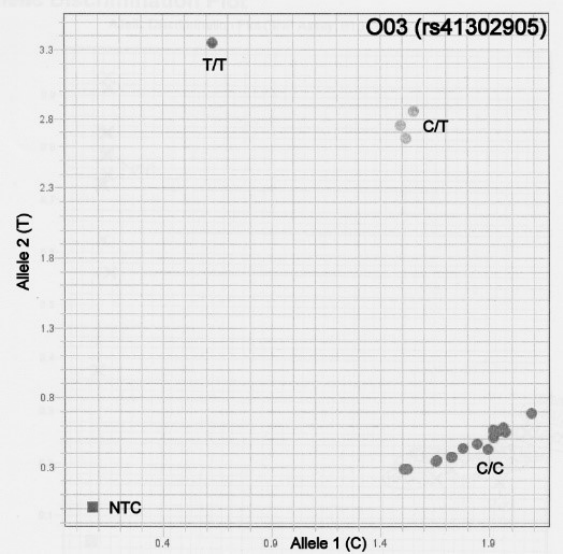

Figure 1: Allelic discrimination plot.

Exemplary portrayal of an allelic discrimination plot with three clusters (homozygous allele 1 = C/C, heterozygous allele 1/allele 2 = C/T, homozygous allele 2 = T/T), here illustrated for SNP ABO*O.02 (rs41302905).

Table 12: Evaluation scheme of ABO genotyping.

ABO genotypes	**Allele-specific target**			
ABO A1.01/A1.01*	**G , G**	**C , C**	**C , C**	**G , G**
*ABO*O.01/O.01*	**A , A**	C , C	C , C	G , G
*ABO*B.01/B.01*	G , G	**T , T**	C , C	G , G
*ABO*O.02/O.02*	G , G	C , C	**T , T**	G , G
ABO A2.01/A2.01*	G , G	C , C	C , C	*** , ***
*ABO*A1.01/O.01*	**G , A**	C , C	C , C	G , G
*ABO*B.01/O.01*	**G , A**	**C , T**	C , C	G , G
*ABO*A2.01/O.01*	**G, A**	C , C	C , C	**G , ***
*ABO*A1.01/B.01*	G , G	**C , T**	C , C	G , G
*ABO*A2.01/B.01*	G , G	**C , T**	C , C	**G , ***
*ABO*A1.01/O.02*	G , G	C , C	**C , T**	G , G
*ABO*O.01/O.02*	**G , A**	C , C	**C , T**	G , G
*ABO*B.01/O.02*	G , G	**C , T**	**C , T**	G , G
*ABO*A2.01/O.02*	G , G	C , C	**C , T**	**G , ***
*ABO*A1.01/A2.01*	G , G	C , C	C , C	**G , ***

The evaluation scheme depicts all 15 genotype patterns possibly resulting from the 5 detected alleles. Both maternal and paternal nucleotides are given for each SNP. Variations of the consensus sequence (gray background) defining the different genotypes are displayed with a light gray background. Deletions are again indicated with an asterisk.

In the case of discrepancies between serological ABO phenotypes and the determined genotypes, blood group genotyping was confirmed using a PCR with sequence-specific primers (PCR-SSP) or repeated using TaqMan-PCR, but with a 5 µl DNA sample instead of 2 µl and a reduced water fraction (from 7 µl to 4 µl) in each reaction mix.

2.7 Principle TaqMan® Real Time PCR

The intensity of the fluorescent signal was proportional to the generation of amplicons, thus, the more the signal increases, the farther away from the zero point the clusters will be depicted in the dot plot analysis. Therefore, a qualitative and quantitative detection at the same time was possible. The data was collected in the exponential phase of the PCR run, where the fresh products doubled precisely, assuming 100% reaction efficiency. In the following linear phase, the reaction slowed down due to consumption of reagents and, lastly, ended in the plateau phase, where no more amplicons were produced. The integration of uracil in the amplicons guaranteed the end of the reaction (ThermoFisher Scientific).

Traditional PCR takes measurements in this plateau phase ("endpoint detection"), but due to different reaction kinetics in each sample, the same initial concentrations would produce different amounts of amplicons. Furthermore, traditional PCR requires the detection and comparison of the amplified band to known standards in gel electrophoresis, which only gives off 'semi-quantitative' results. TaqMan PCR enabled the dropping of these time-consuming post-PCR steps and provides more accurate quantification (ThermoFisher Scientific).

Calculations reveal that the minimal number of probes needed to distinguish all 5 alleles –and thus all 15 resulting genotypes– is at least 8 (MinProb: Analysis and Optimization of Sets of Oligonucleotideprobes by Carlheinz Mueller, University of Ulm, Germany).

2.8 Statistical Analysis of Data

The collected results were documented together with other pseudonymized patient information in a Microsoft Excel database. Mr. Johannes Herrmann provided professional support and performed the first statistical analyses with IBM SPSS Statistics Version 25 for Windows. A p-value less than 0.05 was considered significant.

The graphical illustration was undertaken with GraphPad Prism 8.

Several case definitions were set up to avoid confounding effects of more than one antibody in mothers and to assess possible differences between different antibody specificities causing FNAIT:

- Case definition 1: FNAIT proven or possible (including cases of HPA-1bb mothers and HPA-1ab newborns without detectable anti-HPA-1a antibodies), n = 165.
- Case definition 2: Cases with any anti-HPA antibody specificity or more than one antibody and newborns positive for the corresponding antigen, n = 158.
- Case definition 3: All women who were HPA-1a-negative (HPA-1bb), had an HPA-1a-positive offspring and had anti-HPA-1a antibodies (n=118) or had no anti-HPA-1a antibodies (n=7).
- Case definition 4: All women who were HPA-1a-negative (HPA-1bb), had an HPA-1a-positive offspring and had anti-HPA-1a antibodies (n=118).
- Case definition 5: All women who were HPA-5b-negative (HPA-5aa), had an HPA-5b-positive offspring and had anti-HPA-5b antibodies (n=24).

Regarding influence of fetomaternal ABO incompatibility and ABO blood groups, focus was directed towards case definition 3 and 4, since HPA-1a antigen incompatibility is primarily responsible for FNAIT in Caucasians and ICH as marker for severity only occurred within case definition 4. Therefore, results are reported for case definition 1 to give an overview and for case definition 3 or 4. For case definition 5, most statistics were not able to be computed due to the small number of individuals. Therefore, only descriptive analysis was reported.

The ABO phenotype distribution among the immunized mothers and their children affected by FNAIT was compared to 45295 mainly German individuals within the local bone marrow registry by using a Chi-square (χ^2) test.

The proportion of ABO-incompatible and compatible pregnancies among cases was compared to the data of 522 mother-child-pairs with former suspicion of FNAIT.

Fetomaternal ABO incompatibility, maternal and neonatal phenotypes and allele features were correlated to the severity of FNAIT, defined by platelet count nadir, incidence of intracranial hemorrhage and birth weight of the affected children. Since

some genotypes occurred only rarely, statistics concerning the influence of the underlying genotypes were not performed.

Regarding the incidence of ICH, Fisher's exact tests and χ^2 tests (with continuity correction) were used to assess the effects of fetomaternal ABO incompatibility, maternal and neonatal ABO phenotypes and hetero- or homozygosity for ABO alleles. Regarding gene doses, only *ABO*A1.01* and *ABO*O.01* could be taken into consideration because for *ABO*O.02* and *ABO*B.01*, the number of homozygous individuals was not high enough for valid statistics.

Concerning the magnitude of neonatal thrombocytopenia, Mann-Whitney-U tests were implemented for the assessment of fetomaternal ABO incompatibility and hetero- or homozygosity for allele *ABO*A1.01* and *ABO*O.01*. A Kruskal-Wallis test was conducted to evaluate differences among ABO phenotypes.

Considering the infant's birth weight, data was only available for 96 (58%) of the 165 neonates. A t-test was implemented to assess the effects of fetomaternal ABO incompatibility and *ABO*O.01* allele dose and a Mann-Whitney-U test was performed for assessment of the *ABO*A1.01* allele dose. A Welch-ANOVA was conducted to assess the effect of ABO phenotypes followed by a Games-Howell post-hoc test to see which phenotypes differed significantly.

3 Results

3.1 Retrospective Case-Control Study

3.1.1 ABO phenotype frequencies do not differ between FNAIT cases and controls

The ABO phenotypes of mothers and children affected by FNAIT were deduced from ABO genotypes that are shown in table 13.

Table 13: Distribution of ABO blood group genotypes in mother-child-pairs with a history of FNAIT.

ABO phenotype	ABO genotype Allele 1	Allele 2	Mothers (n = 165)	Children (n = 165)
O	*ABO*O.01*	*ABO*O.01*	54	62
	*ABO*O.01*	*ABO*O.02*	6	4
	*ABO*O.02*	*ABO*O.02*	1	-
A_1	*ABO*A1.01*	*ABO*O.01*	48	44
	*ABO*A1.01*	*ABO*A1.01*	8	9
	*ABO*A1.01*	*ABO*A2.01*	2	6
	*ABO*A1.01*	*ABO*O.02*	2	2
A_2	*ABO*A2.01*	*ABO*O.01*	13	7
	*ABO*A2.01*	*ABO*O.02*	1	1
B	*ABO*B.01*	*ABO*O.01*	18	15
	*ABO*B.01*	*ABO*O.02*	3	2
	*ABO*B.01*	*ABO*B.01*	2	1
AB	*ABO*A1.01*	*ABO*B.01*	6	10
	*ABO*A2.01*	*ABO*B.01*	1	2

Genotype *ABO*A2.01/*A2.01* was not present in any subject.

ABO phenotype frequencies of all mothers and neonates were compared to the ABO phenotype frequencies among the control group (Table 14). The differences did not prove to be statistically significant.

Table 14: ABO phenotype distribution pictured for case definition 1.

ABO phenotype	Mothers (n = 165)	Children (n = 165)	Population (n = 45295)
O	36%	38%	41%
A*	46%	44%	42%
B	14%	11%	12%
AB	4%	7%	5%

Phenotype distribution mothers to controls: χ^2 test, p = 0.507

Phenotype distribution children to controls: χ^2 test, p = 0.585

* The subgroups A_1 and A_2 were aggregated to phenotype A.

The same holds true for the subgroup with case definition 3 (FNAIT due to anti-HPA-1a antibodies). ABO phenotype frequencies did not differ between cases and controls (Table 15).

Table 15: ABO phenotype frequencies for case definition 3.

ABO phenotype	Mothers (n = 124)	Children (n = 124)	Population (n = 45295)
O	35%	39%	41%
A*	47%	44%	42%
B	13%	10%	12%
AB	5%	7%	5%

Phenotype distribution mothers to controls: χ^2 test, p = 0.670

Phenotype distribution children to controls: χ^2 test, p = 0.720

* The subgroups A_1 and A_2 were aggregated to phenotype A.

3.1.2 Fetomaternal ABO incompatibility does not protect against immunization to fetal platelet antigens by pregnancy

ABO-incompatible pregnancies are defined as pregnancies in which the mother has isohemagglutinins directed against fetal ABO blood group antigens (Table 16).

Table 16: Compatible and incompatible fetomaternal ABO blood group combinations.

Maternal blood group	Fetal blood group	
	compatible	incompatible
O	O	A, B, AB
A	A, O	B, AB
B	B, O	A, AB
AB	O, A, B, AB	-

The proportion of ABO-incompatible pregnancies in cases was similar to our control population composed of 522 mother-child-pairs with FNAIT excluded (Figure 2):

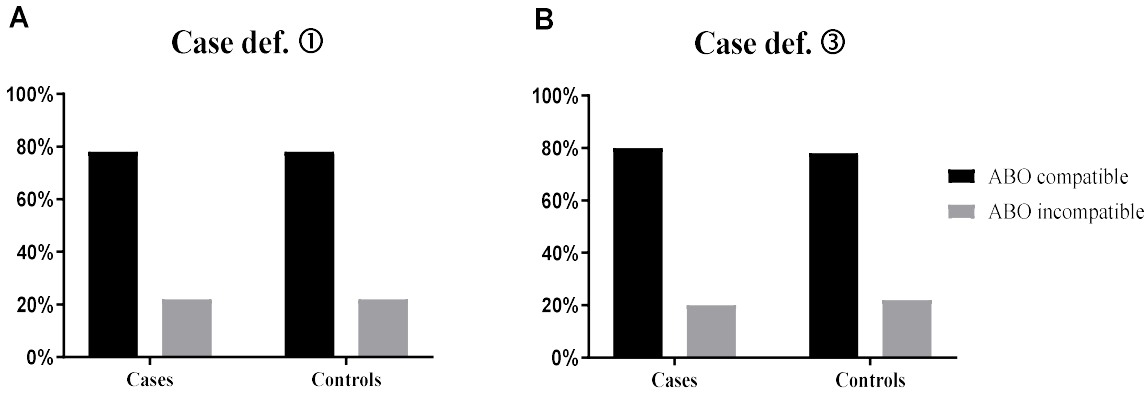

Figure 2: Distribution of ABO-compatible and ABO-incompatible pregnancies

Distribution of ABO-compatible and ABO-incompatible pregnancies for case definitions 1 (all FNAIT cases; illustration A) and 3 (FNAIT cases due to anti-HPA-1a antibodies, illustration B) among cases and controls (χ^2 test, $p > 0.05$).

