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What's with the boys? Lower birth weight in boys from HPA-1a alloimmunized pregnancies – New insights from a large prospective screening study in Poland

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ABSTRACT

Fetomaternal incompatibility in human platelet antigens (HPAs) can cause maternal alloimmunization, which in turn may lead to thrombocytopenia with or without intracranial hemorrhage (ICH) in the fetus or newborn. Retrospective studies suggest that boys from alloimmunized mothers may have higher risk of ICH and lower birth weight than girls. The objective of this study was to assess how maternal HPA-1a alloimmunization, sex of the neonate and birth weight relates in a large prospective cohort. Through a national screening study in Poland (PREVFNAIT) involving HPA-1 typing of 24,259 pregnant women during 2013–2017, 606 HPA-1a negative pregnant women and their offspring were identified and included. Various multivariate models were used to assess if and how maternal HPA-1a alloimmunization status was associated with birth weight and risk of having a small for gestational age (SGA) neonate, and if and how sex of the neonate mattered. Most immunized pregnancies had male fetuses (69 %). Women carrying a male fetus had increased likelihood of having an SGA newborn if they were HPA-1a alloimmunized compared to non-immunized mothers. Increasing maternal anti-HPA-1a antibody levels were significantly associated with reduced birth weight and SGA risk among male-fetus pregnancies, but not if the fetus was female. In conclusion, anti-HPA-1a antibodies in a male fetus pregnancy is associated with increased risk of SGA and lower birth weight, especially if the antibody level is high. Sex of the fetus may therefore be considered as a new clinical predictor of more severe FNAIT neonatal outcome.

1. Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is clinically important because of the potential to cause severe intracranial hemorrhage (ICH) in the fetus or neonate (Ghevaert et al., 2007; Kamphuis et al., 2014; Tiller et al., 2013). Incompatibility in human platelet antigens (HPAs) between the mother and fetus can cause maternal alloimmunization, leading to FNAIT. Fetomaternal incompatibility in the HPA-1 system where the mother is HPA-1a negative accounts for around 85 % of all FNAIT cases (Kamphuis et al., 2010; Kjeldsen-Kragh et al., 2007; Mueller-Eckhardt et al., 1989; Davoren et al., 2004). In this situation, maternal alloantibodies of IgG class targeting the paternally inherited HPA-1a antigen on fetal platelets cross the placenta and can lead to thrombocytopenia with or without bleeding in the fetus or newborn. In a Caucasian population, approximately 2 % of women are HPA-1a negative and thus at risk of having a child with FNAIT (Kamphuis et al., 2010; Kjeldsen-Kragh et al., 2007), and FNAIT occurs in one per 1100 births (Kamphuis et al., 2014; Kjeldsen-Kragh et al., 2007). There is currently no screening programme or prophylaxis for FNAIT. Apart from a previous obstetric history of FNAIT, targeted antenatal

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management is hampered by the lack of clinical predictors to identify the pregnancies at increased risk of severe neonatal outcome.

The risk of ICH is known to be much higher when a mother has previously had a fetus/ neonate with ICH caused by FNAIT, and lower when a previous FNAIT child has not had brain bleeding. High-dose intravenous immunoglobulin (IVIg) has for decades been used as an attempt to prevent severe FNAIT in all HPA-1a-alloimmunised pregnant women. Despite widespread use, IVIg is administered off-label for this condition, and the efficacy of IVIg has never been tested in a placebo-controlled clinical trial. Norway has not used IVIg treatment as a main part of the FNAIT management strategy. In Norway, antenatal IVIg is only recommended in pregnancies where a previous child had ICH (high-risk), and is generally not given in other HPA-1a-alloimmunised pregnancies (low-risk). The neonatal outcome of non-IVIg treated low-risk pregnancies in Norway was recently assessed and did not seem to be less favourable than in an IVIg-treated control group (Ernstsen et al., 2022).

In addition to potentially life-threatening intracranial bleeding, studies have indicated that platelet alloantibodies may influence placenta function and fetal growth. Previous retrospective studies have shown associations between maternal anti-HPA-1a antibodies and reduced birth weight, mainly in boys (Tiller et al., 2013; Tiller et al., 2012; Xue et al., 2022). The proposed mechanisms behind this association is that anti-HPA-1a antibodies might influence placental development and -function through binding to HPA-1a antigen epitopes expressed on extravillous throphoblasts as part of the vitronectin receptor (Eksteen et al., 2017; Kumpel et al., 2008; Vanderpuye et al., 1991). The exact mechanisms are not known, but we recently found a strong association between maternal HPA-1a alloimmunization and chronic placental inflammations, particularly chronic histiocytic intervillositis (Nedberg et al., 2021). Further, a higher degree of classical pathway-induced complement activation was reported present in placentas from pregnancies with untreated FNAIT (De Vos et al., 2021). There are indications on more severe clinical course of FNAIT in male fetuses related to cerebral bleeding (Tiller et al., 2013). How sex of the fetus may influence clinical course of alloimmunized pregnancies is currently not understood. Whether antenatal IVIg in HPA-1a alloimmunized pregnancies has any effect on fetal growth and/ or placental function is not known.

The objective of this study was to examine associations between maternal HPA-1a alloimmunization, sex of the fetus and birth weight in a prospective setting.

2. Materials and methods

The PREVFNAIT project involved HPA-1 genotyping of 24,259 pregnant Polish women, aiming to identify HPA-1a-negative mothers at risk of having a neonate with FNAIT (Dębska et al., 2018). Participants were primarily recruited during their routine visit to a hospital or outpatient department. Some of the women (n = 23) were included in the study because they previously had a baby with FNAIT or because they delivered a baby with clinical symptoms of thrombocytopenia during the study period ("non-prospective group"). All laboratory and diagnostics tests were performed at the Institute of Hematology and Transfusion Medicine (IHTM) in Warsaw (Poland).

HPA-1 antigen typing was done by phenotyping using flow cytometry or genotyping by TaqMan allele discrimination using DNA isolated from blood samples (Orzinska et al., 2018; Dębska et al., 2018). Testing for anti-HPA-1a antibodies was performed in maternal plasma samples by the Monoclonal Antibody Immobilization of Platelet Antigens (MAIPA) assay and by quantitative MAIPA. (Kiefel et al., 1987; Killie et al., 2010). In addition, plasma samples from mothers who delivered newborns with severe thrombocytopenia, who tested negative in MAIPA during pregnancy, were retrospectively tested with LIFECODES Pak Lx (Immucor, Peachtree Corners, GA) on a Luminex 200 instrument (Luminex Corp., Austin, TX).

There was no clinical protocol connected to the PREVNAIT study. All identified HPA-1a alloimmunized women received obstetrical follow-up according to clinical status. Some HPA-1a alloimmunized women were treated antenatally with intravenous immunoglobulins (IVIg). The decision on treatment was based on previous FNAIT history, and in the group of prospectively screened women on high and increasing anti-HPA-1a concentrations, or by a low platelet count in the fetus in pregnancies examined by fetal blood sampling (FBS). Intrauterine platelet transfusions were given after FBS in all cases (Dębska et al., 2018).

All HPA-1a-negative women with available data on HPA-1a alloimmunization, tested by MAIPA and/or the Pak Lx assay were included. We defined a woman as alloimmunized if anti-HPA-1a antibodies were detected in at least one maternal sample taken during pregnancy or within the first six days after delivery. Pregnancies were included as non-immunized for the purpose of this study if maternal anti-HPA-1a antibodies were only detected $>$ 6 days postpartum, and not during pregnancy. If we could not assign the pregnancy as immunized or not during pregnancy, the pregnancy was not included (n = 6 pregnancies). We excluded pregnancies with HPA-1 compatible neonates, as well as pregnancies where neonatal HPA-1 status was missing, unless the father was confirmed to be HPA-1aa. Pregnancies where the women wished to withdraw from the study, failed to attend to follow-up, or ended with miscarriage were also excluded.