3.2 Retrospective Cohort Study

3.2.1 Fetomaternal ABO incompatibility and FNAIT severity

3.2.1.1 Fetomaternal ABO incompatibility is not associated with fetal/neonatal ICH

The incidence of ICH in neonates suffering from FNAIT was compared between ABO-compatible and ABO-incompatible pregnancies. No association between ABO incompatibility and ICH was observed (Figure 3). No ICH was observed in case definition 5 (suspected FNAIT, detection of anti-HPA-5b antibodies).

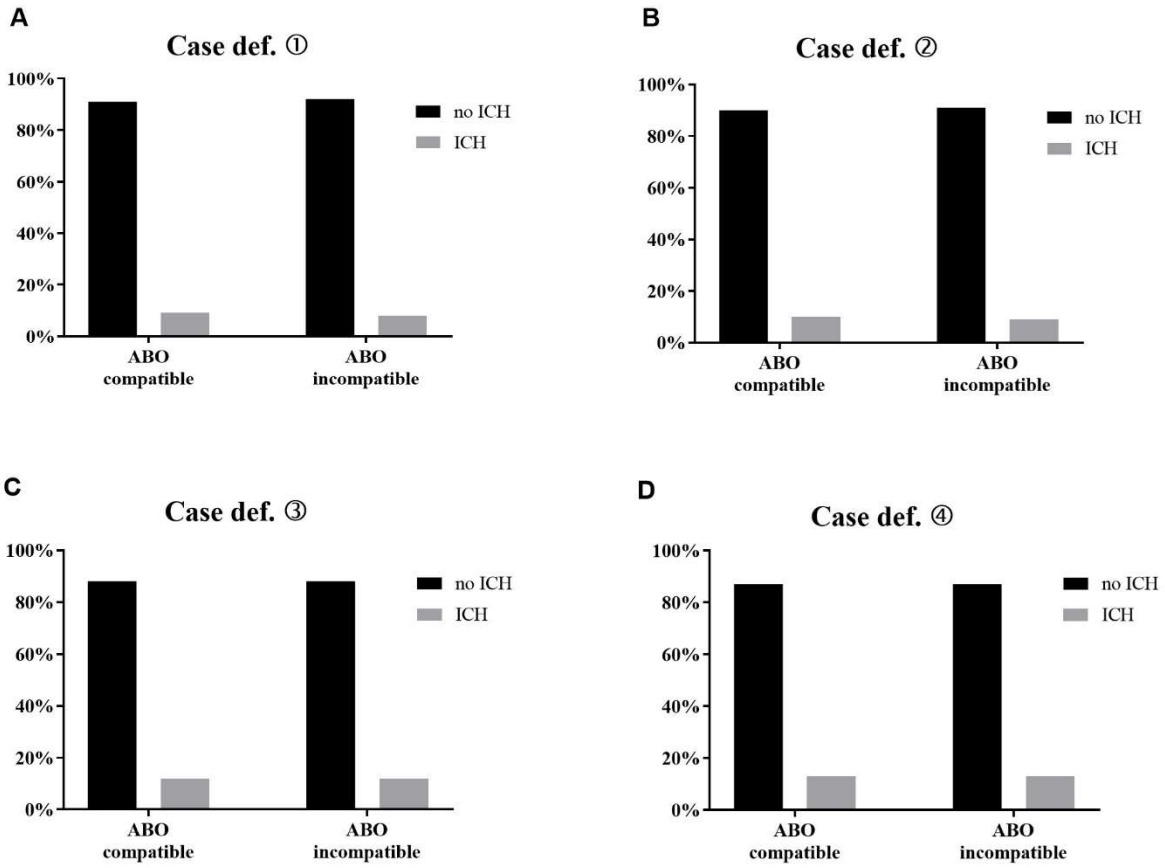

Figure 3: Fetomaternal ABO incompatibility and neonatal ICH incidence.

Comparison of the incidence of ICH in neonates suffering from FNAIT among ABO-compatible and ABO-incompatible pregnancies for case definitions 1 – 4 (illustrations A – D).

A) n = 165, two-sided Fisher's exact test, p = 1.000
B) n = 157, two-sided Fisher's exact test, p = 1.000
C) n = 124, two-sided Fisher's exact test, p = 1.000
D) n = 118, two-sided Fisher's exact test, p = 1.000

3.2.1.2 Fetomaternal ABO incompatibility is not associated with neonatal platelet count nadir

There was no significant difference between ABO-compatible or incompatible pregnancies regarding the magnitude of neonatal thrombocytopenia (Figure 4). Case definition 5 not illustrated due to low number of individuals (n = 21, Mann-Whitney-U test, p = 0.052).

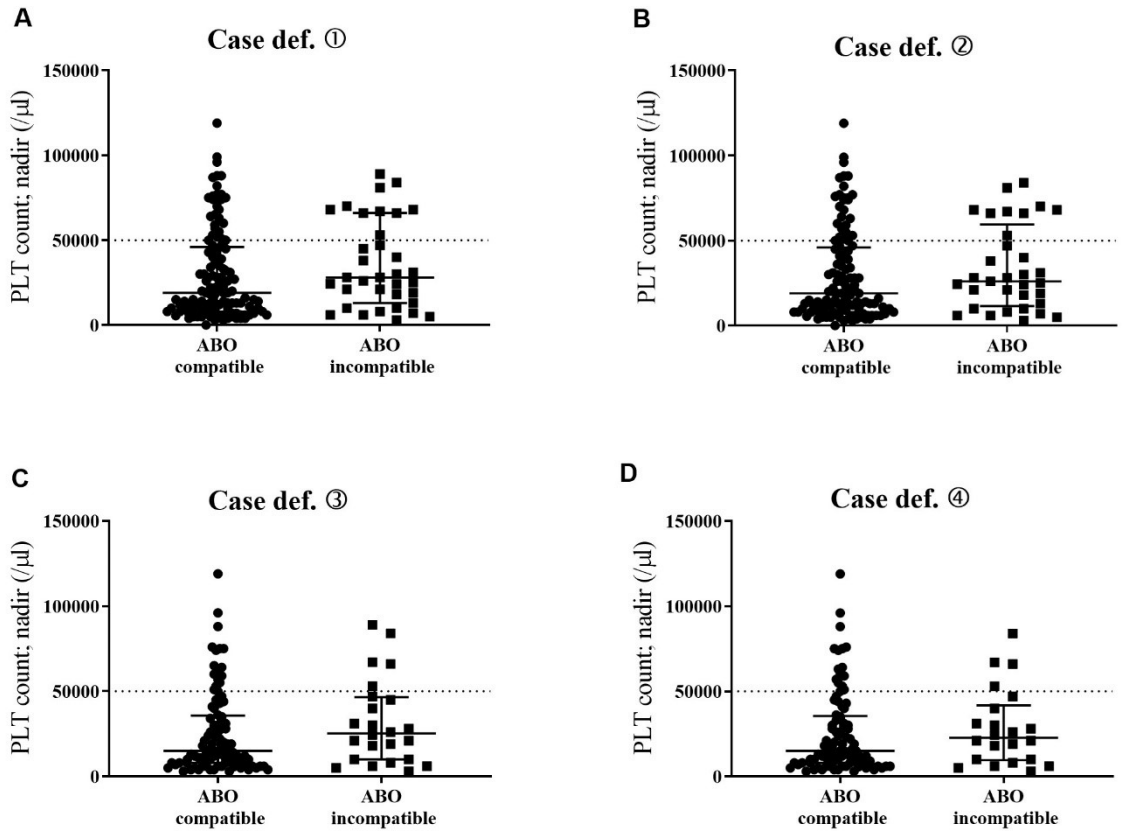

Figure 4: Fetomaternal ABO incompatibility and neonatal platelet count nadir.

Comparison of platelet counts nadir in neonates suffering from FNAIT among ABO-compatible and ABO-incompatible pregnancies for each case definition (illustrations A – D). Dotted line threshold of severe FNAIT (PLT count <50000/µl). Interquartile range and median displayed.

A) n = 156, Mann-Whitney-U test, p = 0.113
B) n = 150, Mann-Whitney-U test, p = 0.212
C) n = 120, Mann-Whitney-U test, p = 0.190
D) n = 115, Mann-Whitney-U test, p = 0.401

3.2.1.3 Fetomaternal ABO incompatibility is not associated with neonatal birth weight

No significant differences between ABO-compatible or incompatible pregnancies regarding the neonatal birth weight were found (Figure 5). Case definition 5 not illustrated due to low number of individuals (n = 12, t-test, p = 0.809).

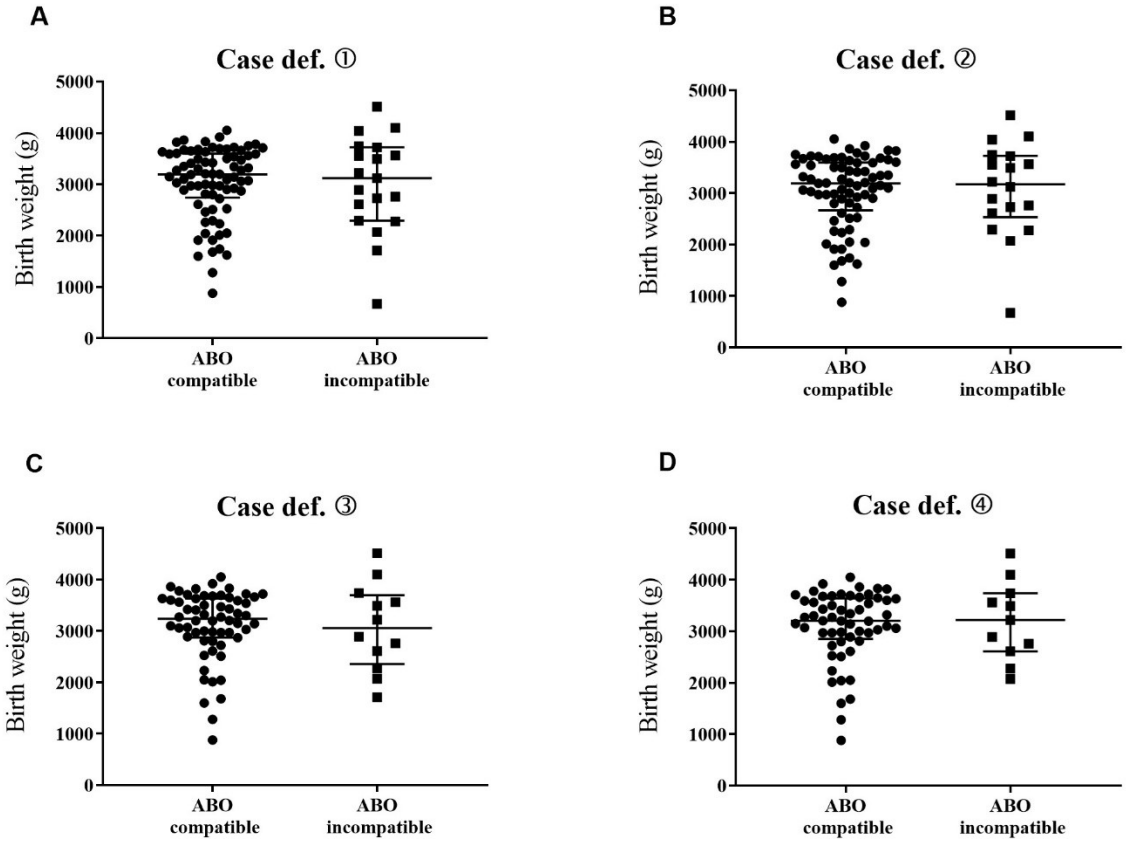

Figure 5: Fetomaternal ABO incompatibility and neonatal birth weight.

Comparison of birth weight in neonates suffering from FNAIT among ABO-compatible and ABO-incompatible pregnancies for each case definition (illustrations A – D). Interquartile range and median displayed.

A) n = 95, t-test, p = 0.836
B) n = 91, t-test, p = 0.799
C) n = 72, t-test, p = 0.866
D) n = 68, t-test, p = 0.654

3.2.1.4 Fetomaternal incompatibility for blood group A_1 is not associated with FNAIT severity

The A antigen is only barely detectable on platelets from A_2 donors (Curtis et al. 2000). Thus, we analyzed the subgroup of mothers with anti-A antibodies and neonates with phenotype A_1. According to this definition of ABO incompatibility, FNAIT severity did not differ between ABO-incompatible and compatible pregnancies (Figure 6).