Clinical data were obtained from the medical records. Data on maternal smoking habits during pregnancy, previous pregnancy history, general health and lifestyle were provided through self-report. Gestational age at time of delivery was calculated from ultrasonographically determined pregnancy due date using the formula: 280 – (pregnancy due date – delivery date (difference in days))/7. In cases where ultrasonographically determined due dates were missing, pregnancy due date was calculated using last menstrual period: date of last menstrual period + 280 days. Miscarriage was defined as spontaneous loss of a fetus before 20 weeks of gestation and stillbirth as fetal death after 20 weeks according to the definition in Poland. Based on gestational age at delivery, birth weight and sex of the neonate, a z-score was calculated for each pregnancy, based on the standard of Skjaerven (Skjaerven et al., 2000). Small for gestational age (SGA) was defined as birth weight less than the 10th percentile. Birth weight $<$ 10 percentile was defined as a z-score < -1.285.

3. Ethics

The study was approved by The Bioethical Committee at the Institute of Hematology and Transfusion Medicine, Warsaw (Approval no: 38/2013), and the PREVFNAIT biobank established in Norway was approved by the Regional Committee for Medical Research Ethics, North Norway (REK 2014/83). Informed written consent was obtained from all participants.

4. Statistics

All statistical analysis was performed using SPSS (Version 29.0 SPSS, SPSS Inc., Chicago, IL). An independent sample t-test was used to compare means for continuous variables between immunized and non-immunized women. For assessing the relationship between non-continuous variables, a Chi-squared test was used.

A multivariable linear regression model was used to assess whether maternal anti-HPA-1a antibodies (yes/no) during pregnancy were associated with birth weight as continuous variable. The dependent variable in the model was birth weight. Covariates were chosen based on 1) general maternal and obstetrical covariates that are known to influence birth weight (maternal age, parity, gestational age at delivery, sex of the newborn) and 2) FNAIT specific covariates (HPA-1 alloimmunization status yes/no or anti-HPA-1a antibody level during pregnancy, depending on model used). Maternal age, parity (dichotomized as nulli- or multiparous), gestational age at time of delivery, sex of the neonate

and maternal anti-HPA-1a antibody status during pregnancy (immunized/ non-immunized) were included as independent variables. A binary logistic regression model was used to assess whether HPA-1a immunization status was associated with risk of SGA (yes/no), including maternal age at delivery, parity (nulli- or multipara) and immunization status as covariates, and this model was applied for the whole study population but also stratified on boys and girls separately. None of the participants reported that they smoked during pregnancy, and this covariate was therefore not included in the model. We did not have complete data on some maternal risk factors known to be associated with lower birth weight, such as pre-pregnancy BMI, preeclampsia, gestational hypertension, gestational diabetes or other co-morbidities during pregnancy, and could therefore not adjust for these co-variates (Kramer, 1987; Li et al., 2021).

For sub-analyses of the immunized group only, the association between maternal anti-HPA-1a antibody level and birth weight was tested using the highest level of anti-HPA-1a antibody level measured during each pregnancy as a continuous variable. In some pregnancies, detectable, but non-quantifiable anti-HPA-1a antibodies were detected in MAIPA ("weak response"). In order not to exclude pregnancies with weak antibody responses, they were designated an arbitrary value of 0.01 IU/ml (n = 4 pregnancies). In pregnancies where anti-HPA-1a antibodies could be detected solely by Pak Lx, and not by MAIPA, these were not included in analyses of antibody levels (n = 5 pregnancies). Some of the samples were also tested by quantitative MAIPA in Tromsø, Norway, and for the purpose of this study, we included antibody level values from the Tromsø MAIPA when these values were not available from the Polish laboratory (n = 2 pregnancies). If the pregnant woman received antenatal IVIg, we included the highest anti-HPA-1a antibody level measured before IVIg treatment was commenced, if available. To assess how maternal alloimmunization state (immunized or not) was related with sex of the neonate, a binary logistic regression model was used. The dependent variable was maternal HPA-1a alloimmunization (yes/no), while maternal age, parity (nulli- or multipara), gestational age at time of delivery and sex of the neonate were included as covariates. Some of the immunized women received antenatal IVIg-treatment during pregnancy. Due to a high degree of correlation between maternal anti-HPA-1a antibody values and IVIg treatment, explained by the fact that 81 % of IVIg treated pregnancies were recruited non-prospectively (i.e. they have a previous known FNAIT pregnancy and therefore higher antibody levels in the next pregnancy), we could not adjust for antenatal IVIg treatment in addition to maternal anti-HPA-1a antibody levels in the same models.

For all the statistical models described, each neonate was included as a single case, including twins (n = 8 pregnancies and n = 16 neonates) and women who gave birth more than once (n = 14 neonates) during the study period.

5. Results

5.1. General characteristics of the study population

From a total of 24,259 women screened, 606 (2.5 %) women were HPA-1a negative. We excluded 165 women/ pregnancies due to known or possible HPA-1 compatibility, missing data regarding immunizing status during pregnancy, stillbirth/miscarriage, lost to follow up or withdrawal from the study. A total of 441 HPA-1a negative women were included for analysis. These 441 participants completed 455 pregnancies and resulted in 463 neonates during the study period.

Maternal anti-HPA-1a antibodies during pregnancy or within six days after birth were detected in 53 (12 %) of the pregnancies and included as immunized. The remaining 402 pregnancies, including nine pregnancies with anti-HPA-1a antibodies detected postpartum only, were included as non-immunized. Data on gestational age at delivery, sex of the neonate and birth weight was obtained in all immunized pregnancies and for 95 % of non-immunized pregnancies. Description of the study population is outlined in Fig. 1.

There were significantly more multiparous women in the immunized group compared to the non-immunized group (Table 1, chi-square test, p = 0.03). This is not surprising since the chance of a mother being alloimmunized increases with each pregnancy. Neonates from the immunized group were delivered at an earlier gestational age compared to non-immunized pregnancies (Table 1, Independent sample t-test, p < 0.001). Further general maternal and neonatal characteristics are given in Table 1.

5.2. Maternal HPA-1a alloimmunization, sex of the fetus and birth weight

The SGA frequency among immunized pregnancies was 13 % and not significantly different from the non-immunized group in neither univariate (Table 1) nor multivariate analyses (binary logistic regression, p = 0.7) for the whole study population. However, all seven SGA neonates from the immunized pregnancies were boys. Birth weights were not significantly lower among neonates from the immunized group when assessed unadjusted (Table 1, independent sample t-test, p = 0.06) or when adjusting for confounders (linear regression, p = 0.6). There were more boys in the immunized group (69 %) compared to neonates from non-immunized pregnancies (Table 1, chi-square test, p = 0.003). Anti-HPA-1a antibodies were also more often detected in pregnancies with a male fetus; there was a significantly higher likelihood of the mother having detectable anti-HPA-1a antibodies during pregnancy if the fetus was male (OR 0.372) binary logistic regression, p = 0.002).

5.2.1. Maternal antibody levels

Maternal antibody levels during pregnancy were not significantly associated with birth weight or SGA frequency when analyzing boys and girls together (linear regression and binary logistic regression, p = 0.6, data not shown). Notably, among pregnancies with male fetuses, the maternal anti-HPA-1a antibody levels were significantly higher in SGA pregnancies compared to non-SGA pregnancies (25 IU/ml vs 9 IU/ml, independent samples t-test, p = 0.04). Further, birth weight in boys was significantly and inversely associated with increasing anti-HPA-a antibody levels when adjusted for confounders (linear regression, p = 0.01). There was no equivalent association between maternal antibody levels and birth weights among girls (data not shown). Mean and median anti-HPA-1a antibody levels during pregnancy were higher among alloimmunized pregnant mothers carrying a girl compared with male fetus alloimmunized pregnancies, but the difference was not significant (data not shown).