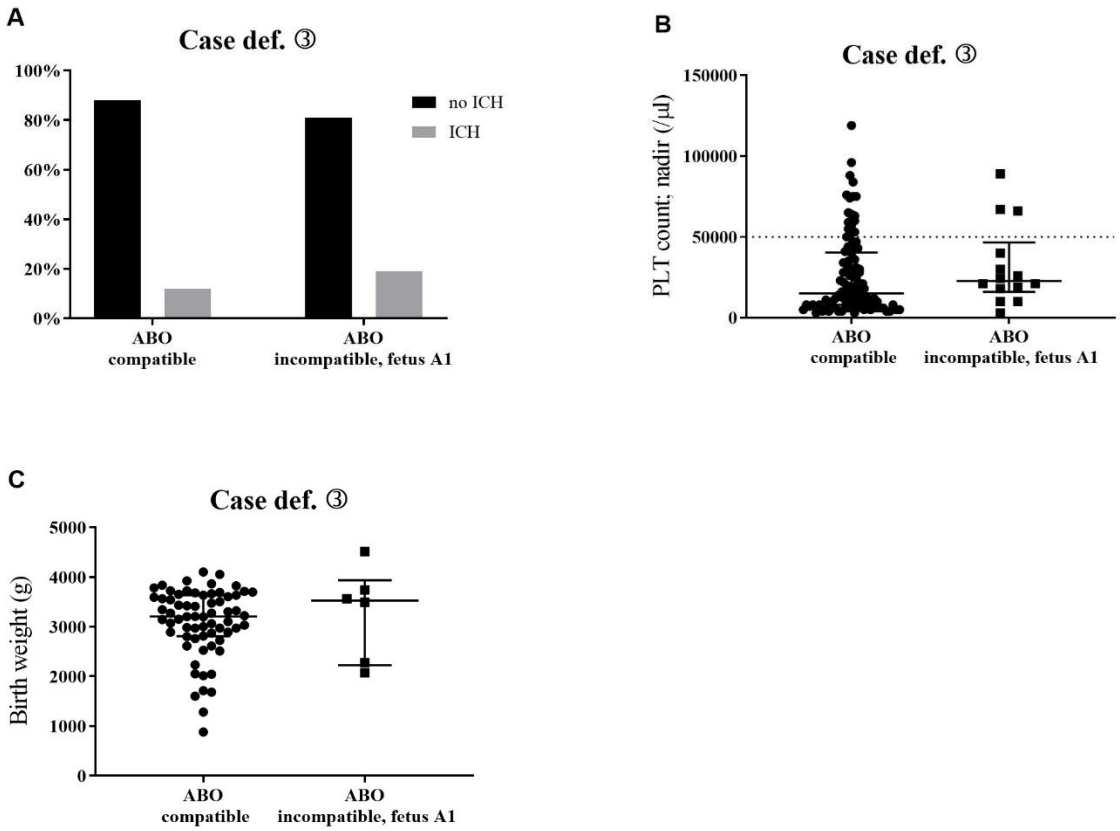

Figure 6: Association of platelet-adjusted ABO incompatibility and FNAIT severity.

Pictures A – C provide an overview on the influence of platelet-adjusted ABO incompatibility on neonatal ICH, platelet count nadir and birth weight for case definition 3. Interquartile range and median are displayed.

A) n = 124, two-sided Fisher's exact test, p = 1.000
B) n = 120, Mann-Whitney-U test, p = 0.223
C) n = 72, t-test, p = 0.539

3.2.2 Maternal ABO phenotypes and FNAIT severity

3.2.2.1 Maternal ABO phenotypes are not associated with neonatal ICH

The comparison of maternal ABO phenotypes and the occurrence of neonatal ICH disclosed no significant associations. The cases with maternal blood group AB were excluded due to the low number of individuals (Figure 7).

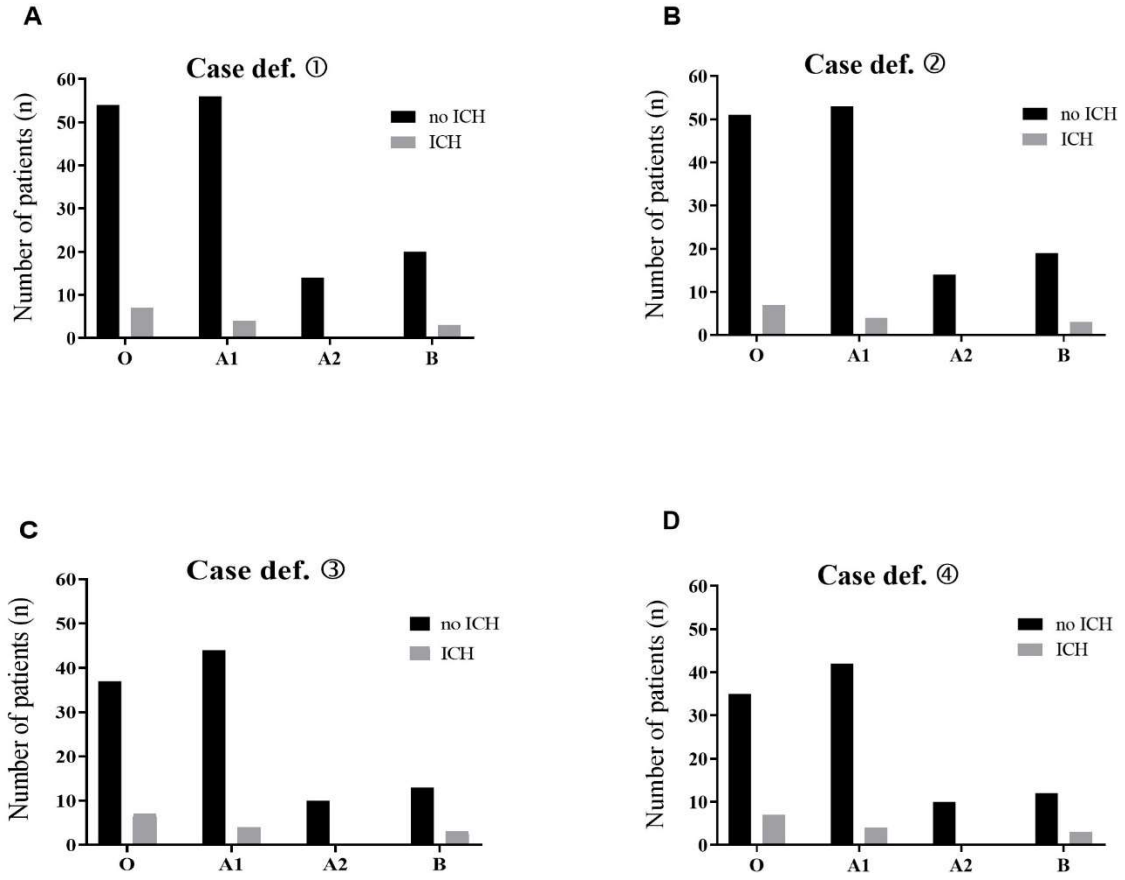

Figure 7: Maternal ABO phenotypes and neonatal ICH incidence.

Distribution of maternal ABO phenotypes and the occurrence of ICH in neonates suffering from FNAIT for case definitions 1 - 4 (illustrations A – D).

A) n = 158, two-sided Fisher's exact test, p = 0.530
B) n = 151, two-sided Fisher's exact test, p = 0.506
C) n = 118, two-sided Fisher's exact test, p = 0.405
D) n = 113, two-sided Fisher's exact test, p = 0.355

3.2.2.2 Maternal ABO phenotypes are not associated with neonatal platelet count nadir

The maternal ABO phenotypes were compared to the platelet count nadir in their neonates. There were no significant associations between the magnitude of neonatal thrombocytopenia and the maternal ABO phenotype (Figure 8). The cases with maternal blood group AB were excluded due to the small number of individuals.

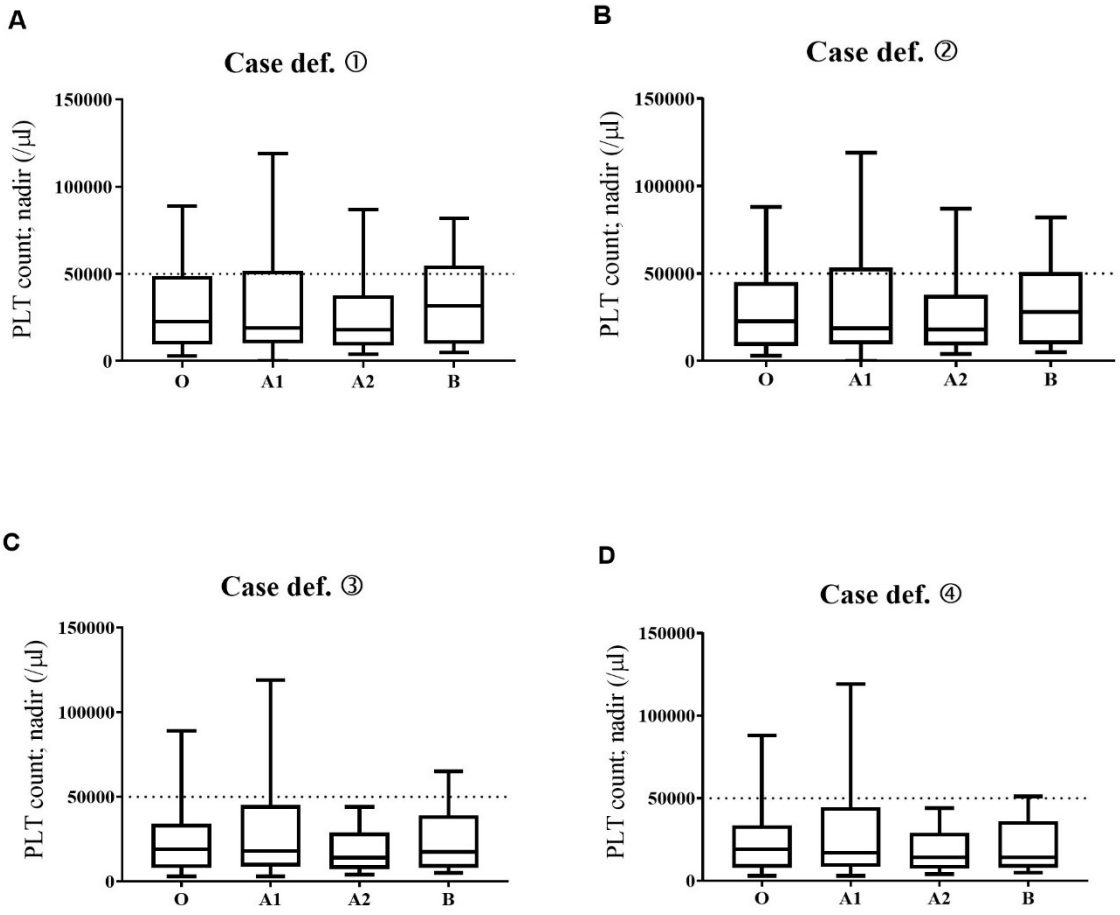

Figure 8: Maternal ABO phenotypes and neonatal platelet count nadir.

Distribution of maternal ABO phenotypes and platelet count nadir in neonates suffering from FNAIT for case definitions 1 - 4 (illustrations A – D). Dotted line threshold of severe FNAIT (PLT count <50000/µl). Logarithmic box and whisker plot; vertical line inside the box equals the median.

A) n = 149, Kruskal-Wallis test, p = 0.856
B) n = 144, Kruskal-Wallis test, p = 0.914
C) n = 114, Kruskal-Wallis test, p = 0.874
D) n = 110, Kruskal-Wallis test, p = 0.851

In case definition 5 (FNAIT suspected, anti-HPA-5b antibodies detected), the median platelet count nadir for O, A_1, A_2 and B was 39000, 64500, 77500, 70000/µl. 1 out of 8 (12.5%) phenotype A mothers and 4 out of 7 (57%) phenotype O mothers gave birth to children with severe instead of moderate thrombocytopenia (OR 0.1071, 95% CI 0.0082 – 1.4071).

3.2.2.3 Maternal phenotype A is associated with neonatal birth weight

The conducted Welch-ANOVA depicted a statistically significant difference in mean birth weight levels for the different maternal ABO phenotypes for case definitions 1 and 2. The subsequent Games-Howell post-hoc test revealed a significant difference between maternal phenotypes A_1 and A_2. On average, children of A_2 mothers were 679 g for case def. 1 (95% CI: 140.48 – 1217.00, p = 0.010) and 642 g for case def. 2 (95% CI: 106.05 – 1178.81, p = 0.015) heavier than children from A_1 mothers. The children of A_2 mothers had a mean birth weight of 3551 g (for case def. 1 and 2) and of A_1 mothers 2872 g (case def. 1) and 2908 g (case def. 2) respectively (Figure 9).
Case definition 5 not illustrated due to low number of individuals.

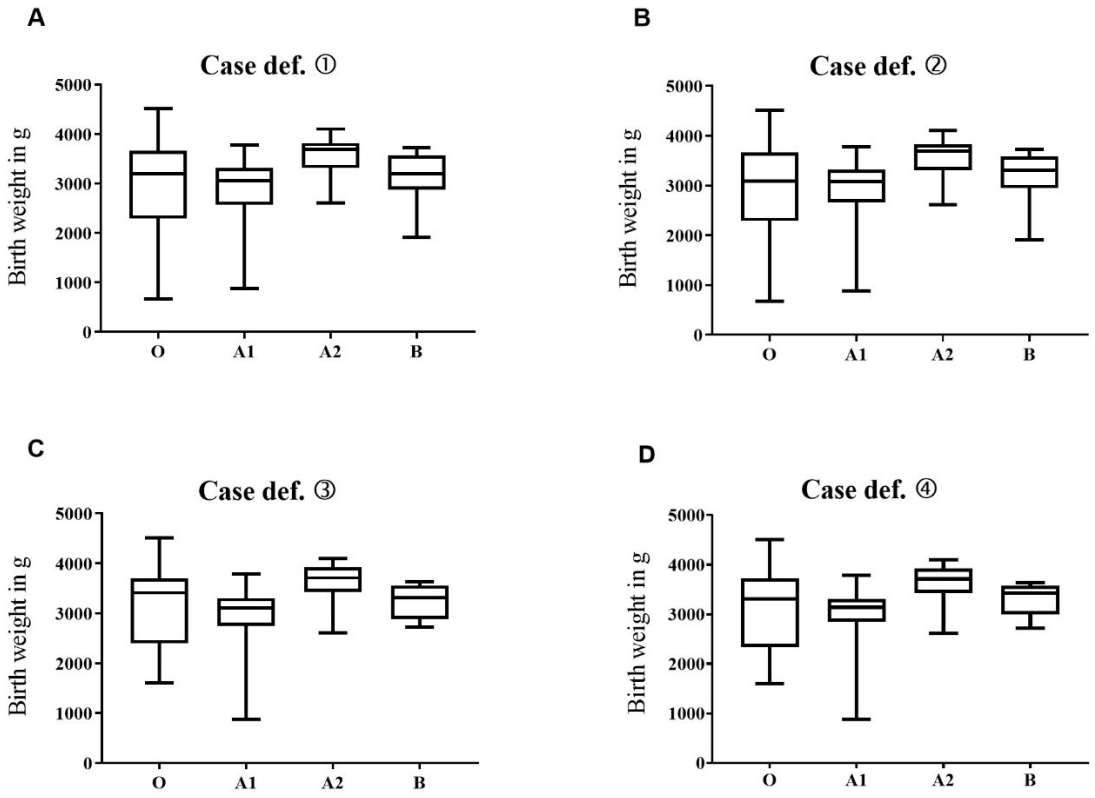

Figure 9: Maternal ABO phenotypes and neonatal birth weight.

Distribution of maternal ABO phenotypes and birth weight in neonates suffering from FNAIT for case definitions 1 - 5 (illustrations A – D). Logarithmic box and whisker plot; vertical line inside the box represents the median.

A) n = 89, Welch-ANOVA, p = 0.012
B) n = 86, Welch-ANOVA, p = 0.016
C) n = 67, Welch-ANOVA, p = 0.130
D) n = 64, Welch-ANOVA, p = 0.066

3.2.3 Maternal ABO gene dose and FNAIT severity

According to a study of Ahlen et al. (Ahlen et al. 2012), the ABO genotype was associated with FNAIT severity. Among mothers with blood group A, the frequency of newborns with severe NAIT was lower in pregnancies where the mother carried only one A allele, and higher where mothers carried two A alleles. To analyze the possible association between maternal ABO genotype and neonatal outcomes, mothers were stratified according to zygosity for A and O alleles (*ABO*A1.01* and *ABO*O.01* alleles). For alleles *ABO*O.02* and *ABO*B.01*, the number of homozygous mothers was too small for valid statistics.

3.2.3.1 Maternal ABO gene dose is not associated with neonatal ICH

There was no significant difference of the incidence of ICH in neonates suffering from FNAIT born to mothers that were hetero- or homozygous for *ABO*A1.01* or *ABO*O.01* (Figures 10 and 11).

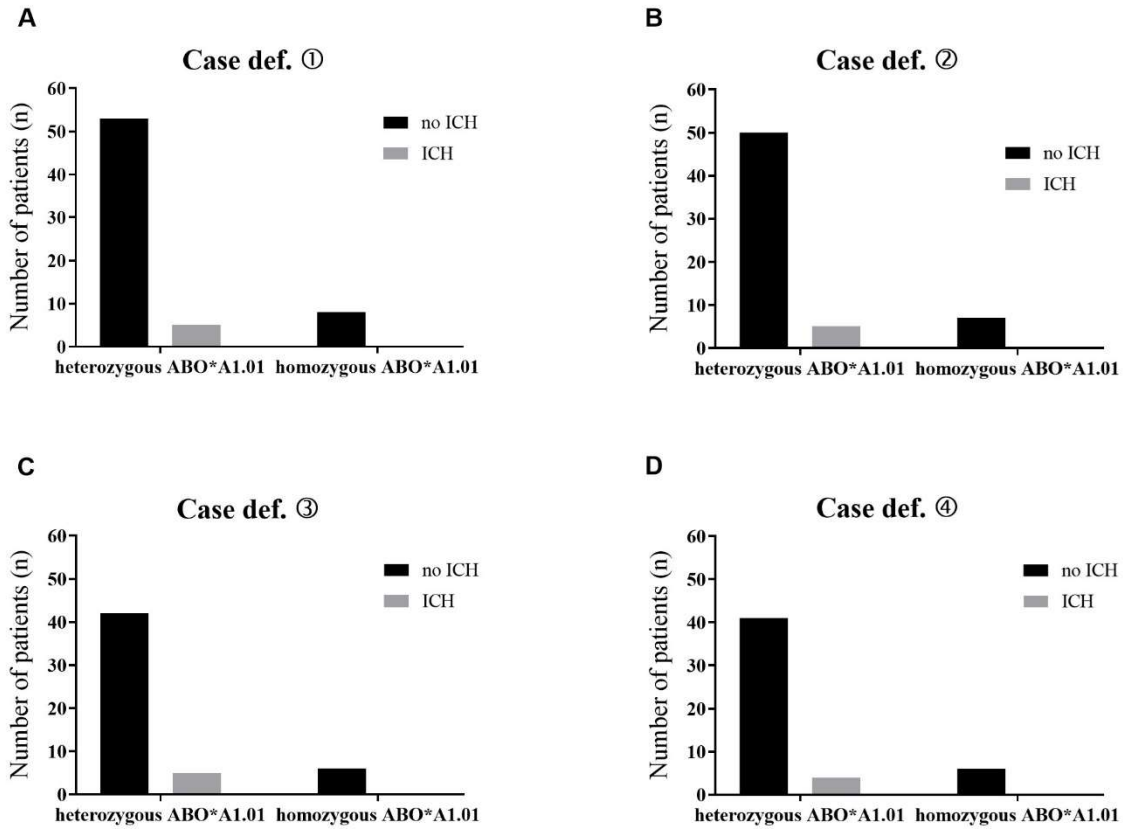

Figure 10: Maternal hetero- or homozygosity for allele *ABO*A1.01* and neonatal ICH incidence.

Comparison of maternal *ABO*A1.01* hetero- or homozygosity and the occurrence of ICH in neonates suffering from FNAIT for case definitions 1 – 4 (illustrations A – D). Heterozygous mothers carry one *ABO*A1.01* allele on one haplotype and one *ABO*O.01* or *ABO*A2.01* allele on the other haplotype.

A) n = 66, two-sided Fisher's exact test, p = 1.000
B) n = 62, two-sided Fisher's exact test, p = 1.000
C) n = 53, two-sided Fisher's exact test, p = 1.000
D) n = 51, two-sided Fisher's exact test, p = 1.000

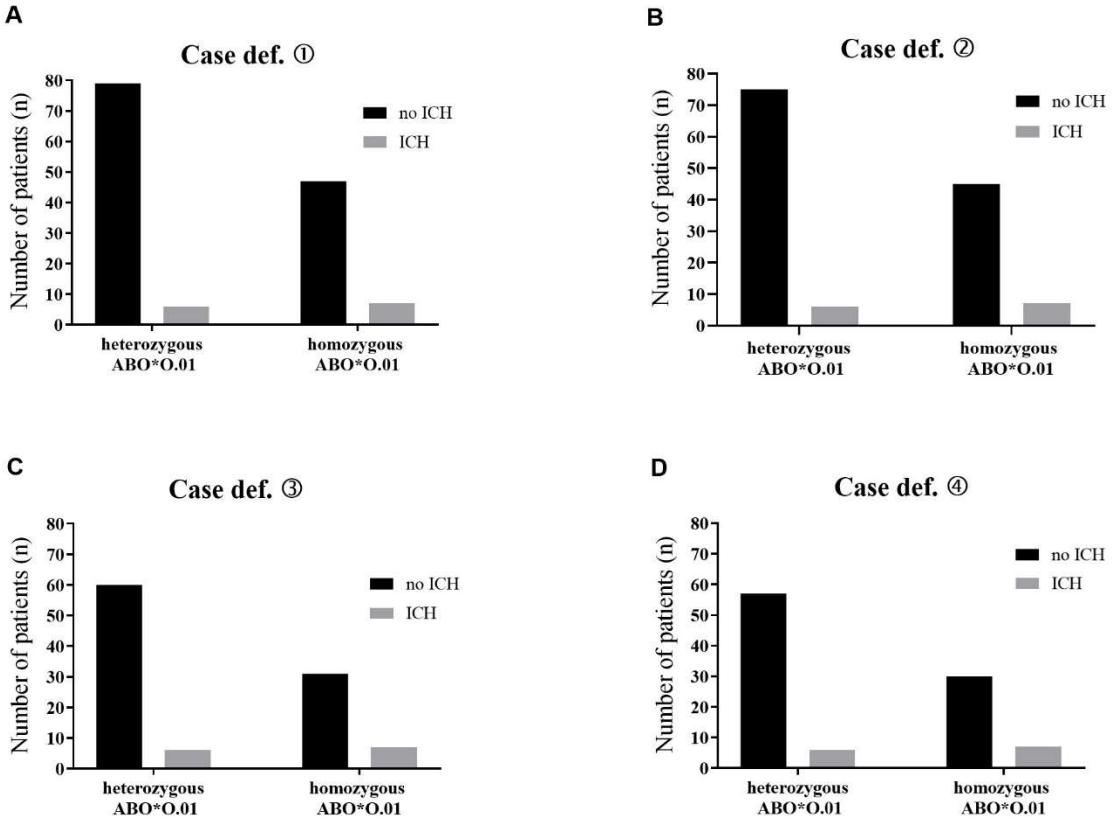

Figure 11: Maternal hetero- or homozygosity for allele *ABO*O.01* and neonatal ICH incidence.

Comparison of maternal *ABO*O.01* hetero- or homozygosity and the occurrence of ICH in neonates suffering from FNAIT for case definitions 1 – 4 (illustrations A – D). Heterozygous mothers carry one *ABO*O.01.01* or one *ABO*O.01.02* allele, whereas homozygous mothers carry two (*ABO*O.01.01* and *ABO*O.01.02* not discriminated).

A) n = 139, χ^2 test with continuity correction, p = 0.386
B) n = 133, χ^2 test with continuity correction, p = 0.396
C) n = 104, two-sided Fisher's exact test, p = 0.220
D) n = 100, two-sided Fisher's exact test, p = 0.222

3.2.3.2 Maternal ABO gene dose is not associated with neonatal platelet count nadir

There was no significant difference in the platelet count nadir in neonates suffering from FNAIT born to mothers that were hetero- or homozygous for *ABO*A1.01* or *ABO*O.01* (Figure 12 and 13).

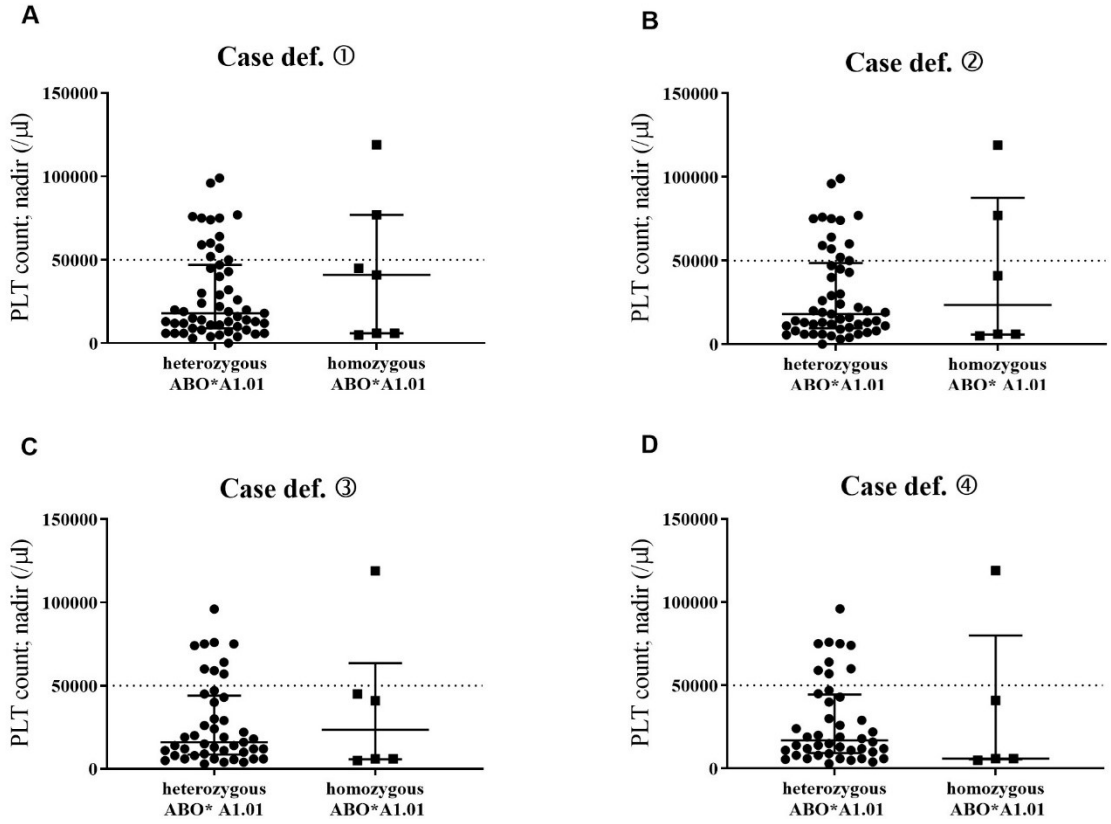

Figure 12: Maternal hetero- or homozygosity for allele *ABO*A1.01* and neonatal platelet count nadir.