5.2.2. Antenatal IVIg treatment

Antenatal intravenous immunoglobulin (IVIg) treatment was administered in 16 (30 %) of the 53 pregnancies from HPA-1a immunized women (including one twin pregnancy), of which 13 (81 %) were recruited non-prospectively. There was a non-significant tendency toward predominance of female-fetus immunized pregnancies exposed to antenatal IVIg (8/17,47 %) compared to 9/38 (23 %) of male-fetus pregnancies (Chi-square test, p = 0.08).

Non-prospectively recruited pregnancies had borderline significantly higher anti-HPA-1a antibody levels compared to prospectively recruited pregnancies (Mann-Whitney U test, p = 0.08). There was no association between IVIg-treatment and birth weight (data not shown).

6. Discussion

This is the first prospective study to specifically address birth weight and sex of the neonate in relation to maternal HPA-1a alloimmunization. In this study including more than 600 HPA-1bb pregnant women and their offspring, we found that higher levels of maternal anti-HPA-1a antibodies during pregnancy was associated with increased risk of having an SGA neonate – but only if the fetus was a boy. Similar

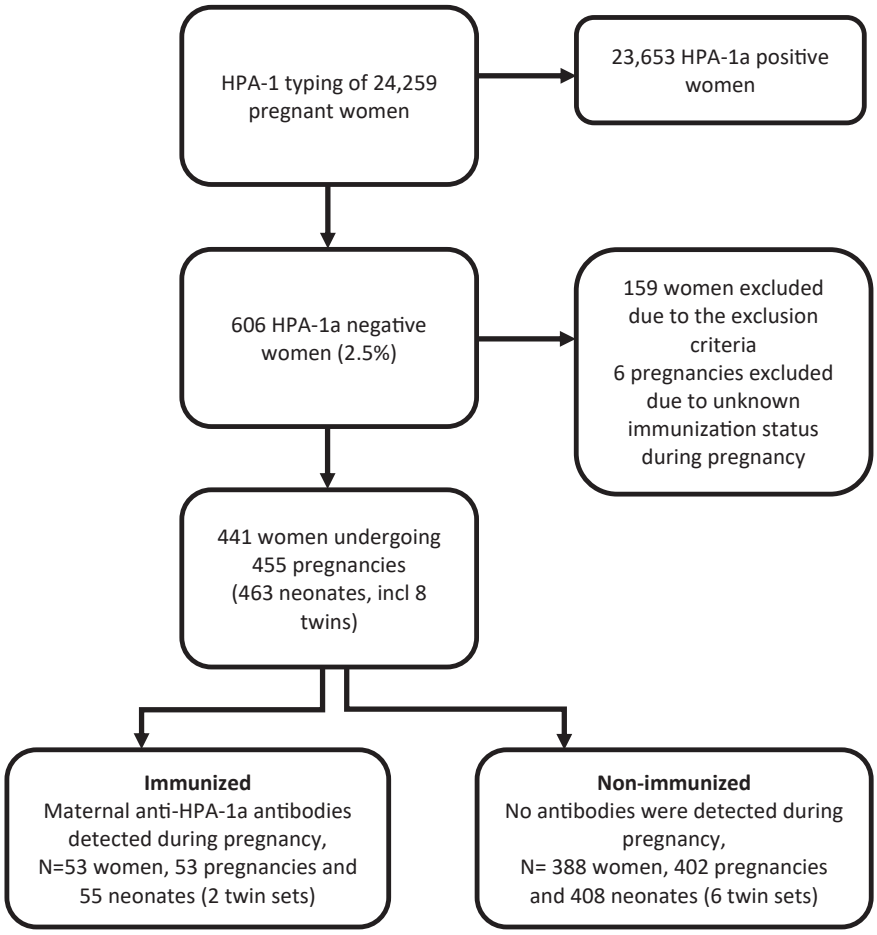

Fig. 1. Overview of study population.

Table 1
General characteristics.