Comparison of maternal *ABO*A1.01* hetero- or homozygosity and platelet count nadir in neonates suffering from FNAIT for case definitions 1 – 4 (illustrations A – D). Heterozygous mothers carry one *ABO*A1.01* allele on one haplotype and one *ABO*O.01* or *ABO*A2.01* allele on the other haplotype. Dotted line threshold of severe FNAIT (PLT count <50000/µl). Interquartile range and median displayed.

A) n = 62, Mann-Whitney-U test, p = 0.748
B) n = 59, Mann-Whitney-U test, p = 0.985
C) n = 51, Mann-Whitney-U test, p = 0.892
D) n = 49, Mann-Whitney-U test, p = 0.536

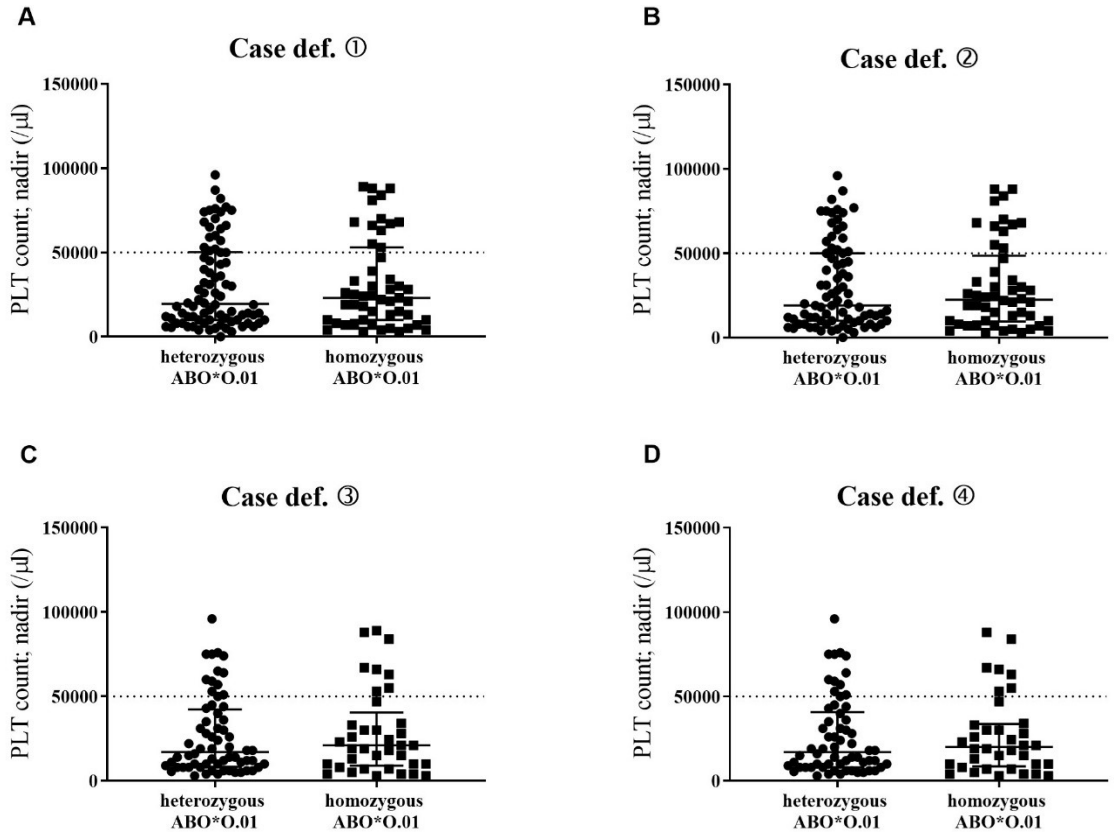

Figure 13: Maternal hetero- or homozygosity for allele *ABO*O.01* and neonatal platelet count nadir.

Comparison of maternal *ABO*O1.01* hetero- or homozygosity and platelet count nadir in neonates suffering from FNAIT for case definitions 1 – 4 (illustrations A – D). Heterozygous mothers carry one *ABO*O.01.01* or one *ABO*O.01.02* allele, whereas homozygous mothers carry two (*ABO*O.01.01* and *ABO*O.01.02* not discriminated. Dotted line threshold of severe FNAIT (PLT count <50000/µl). Interquartile range and median displayed.

A) n = 133, Mann-Whitney-U test, p = 0.895
B) n = 129, Mann-Whitney-U test, p = 0.983
C) n = 101, Mann-Whitney-U test, p = 0.778
D) n = 98, Mann-Whitney-U test, p = 0.897

3.2.3.3 Maternal ABO gene dose is not associated with neonatal birth weight

There was no significant difference in the birth weight of neonates suffering from FNAIT born to mothers that were hetero- or homozygous for *ABO*A1.01* or *ABO*O.01* (Figure 14 and 15).

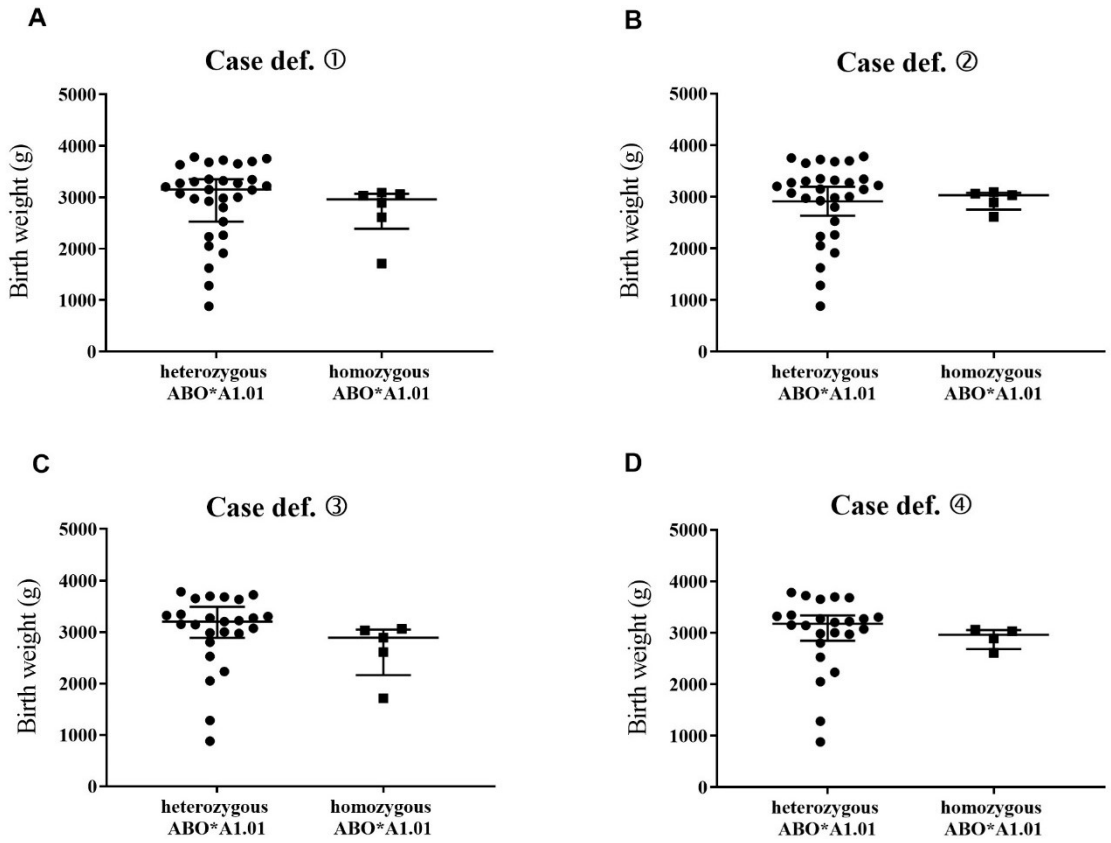

Figure 14: Maternal hetero- or homozygosity for allele *ABO*A1.01* and neonatal birth weight.

Comparison of maternal *ABO*A1.01* hetero- or homozygosity and birth weight of neonates suffering from FNAIT for case definitions 1 – 4 (illustrations A – D). Heterozygous mothers carry one *ABO*A1.01* allele on one haplotype and one *ABO*O.01* or *ABO*A2.01* allele on the other haplotype. Interquartile range and median displayed.

A) n = 37, Mann-Whitney-U test, p = 0.184
B) n = 35, Mann-Whitney-U test, p = 0.418
C) n = 30, Mann-Whitney-U test, p = 0.083
D) n = 28, Mann-Whitney-U test, p = 0.231

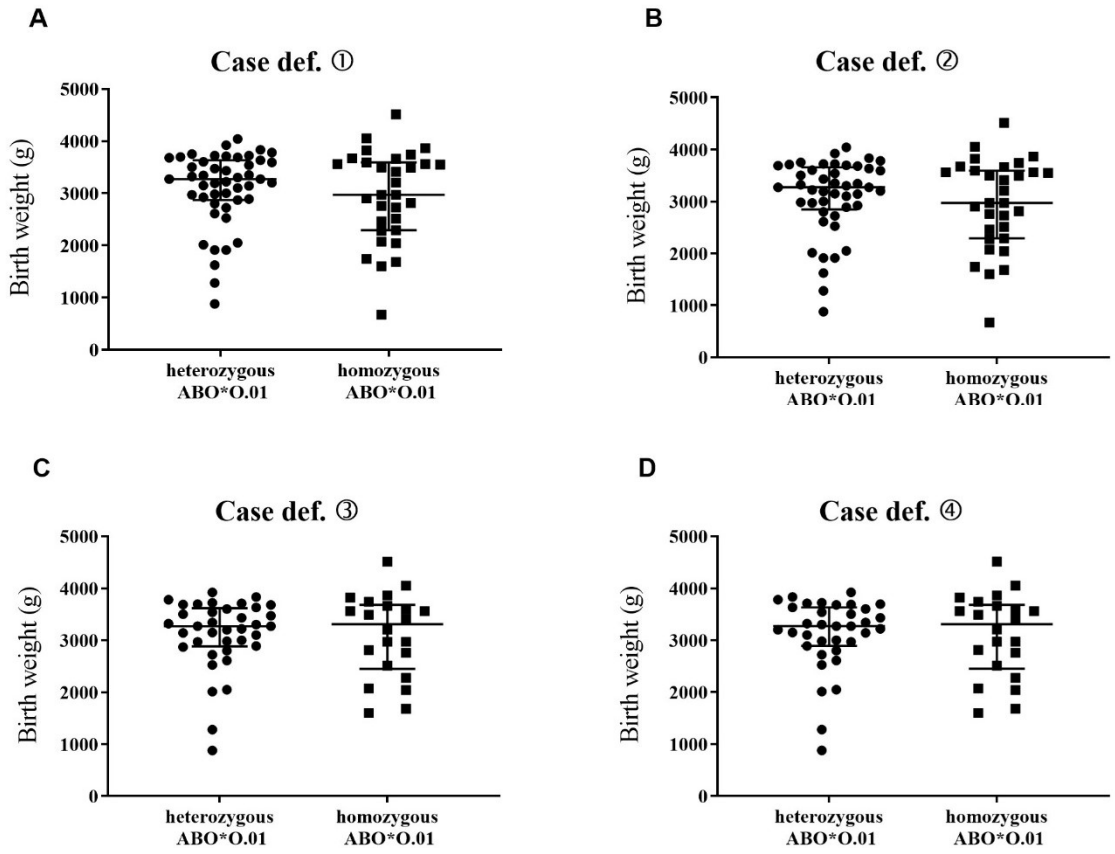

Figure 15: Maternal hetero- or homozygosity for allele *ABO*O.01* and neonatal birth weight.

Comparison of maternal *ABO*O1.01* hetero- or homozygosity and birth weight of neonates suffering from FNAIT for case definitions 1 – 4 (illustrations A – D). Heterozygous mothers carry one *ABO*O.01.01* or one *ABO*O.01.02* allele, whereas homozygous mothers carry two (*ABO*O.01.01* and *ABO*O.01.02* not discriminated). Interquartile range and median displayed.

A) n = 78, t-test, p = 0.469
B) n = 76, t-test, p = 0.490
C) n = 59, t-test, p = 0.946
D) n = 57, t-test, p = 0.961

3.2.4 Neonatal phenotype O is associated with ICH

We analyzed the possible association between neonatal ABO phenotype and neonatal outcomes. Results showed a significant difference of the incidence of ICH in newborns stratified according to ABO phenotype (Figure 16). Further tests revealed that ICH occurred significantly more often in neonates with phenotype O compared to those with phenotype A (χ^2 (1) = 5.336, p = 0.021 with continuity correction, φ = 0.264). Newborns grouped according to ABO phenotype did not show significant differences in platelet count nadir and birth weight.

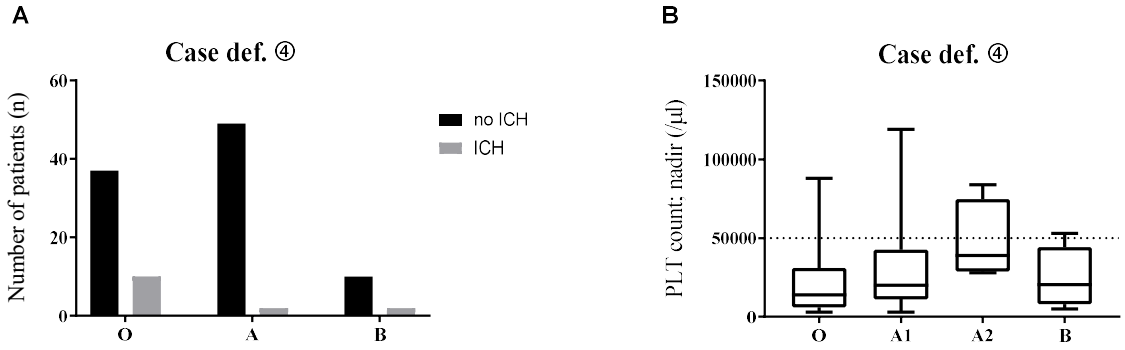

Figure 16: Neonatal ABO phenotypes and ICH or platelet count nadir.

Pictures A and B provide an overview on the influence of the neonatal ABO blood group on occurrence of ICH and platelet count nadir for case definition 4. In picture B interquartile range and median displayed.

A) n = 110, χ^2 test, p = 0.035
B) n = 107, Kruskal-Wallis test, p = 0.067

3.2.5 Neonatal ABO gene dose and FNAIT Severity

3.2.5.1 Neonatal ABO gene dose is not associated with ICH

There was no significant difference of the incidence of ICH in neonates hetero- or homozygous for alleles *ABO*A1.01* or *ABO*O.01* (Figure 17).

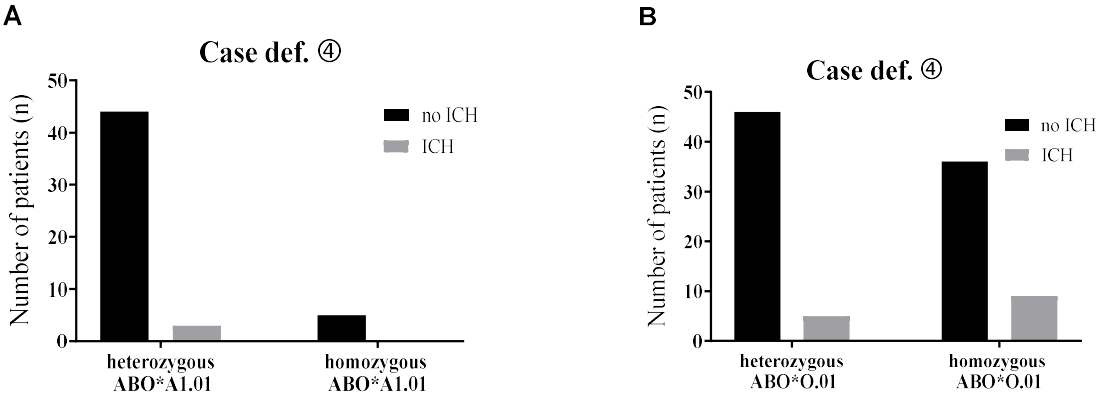

Figure 17: Neonatal hetero- or homozygosity and ICH.

Incidence of ICH in FNAIT neonates hetero- or homozygous for allele *ABO*A1.01* or *ABO*O.01* for case definitions 4 (illustrations A and B). A) Heterozygous newborns carry one *ABO*A1.01* allele on one haplotype and one *ABO*O.01* allele on the other haplotype. B) Heterozygous newborns carry one *ABO*O.01.01* and one *ABO*O.01.02* allele.

A) n = 52, two-sided Fisher's exact test, p = 1.000
B) n = 96, χ^2 test with continuity correction, p = 0.262

3.2.5.2 Neonatal ABO gene dose is not associated with neonatal platelet count nadir

There was no significant difference in platelet counts in neonates hetero- or homozygous for alleles *ABO*A1.01* or *ABO*O.01* (Figure 18).

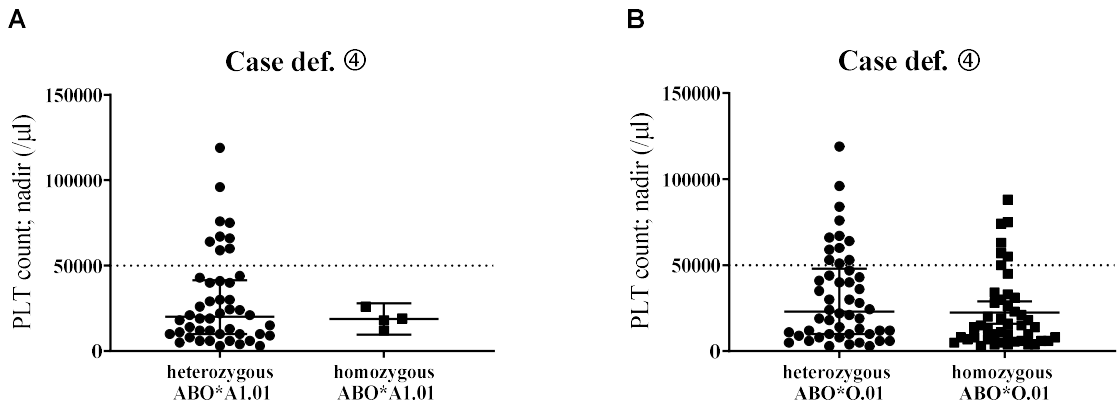

Figure 18: Neonatal hetero- or homozygosity and platelet count nadir.

Platelet count of FNAIT neonates hetero- or homozygous for allele *ABO* A1.01* and *ABO*O.01* for case definitions 4 (illustrations A and B). A) Heterozygous newborns carry one *ABO*A1.01* allele on one haplotype and one O allele on the other haplotype. B) Heterozygous newborns carry one *ABO*O.01.01* and one *ABO*O.01.02* allele. Dotted line threshold of severe FNAIT (PLT count <50000/µl). Interquartile range and median displayed.

A) n = 50, Mann-Whitney-U test, p = 0.870
B) n = 95, Mann-Whitney-U test, p = 0.072

3.2.5.3 Neonatal ABO gene dose is not associated with birth weight

There was no significant difference in birth weight in neonates hetero- or homozygous for alleles *ABO*O.01* (Figure 19). Birth weight data was not available for neonates homozygous for allele *ABO*A1.01*, therefore statistics for *ABO*A1.01* could not be computed.

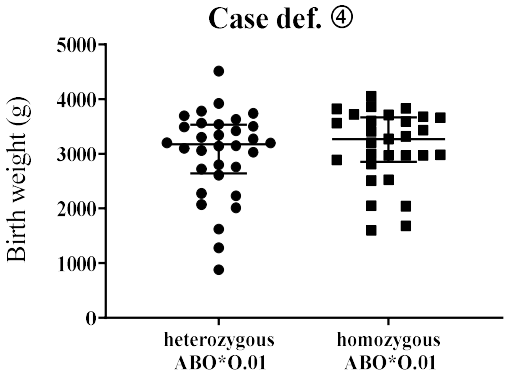

Figure 19: Neonatal hetero- or homozygosity and birth weight.

Birth weight of FNAIT neonates hetero- or homozygous for allele *ABO*O.01* for case definition 4. Heterozygous newborns carry one *ABO*O.01.01* and one *ABO*O.01.02* allele. Heterozygous newborns carry one *ABO*O.01.01* and one *ABO*O.01.02* allele.

n = 61, t-test, p = 0.481

4 Discussion

This is the largest study examining the significance of fetomaternal ABO blood group incompatibility or compatibility and the possible association of maternal and neonatal ABO blood groups with incidence and severity of FNAIT. 165 mother-child pairs with confirmed FNAIT, of which 118 cases with FNAIT due to anti-HPA-1a, the most important antibody specificity causing FNAIT in Caucasians, were included. Since almost all examined mothers were HLA-DRB3*01:01 positive, we did not stratify the cohorts according to the presence or absence of HLA-DRB3*01:01. All cases were categorized adjusted to their antibody specificity, in order to avoid confounding effects such as differences in clinical presentation of FNAIT due to antibody entities, e.g., higher neonatal platelet counts in FNAIT cases presumptively induced by anti-HPA-5b than in FNAIT cases due to anti-HPA-1a (Alm et al. 2022; Ghevaert et al. 2007b). Distribution of antibody specificities was similar to the distribution observed in 200 FNAIT cases in the United Kingdom (Ghevaert et al. 2007b).

4.1 ABO Phenotype Distribution

ABO phenotype distribution in cases resembled a large control group consisting of 45295 mainly German individuals within the local bone marrow registry. Therefore, it can be assumed that the ABO phenotype distribution of examined mothers and neonates corresponds to that of the general population. This is in conformity with the findings of Bertrand et al. in a French study and of Ahlen et al. in a Norwegian study, and indicates that the risk of HPA-alloimmunization is independent from maternal ABO blood groups (Ahlen et al. 2012; Bertrand et al. 2011) and that neonatal ABO blood groups do not affect or trigger HPA-alloimmunization.

4.2 Fetomaternal ABO Incompatibility and FNAIT

For HDFN, the red blood cell counterpart of FNAIT, a preventive effect of fetomaternal ABO incompatibility on alloimmunization against RhD- and non-D-antigens is well-known (Bowman 1997; Levine 1943, 1959; Zwiers et al. 2018). Whether ABO incompatibility has a preventive effect on FNAIT also, was tested in this study by comparing the frequencies of ABO incompatible pregnancies among FNAIT cases and

a control population consisting of 522 pregnancies resulting in thrombocytopenic children with unconfirmed suspect of FNAIT.

When Gratwohl and Shulman came to the conclusion that fetomaternal ABO compatibility was required to develop FNAIT, they were only able to examine the blood groups of 25 FNAIT families (Gratwohl and Shulman 1977). Since ABO compatibility is by far more frequent than ABO incompatibility in pregnancies (Akanmu et al. 2015; Cariani et al. 1995), the findings of Gratwohl and Shulman could be accidental, no p-values are reported.

The frequency of ABO incompatible pregnancies in this study was not reduced among FNAIT cases and a significant association was not found. 22% of FNAIT pregnancies were ABO-incompatible, identical to 22% ABO-incompatible pregnancies among the control population. Clarke and Cariani et al. reported similar proportions of ABO-incompatible pregnancies for the British (Clarke 1973) and Venezuelan (Cariani et al. 1995) population.

The question is why ABO incompatibility protects against maternal anti-D-immunization, but not against anti-HPA-1a-immunization.

Curtis at al. proposed that the reduced ABO antigen amount on platelets compared to RBCs fails to trigger the maternal “rapid clearance system” and therefore enables maternal alloimmunization against fetal HPA, before platelets are degraded in maternal circulation (Curtis et al. 2000).

However, the ABO antigen amount on fetal RBCs is also weaker than that of adults, but ABO incompatibility still protects against anti-RhD immunization (Fischer 1961). Furthermore, type II high-expressers with elevated ABO antigen amounts on platelets (Miserre et al. 2022) and erythrocytes already exist in the fetal/neonatal population, which leads to the conclusion that maternal isohemagglutinins should also bind to incompatible fetal platelets in vivo (in case of fetal transplacental hemorrhage).

Taken together, protection of ABO incompatibility against HPA-1a immunization would be expected if fetal platelets enter maternal circulation, but was not verifiable in this study.

The lack of the assumed protective effect contributes to the hypothesis that, due to anti-HPA-1a, the true immunogenetic agent in FNAIT are particles of the syncytiotrophoblast instead of fetal platelets (Kumpel and Manoussaka 2012), since syncytiotrophoblast cells lack ABO blood group antigens (Goto et al. 1976; Thiede et al. 1965). This early confrontation of primigravidae with an alloantigen on trophoblast cells could cause immunization already in the first pregnancy, which should be considered in the development of anti-HPA-1a immunoprophylaxis.