Characteristics	Immunized group (n = 55 neonates)	Non-immunized group (n = 408 neonates)	p-value
Maternal age in years, mean (SD)	31.8 (4.9)	31.2 (4.5)	0.3
Parity, nullipara %	24 (44)	239 (59)	0.031
Gestational age at delivery, days (SD)	268 (18)	273 (14)	< 0.001
Sex of the newborn, *n* boys (%)	38 (69)	187 (46)	0.003
Birth weight, grams (SD)	3168 (633)	3319 (548)	0.06
Small for gestational age (SGA), *n* (%)	7 (13)	44 (11)	0.8
Placental weight, grams (SD)	574 (159)*	587 (155)**	0.7
PW/BW, mean (SD)	0.19 (0.06)*	0.17 (0.05)**	0.20

*Data available for 25 cases.
**Data available for 183 cases.

associations between maternal anti-HPA-1a antibodies and reduced birth weight in boys have been observed previously in retrospective populations (Tiller et al., 2013; Tiller et al., 2012; Xue et al., 2022). This could indicate that male fetuses are more susceptible to fetal growth restriction in relation to platelet alloimmunization, or that the effect of immunization on the fetus is different between boys and girls. The excess of boys previously described among neonates with ICH among FNAIT cases supports this idea and also that male fetuses are more susceptible to a worse neonatal outcome of FNAIT in general (Tiller et al., 2013). Our study suggests that being pregnant with a male fetus is an independent clinical predictor of a more severe clinical course in HPA-1a alloimmunized pregnancies.

Fetal sex-dependent differences are found in many aspects of maternal fetal medicine (Di Renzo et al., 2007). A higher incidence of preterm born boys, as well as better outcome for preterm-born girls are well known phenomena, despite not yet fully understood. An increased vulnerability to infections in women carrying male fetuses has been suggested. (Mcgregor et al., 1992) Another example is that women carrying male fetuses are found to have higher rates of gestational diabetes mellitus and higher perinatal complication rates. (Bracero et al., 1996) Male sex as an independent risk factor for adverse pregnancy outcome is therefore not novel. Relevant for platelet alloimmunization, lesions of chronic placental inflammation has been observed more evident in male fetus pregnancies and it is suggested that this may arise from a maternal immune response against invading interstitial trophoblasts (Ghidini and Salafia, 2005).

Poor fetal growth is associated with increased risk of disease and death both in the newborn period and adulthood (Barker et al., 1993; Strauss, 2000). Identifying pregnancies with increased risk of having an SGA neonate is therefore a highly prioritized task among obstetricians (Aarnoudse-Moens et al., 2009; Clayton et al., 2007). Our data supports that identification and clinical follow-up including serial fetal growth scans of HPA-1a alloimmunized pregnancies with high antibody levels should be prioritized.

Reassuringly, we did not find evidence that anti-HPA-1a antibodies at lower levels influence birth weight, suggesting that alloimmunized pregnancies with low anti-HPA-1a antibody levels do not need to be monitored for fetal growth restriction. In Norway, we use a cut-off anti-

HPA-1a antibody level of more than 3 IU/ml to predict and stratify pregnancies at high risk of severe neonatal thrombocytopenia (Tiller et al., 2020). There was no indication that the same cut-off value could predict risk of SGA in this cohort (data not shown), and more data is needed to identify whether a specific antibody level could be clinically useful to predict risk of fetal growth restriction.