So far, the mechanism underlying the protective effect of ABO incompatibility against RhD immunization remains unclear. Taking into consideration that in cases of ABO incompatible pregnancies, fetal RBCs are still detectable in maternal circulation postpartum, albeit at a lower incidence and degree compared to compatible pregnancies, (Cohen and Zuelzer 1967), it seems unlikely that the “rapid clearance system” is the only mechanism of protection. Regarding antigen-antibody dynamics in vivo, in animal models of antibody-mediated immune suppression (AMIS) mainly two other mechanisms are proposed predominantly next to the antigen clearance hypothesis (IgG-induced acceleration of antigen removal by the mononuclear phagocytic system): the FcγRIIB-mediated B-cell inhibition hypothesis (initiation of inhibitory signaling in B-cells by antigen-antibody mediated cross-linking of B-cell receptor and FcγR); and the steric hindrance hypothesis (interruption of recognition of corresponding epitopes) (Brinc and Lazarus 2009). Crow et al. demonstrated that injection of pre-sensitized (IgG-coated) platelets could prevent alloimmunization; this monoclonal antibody-mediated suppression of the alloimmune response to platelet transfusion was antigen specific and independent of FcgammaRII-mediated immune regulation (Crow et al. 2000), showing that murine models of AMIS cannot be extrapolated to humans inevitably.

Regarding FNAIT severity, an association between ABO incompatibility and occurrence of ICH, magnitude of thrombocytopenia, or birth weight was not evident in this study, neither for the classical ABO-incompatibility definition nor for the platelet - adjusted definition. Ahlen et al. found no association between ABO incompatibility (platelet-adjusted definition) and frequency of severe NAIT in blood group O mothers either (Ahlen et al. 2012).

4.3 Maternal ABO Phenotypes and FNAIT Severity

An association between maternal ABO phenotypes and the occurrence of ICH in neonates was not observed. Other studies on the possible association between maternal blood groups and fetal/neonatal ICH in FNAIT cases were not accessible. When it is assumed that neonatal ICH in FNAIT cases is most likely caused by impairment of angiogenesis through anti-αvβ3 (Santoso et al. 2016), testing for an association between maternal ABO phenotypes and anti-HPA-1a subtype might be interesting.

Likewise, an association between maternal ABO phenotypes and the occurrence of severe thrombocytopenia was not found. This is in accordance with the results of Bertrand et al.'s retrospective study with 75 FNAIT cases, in which a significant difference between maternal blood groups A and O and alloantibody concentration after delivery or severe thrombocytopenia was not evident (Bertrand et al. 2011).

In a contrasting study by Ahlen et al., immunized women with blood group A had a higher risk for receiving a child with severe thrombocytopenia (46.6% severe FNAIT) compared to blood group O women (20% severe FNAIT), while moderate FNAIT seemed to occur equally among women with these blood groups. 75 HPA-1-incompatible pregnancies which did not result in FNAIT and 83 FNAIT cases were examined and mean platelet counts at delivery were significantly higher in newborns received by women with ABO type O than A (one-way ANOVA with Bonferroni correction, test for Gaussian distribution not mentioned) (Ahlen et al. 2012). According to Ahlen et al. and Bertrand et al., divergent results might be due to different study designs (prospective vs. retrospective, different definitions for ABO incompatibility), furthermore affected by different composition of collectives (FNAIT cases and non-FNAIT-cases) and statistical analysis. In this study, only non-parametric tests were used to assess the influence of ABO types on thrombocytopenia, because platelets among FNAIT cases were not parametrically distributed.

Additionally, maternal titers of isohemagglutinins would have been of interest due to their influence on blood group antigens carried on fetal platelets, particularly because Kato et al presented a FNAIT case due to maternal high-titer anti-A antibodies, without HPA-incompatibilities. In this context, ABO antigen-presenting levels on fetal platelets would be of relevance too (Kato et al. 2013).

A Welch-ANOVA with consecutive Games-Howell post-hoc Test revealed a significant difference in birth weight of neonates from mothers with phenotype A_1 and A_2. Children of A_2 mothers were over 600 g heavier than children of A_1 mothers. It is already known that the activity of A glycosyltransferase, the amount of A antigen expressed per unit von Willebrand factor (vWF) and the plasma levels of vWF and coagulation factor VIII (FVIII) are higher in individuals with genotype *ABO*A1.01/O.01.01* than *ABO*A2.01/O.01.01* (O'Donnell et al. 2002). The by over 20% higher vWF plasma levels (Gill et al. 1987) (and thus FVIII levels (Orstavik et al. 1985)) in blood group A individuals compared to blood group O individuals are also used to partly explain the significantly higher risk for preeclampsia in blood group A and AB mothers (not B mothers), than blood group O mothers (Hiltunen et al. 2009; May 1973; Phaloprakarn and Tangjitgamol 2013). Elevated vWF levels can contribute to a prothrombotic state (Sonneveld et al. 2014; Tóth et al. 2017; Zhou and Welsby 2014) and, together with placental fibrin depositions, impair placental microcirculation, which can lead to preeclampsia and IUGR (Franchini et al. 2016; Kanfer et al. 1996). Thus, it is possible that the higher vWF levels in A_1 mothers contribute to the reduced neonatal birth weight, compared to A_2 mothers.

The almost significant trend of A_2 mothers also having heavier children than O mothers was, therefore, rather unexpected. Assuming small for gestational age births (SGA) partly share the underlying pathogenesis of preeclampsia, it was speculated that maternal phenotype A may also have a higher risk for SGA infants than maternal blood group O. However, neither a study on 5320 Thai women by Phaloprakarn et al. (Phaloprakarn and Tangjitgamol 2013), nor a study on 4250 Scottish women by Clark et al. (Clark et al. 2008), could find an association between maternal ABO blood groups and risk for low birth weight, IUGR (defined at <5th centile) or SGA. However, recently Beyazit et al. showed a higher risk for low neonatal birth weight for women with blood group B, the underlying mechanism is still unknown. It is commonly acknowledged that a direct association between ABO blood groups and immune system genes must exist (Beyazit et al. 2017).

Since anti-HPA-1a subtype anti-β3 antibodies and uterine natural killer cells are partly held responsible for IUGR and miscarriage in FNAIT cases (Eksteen et al. 2017; Yougbaré et al. 2015; Yougbaré et al. 2017) and a negative correlation between anti-

HPA-1a antibody levels and birthweight was described (Tiller et al. 2012), knowledge of antibody subtype and antibody levels and consideration of gestational age at birth could be helpful to reassess these findings. The significant results applied to case definitions 1 and 2 only, comprising antibodies of all specificities (mainly anti-HPA-1a and anti-HPA-5b) and making antibody specificity as an underlying cause rather unlikely.

4.4 Maternal ABO Gene Dose and FNAIT Severity

Whether the mother was heterozygous or homozygous for allele *ABO*A1.01* or *ABO*O.01* had no effect on the fetal/neonatal occurrence of ICH, the magnitude of thrombocytopenia or on neonatal birth weight.

Ahlen et al. reported maternal blood group A to be fraught with risk for severe courses of FNAIT. This effect was shown to be dependent on the gene dose, regardless if the allele was *ABO*A1.01* or *ABO*A2.01*. Frequency of severe thrombocytopenia was 69% when the mother carried two A alleles compared to 42% when the mother only carried one A allele. An obvious, but not significant, difference between the mean platelet count in FNAIT neonates of homozygous *ABO*O.01* mothers (83.2 x 10^9/L) and *ABO*O.01.02* positive blood group O mothers (43.7 x 10^9/L) was also described. Their findings led Ahlen et al. to hypothesize a genetic linkage between ABO alleles and the development of severe FNAIT, potentially due to a linkage of ABO alleles to genes encoding immunoregulatory factors (Ahlen et al. 2012). This hypothesis was not supported by the results of Bertrand et al. (Bertrand et al. 2011) nor in this study.

For alleles *ABO*O.02*, *ABO*B.01* and *ABO*A2.01* no valid statistics could be computed, due to the low or missing number of homozygous mothers. The rare *ABO*O.02* does not evolve from a deletion, in contrast to *ABO*O.01.01* and *ABO*O.01.02* (Yamamoto et al. 1993). *ABO*O.02* is distinguished by its inactivated *ABO*A1.01* glycosyltransferase, which might be able to produce small amounts of *ABO*A1.01* antigen (Seltsam et al. 2005; Yazer et al. 2008), whereas *ABO*O.01* have a truncated protein in common. Genotype *ABO*O.01/O.01* and *ABO*O.01/O.02* were compared directly to assess the effect of *ABO*O.02* on phenotype O (data not shown) and the results argue more against, than for, a genetic linkage between ABO genotypes and severity of FNAIT. For valid statistics the number of individuals with ABO*O.02

must be significantly higher. The rare occurrence of genotype *ABO*O.01/O.02* in the studied individuals is in harmony with the general Caucasian population (Umbria et al. 2014; Wagner et al. 2005).

4.5 Neonatal ABO Blood Groups and FNAIT Severity

On closer consideration, table 2 shows that 10 of 15 children affected by ICH have blood group O. Since all children with ICH were born of mothers immunized against anti-HPA-1a, with detectable anti-HPA-1a antibodies, it seemed justified to examine collective 4 (cases exactly due to anti-HPA-1a) in order to detect a possible association of neonatal ABO blood groups and FNAIT severity.

10 out of 47 neonates with blood group O suffered from ICH, compared to 2 out of 51 neonates with blood group A. Tests confirmed a significant association between neonatal phenotype O and occurrence of neonatal ICH. However, this result is limited by the small proportion of children with ICH studied (n = 15) and could not be replicated in an independent cohort of FNAIT cases (inclusion criteria: period 1991–1999; ICH of the fetus/newborn; mother HPA-1bb; newborn HPA-1ab; solely maternal anti-HPA-1a antibody detected; ABO blood group determined. 4 of 10 (40%) newborns were blood group O). It can be assumed that the association of blood group O newborns with ICH in the initial cohort is most likely due to a type 1 error, taking into account that the gene dose of *ABO*O.01* had no effect on the occurrence of ICH as well. There were also no significant differences between hetero- or homozygous neonates regarding magnitude of thrombocytopenia or birth weight; a genetic linkage between ABO alleles and platelet counts or neonatal birth weight is not known.

While the association between non-blood group O and thrombotic events has been in focus of multiple studies and is widely accepted (Wu et al. 2008), the association between blood group O and bleeding propensity is still unclear. Reports on the role of ABO as prognostic factor for different diseases are especially scarce for neonates.

In a study examining the association of ABO blood groups and response to inhaled nitric oxide (iNO) in neonates with persistent pulmonary hypertension of the newborn (PPHN), El-Ferzli et al. observed a greater response to iNO in adults and neonates with blood group O and A than B and AB. They hypothesize a genetic linkage of the ABO

gene locus with vasoregulatory genes or direct effect of blood group antigens (El-Ferzli et al. 2012) expressed on ion-channels, transporters (Cartron and Colin 2001) etcetera on vascular reactivity, e.g., vascular tone.

For adults taking oral anticoagulants, results on blood group O being a risk factor for bleeding complications or for more severe hemorrhage are ambivalent (Dentali et al. 2013a; Franchini et al. 2013; Garcia et al. 2006). A literature meta-analysis by Dentali et al. identifies ABO blood group O as a potential risk factor for bleeding when taking oral anticoagulants (Dentali et al. 2013a), however they find no association between ABO blood groups and risk for spontaneous ICH (Dentali et al. 2013b). Regarding the "pediatric population", Tatar Aksoy et al. depict no significant association between neonatal ABO blood groups and occurrence of intraventricular hemorrhage (IVH) in 126 extremely low birth weight infants (ELBW, birth weight < 1000g) (Tatar Aksoy et al. 2013).

No significant differences between neonatal ABO phenotypes regarding magnitude of thrombocytopenia or birth weight were found. This was to be expected, because to date, the same criteria for thrombocytopenia are applied independently of ABO blood groups if ABO blood group antigens do not affect the platelet count significantly. Data on a potential relationship between ABO phenotypes and birthweight is not available and even for adults, the data on ABO phenotypes and BMI is inconsistent (Smith et al. 2018).

4.6 Strength and Weaknesses

The main strength of this study is the large cohort of FNAIT cases with full details on blood groups of mothers and children. Due to the retrospective study design needed to examine a larger number of these rare FNAIT cases, data was partly underreported and possible confounders were not documented (e.g. the administration of Rh immunoprophylaxis, which is known to reduce the risk for anti D- and non-Rh alloimmunizations (Zwiers et al. 2018)). Results were not adjusted to further covariates, e.g., underlying diseases of the mother that could possibly affect pregnancy outcomes (for example preeclampsia), maternal age and gestational age at time of delivery, parity, or maternal antibody levels.

Fetal sex was not considered in statistics. Male sex is an independent risk factor for unfavorable pregnancy outcomes (Sheiner et al. 2004; Sheiner 2007) and maternal anti-D alloimmunization is thought to have a more severe effect on male than female fetuses (Ulm et al. 1999). For FNAIT, a significant association between reduced birth weight and male gender was reported (Tiller et al. 2012). Regarding the neonatal platelet count in FNAIT cases, fetal sex had no significant influence (Tiller et al. 2016). What remains remarkable is that 113 of the 165 examined pregnancies in this study resulted in a male newborn (68.5%), in contrast to only 51 female newborns (30.9%) and one intersex newborn (0.6%, later recorded as male). In Hessen, of 60988 live born children in 2017, 31240 (51%) were male and 29748 (49%) female (Statistisches Bundesamt) and the general German sex ratio is 1.05 males per female (Central Intelligence Agency 2019). Of the 15 examined children with ICH, 12 were males and 3 females. The obvious overrepresentation of male sex in the studied FNAIT cohort should be examined and tested against coincidence. There are no hints that the coexistence of female sex and blood group O is equal for the assumed risk factor male sex.