Due to a large cohort of unselected pregnant women combined with a prospective study design, our data may be considered representative of a Caucasian pregnant population. The quality of the study is further strengthened by comprehensive laboratory, as well as clinical, data. The large control group of pregnancies from non-immunized HPA-1bb women greatly increases the quality and value of our results. Although our data is based mainly on prospective cases, the risk of FNAIT was known in 23 pregnancies due to previous obstetric FNAIT history. We found that these "non-prospective" pregnancies had higher anti-HPA-1a antibody levels, supporting the fact that most of the IVIg-treated women had clinically recognized FNAIT in their obstetric history and thus belonged in the more severe spectrum of HPA-1a alloimmunization. The inclusion of non-prospective pregnancies may influence the overall incidence of maternal HPA-1a alloimmunization but should not influence the distribution of fetal sex or how maternal anti-HPA-1a antibodies are related to birth weight, which was the focus in this work. Although the PREVFNAIT study screened more than 24,000 pregnant women, the sample size of HPA-1a alloimmunized pregnancies was only 53 and limits power of data analysis. Especially, the stratified analysis by sex could be unstable and in theory by chance. The consistency of similar findings from other previous HPA-1a alloimmunized cohorts, (Tiller et al., 2013, 2012; Xue et al., 2022) however, supports that our findings mirror a true trend. Although we adjusted for many important covariates known to affect birth weight, we did not have complete data on common maternal risk factors, such as preeclampsia, gestational hypertension or gestational diabetes. A higher rate of hypertensive disorders in HPA-1a alloimmunised pregnant women was observed in the recent Dutch HPA-1 screening study, (De Vos et al., 2023) suggesting that a possible confounding effect cannot be ruled out. Although very interesting, the Dutch observation needs to be specifically addressed and confirmed in future studies.

There were 14 sibling pairs and 8 twin pairs included in our study population of 455 included pregnancies, encompassing 9.7 % of the study population. The birth weights among siblings and twins, are not unrelated. We chose to include these pregnancies as independent cases in the statistical analysis, as less than 10 % of the measurements were repeated, and since only three of the siblings included belonged the smaller group of immunized pregnancies.

None of the participating pregnant women reported smoking during pregnancy. This contrasts the rather high frequency of smokers among pregnant women in Poland described by others (Wojtyła and Wojtyła--Buciora, 2017), and thus an under-reporting in our cohort is possible. On the other hand, most PREVFNAIT participants came from urban areas in Poland where the smoking rate is much lower than in rural areas. A bias toward pro-health behavior in PREVFNAIT participants as part of being part of a study during pregnancy is also possible. Nonetheless, there is no reason to think that the self-reporting of smoking should differ between women with and without anti-HPA-1a antibodies.

The relative overweight of female-fetus immunized pregnancies exposed to antenatal IVIg is conspicuous given the overweight of boys among immunized pregnancies and lack of associations with birth weight among girls. If antenatal IVIg-treatment counteracts anti-HPA-1a antibody effect on birth weight, we cannot rule out that also girls' birth weight may have been lower if the mother had not received IVIg. In support of this, it has been reported that IVIg may affect the anti-HPA-1a antibody level (Bertrand et al., 2006) thus masking a possible dose-response association in our data. Thus, more data on non-IVIg treated alloimmunized pregnancies is needed to settle this issue.

The observation that most of the neonates in the immunized group were boys (69 %) deserves to be commented. We found that the likelihood of maternal HPA-1a antibodies being detected during pregnancy was higher if the neonate was a boy. The interpretation of this findings is challenging: It could theoretically indicate that sex of the fetus affects whether the mother *becomes* alloimmunized in the first place. The concept that the maternal immune response may differ whether the fetus is male or female is currently being explored. Sex of fetus has been found to influence adaptive and innate immune response to COVID-1, where male placentas produced more proinflammatory molecules than female placentas (Bordt et al., 2021). Male fetal sex gender as a trigger of alloimmunization has however not been suggested before. Since we do not know the sex of the fetus in any previous pregnancies, we cannot conclude from our data that sex of the fetus influences the risk of becoming alloimmunized. However, the association between maternal antibody status during pregnancy and sex of neonate was significant also when we analyzed only for first-borns, suggesting that this idea is possible. This should be studied further, perhaps by combining data from other previous prospective screening studies on FNAIT.

7. Conclusion

Male neonates are more susceptible to being SGA than girls when the mother has high anti-platelet antibodies during pregnancy. Sex of the fetus and maternal anti-HPA-1a antibody levels may be integrated as part of antenatal risk assessment when planning for management of HPA-1a alloimmunized pregnancies.

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Declaration of Competing Interest

The other authors declare that they have no conflict of interest relevant to this publication.

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