Most of the laboratory methods were already established in the daily routine. MAIPA used for the detection of platelet antibodies is a very specific test and regarded as the gold standard, although it is very elaborate and not infallible. Bakchoul et al. propose that FNAIT might be ruled out in up to 50% of the cases because of false negative results, most probably because MAIPA includes washing steps which impair the detection of low-avidity antibodies and thus reduce the sensitivity. In this study, FNAIT was strongly suspected in 7 cases, but no antibodies were detected. A benefit of Surface Plasmon Resonance (SPR) is the abandonment of any washing steps during antibody detection (Bakchoul et al. 2011). In SPR, HPA are immobilized on a gold sensor surface and maternal serum is injected over the surface. When polarized light hits the sensor surface, electron charge density waves called plasmons are generated. In a specific angle, they cause a reduction of the intensity of the reflected light. This specific angle changes depending on the mass on the sensor surface and thus on the interaction of HPA and maternal anti-HPA. A real-time sensogram reveals the binding and dissociation of the molecules (Nguyen et al. 2015). Although SPR is more sensitive than MAIPA and PIFT, false-negative results cannot be entirely excluded (Bakchoul et al. 2011) and SPR could not succeed over MAIPA in basic routine, but it can possibly contribute to more integral diagnostics in individual cases.

Compared to the already practiced PCR-SSP, the ABO allelic discrimination with TaqMan® real time PCR is a quick and easy method for ABO genotyping, once all probes and positive controls are available. However, adequate probes only exist for common ABO alleles, and positive controls for genotype *ABO*O.02/O.02* are particularly rare. For allele *ABO*B.01*, differentiation between homo- and heterozygous mothers was intensified, because the clusters were arranged too closely in the allelic discrimination plot. This likely occurred because probe "*ABO*B.01*" attached too firmly to the DNA and, in case of heterozygous subjects, complicated the attachment of other probes. Thus, samples in the border zone between these clusters were not automatically determined and their ABO genotyping therefore had to be repeated with the elaborate PCR-SSP.

In the current situation, HLA-DRB3*01:01 to assess the risk for alloimmunization (Delbos et al. 2016; Wienzek-Lischka et al. 2017) and history of affected offspring to assess the risk for severe FNAIT (Bussel et al. 1997; Kamphuis et al. 2010; Kjeldsen-Kragh et al. 2007) are used most in the context of screening and prevention of FNAIT. For index-children, non-invasive markers for severe disease outcomes are missing. The recently suggested association between maternal blood group A and severe neonatal thrombocytopenia (Ahlen et al. 2012) was not confirmed in this study, and no maternal blood group was found to be fraught with risk for alloimmunization or severe FNAIT. Caution is necessary, because the frequency of ABO blood types varies according to ethnic and geographical origin and can affect study results (Clark et al. 2008; Phaloprakarn and Tangjitgamol 2013).

The preventive effect of fetomaternal ABO incompatibility on alloimmunization against antigens on RBCs (Zwiers et al. 2018) seemingly does not apply to FNAIT. Likewise, ABO compatibility does not increase the occurrence of adverse FNAIT outcomes. Therefore, fetomaternal ABO compatibility cannot be used as predictive marker to distinguish the necessary intensity of therapy. Furthermore, maternal or fetal ABO genotyping does not enable non-invasive estimations on the likelihood of severe FNAIT. These observations should be confirmed in an independent, larger, prospective study.

5 Synopsis (English and German)

In fetal and neonatal alloimmune thrombocytopenia (FNAIT), maternal alloantibodies directed against paternally inherited platelet antigens cause fetal/neonatal platelet degradation and interfere with vascular endothelial cells. This results in intracranial hemorrhage (ICH) in approximately 10% of severely thrombocytopenic neonates. Non-invasive monitoring of the fetal platelet count is not possible and prophylaxis of fetal bleeding during pregnancy itself is not harmless either. For HDFN, the red blood cell counterpart of FNAIT, a protective effect of fetomaternal ABO blood group incompatibility against anti-D-immunization is known. For FNAIT, an association between the ABO blood group of the mother and severity of the disease is hypothesized.

ABO genotyping in 165 mother-child pairs with proven FNAIT was performed with in-house TaqMan real time PCR assays to detect the major ABO alleles *ABO*A1.01*, *ABO*A2.01*, *ABO*B.01*, *ABO*O.01* and *ABO*O.02*. The cohorts were stratified according to antibody specificities. Severity of FNAIT was defined through neonatal platelet count nadir, ICH-occurrence, and birth weight.

Distribution of ABO phenotypes among immunized women, their neonates and 45295 first time blood donors were not statistically different. Frequency of ABO-incompatible pregnancies was not reduced among immunized mothers, compared to 522 pregnancies with thrombocytopenic neonates without FNAIT. Fetomaternal ABO incompatibility was not associated with FNAIT severity. ICH frequency was significantly higher in neonates with phenotype O than A, most likely due to a type I error. Neonates of A_2 mothers had a significantly higher birth weight than neonates of A_1 mothers, this finding was only valid without association to any antibody specificity and needs to be replicated in a larger cohort.

A protective effect of fetomaternal ABO incompatibility against anti-HPA-1a immunization was not observed. This may indicate that instead of fetal platelets, particles of the syncytiotrophoblast (which lack ABO antigens) are the cellular source by which immunization is triggered already early in the first pregnancy. This should be regarded in the development of FNAIT prophylaxis. No maternal ABO blood group was associated with risk for alloimmunization or more severe FNAIT. Therefore, risk assessment regarding the outcome and necessary therapy intensity does not seem possible based on maternal blood groups.

Die fetale und neonatale Alloimmunthrombozytopenie (FNAIT) basiert auf einer fetomaternalen Inkompatibilität der humanen Plättchenantigene (HPA). Mütterliche Alloantikörper gegen paternal vererbte Plättchantigene auf den kindlichen Thrombozyten beschleunigen die Degradation letzterer und interferieren mit den kindlichen Endothelzellen. Dies führt bei ca. 10 % der schwer thrombozytopenen Feten/Neonaten zu intrakraniellen Blutungen (ICH). Ein nicht-invasives Monitoring der fetalen Thrombozytenzahlen ist nicht möglich und die Prophylaxe fetaler Blutungen während der Schwangerschaft risikobehaftet. Für die analoge hämolytische Erkrankung des Fetus und Neugeborenen (HDFN) ist ein präventiver Effekt der fetomaternalen ABO-Inkompatibilität gegen die Anti-RhD-Immunisierung bekannt. Für FNAIT wurde ein Zusammenhang zwischen der mütterlichen Blutgruppe und dem Schweregrad beschrieben.

165 Mutter-Kind-Paare mit nachgewiesener FNAIT wurden mittels TaqMan-Echtzeit-PCR ABO-genotypisiert (Detektion der Allele *ABO*A1.01*, *ABO*A2.01*, *ABO*B.01*, *ABO*O.01* und *ABO*O.02*). Die Kohorten wurden entsprechend HPA-Antikörper-Spezifität unterteilt, der FNAIT-Schweregrad definiert durch das Vorkommen von ICH, das Thrombozytopenie-Ausmaß und Geburtsgewicht. Die ABO-Phänotypen-Distribution zwischen immunisierten Frauen, deren Neonaten und 45295 Erstblutspendern war ähnlich. Die Frequenz ABO-inkompatibler Schwangerschaften war, im Vergleich zu 522 Schwangerschaften mit thrombozytopenen Neonaten ohne FNAIT, nicht reduziert. Die fetomaternale ABO-Inkompatibilität war nicht mit dem FNAIT-Schweregrad assoziiert. Die ICH-Frequenz bei Kindern mit Phänotyp O war signifikant höher als bei Phänotyp A, hier ist jedoch von einem statistischen Fehler 1. Art auszugehen. Kinder von A_2-Müttern hatten ein signifikant höheres Geburtsgewicht als Kinder von A_1-Müttern, jedoch nur bei Betrachtung des gesamten Kollektivs (unabhängig von der Antikörperspezifität), so dass die Replikation des Ergebnisses in einer größeren Studie notwendig ist.

Ein protektiver Effekt fetomaternaler ABO-Inkompatibilität auf die Entwicklung von FNAIT wurde nicht nachgewiesen. Dies unterstützt die Vermutung, dass anstelle fetaler Plättchen die Partikel des Synzytiotrophoblasts (welche keine ABO-Antigene tragen) das immunisierende Agens darstellen, und FNAIT bereits früh in der ersten Schwangerschaft verursachen können. Dieser Umstand sollte in der Entwicklung einer Anti-HPA-1a-Prophylaxe zur vorgeburtlichen Therapie berücksichtigt werden. Die mütterliche ABO-Blutgruppe hat keinen Einfluss auf das Auftreten oder den Krankheitsverlauf von FNAIT. ABO-Blutgruppen können daher nicht zur Risikoabschätzung der notwendigen Therapieintensität herangezogen werden.

6 List of Abbreviations

°C	Degree Celsius
AMIS	Antibody-mediated immune suppression
BGMUT	Blood Group antigen gene MUTation
bp	Base pairs
CI	Confidence interval
dbRBC	Database Red Blood Cells
DNA	Deoxyribonucleic acid
e.g.	Exempli gratia
EDTA	Ethylenediaminetetraacetate
ELBW	Extremely low birth weight
FBS	Fetal blood sampling
FcRn	Fragment cristallizable Receptor neonatal
FMH	Fetomaternal hemorrhage
FNAIT	Fetal and neonatal alloimmune thrombocytopenia
FVIII	Factor VIII
Gal	Galactose
GalNAc	N-acetylgalactosamine
GP	Glycoprotein
GTA	α1→3 N-acetylgalactosaminyltransferase
GTB	α1→3 galactosyltransferase
HD	Hemolytic disease
HDFN	Hemolytic disease of fetus and newborn
HLA	Human leucocyte antigen
HPA	Human platelet antigen
HXP	High expresser
i.e.	Id est
ICH	Intracranial Hemorrhage
IgG	Immunoglobulin G
IgM	Immunoglobulin M
iNO	Nitric oxide
ISBT	International Society of Blood Transfusion

ITP	Immune thrombocytopenia
IUGR	Intrauterine growth retardation
IUT	Intrauterine platelet transfusion
IVH	Intraventricular hemorrhage
IVIG	Intravenous immunoglobulin
LXP	Low expressor
MAIPA	Monoclonal antibody immobilization of platelet antigens
MHC	Major histocompatibility complex
min	Minute
NOD-SCID	Non-obese diabetic - severe combined immuno deficiency
NTC	No template control
OD-value	Optical density-value
OR	Odds ratio
p.c.	Post conceptionem
PCR	Polymerase chain reaction
PCR-SSP	Polymerase chain reaction with sequence-specific primers
PIFT	Platelet immunofluorescence test
PLT	Platelet
PPHN	Pulmonary hypertension of the newborn
RBC	Red blood cell
RhIg	Rhesus-immunoglobulin
ROS	Reactive oxygen species
rpm	Rounds per minute
SGA	Small for gestational age
SPR	Surface Plasmon Resonance
UDP-GalNAc	Uridine diphosphate-N-acetylgalactosamine
vWF	Von Willebrand factor

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10 List of Publications, Congressional Contributions

Miserre, L., Wienzek-Lischka, S., Mann, A., Cooper, N., Santoso, S., Ehrhardt, H., Sachs, U. J., & Bein, G (2022). ABO Incompatibility between the Mother and Fetus Does Not Protect against Anti-Human Platelet Antigen-1a Immunization by Pregnancy. *Journal of clinical medicine*, *11*(22), 6811. https://doi.org/10.3390/jcm11226811

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Parts of this dissertation were used in the publication and presentation.

11 Declaration of Academic Honesty

“I hereby declare that I have completed this work independently and without inadmissible assistance or the use of other than the resources quoted. All texts that have been quoted verbatim or by analogy from published and non-published writings and all details based on verbal information have been identified as such. In the analyses that I have conducted and to which I refer in this thesis, I have followed the principles of good scientific practice, as stated in the Statute of Justus Liebig University Giessen for Ensuring Good Scientific Practice, as well as ethical principles and those governing data protection and animal welfare. I give my assurance that third parties have not received from me, either directly or indirectly, any financial remuneration for work in connection with the content of this doctoral thesis and that the work presented has not been submitted in the same or a similar form to another assessment authority in Germany or elsewhere for the purpose of being awarded a doctorate or another assessment procedure. All materials taken from other sources and other persons and used in this thesis or to which direct reference is made has been identified as such. In particular, all those who took part directly and indirectly in the production of this study have been named. I agree to my thesis being subjected to scrutiny by plagiarism detection software or by an internet-based software program.”

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