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Platelet alloimmunization is associated with low grade chronic histiocytic intervillositis - A new link to a rare placental lesion?

Nora Hersoug Nedberg [a,*], Gitta Turowski [b,f], Katarzyna Guz [c], Ewa Przytuła [e], Małgorzata Uhrynowska [c], Borghild Roald [b,f], Anne Husebekk [a], Vasilis Sitras [g], Mona Nystad [h,j], Marzena Dębska [i], Ewa Brojer [c], Heidi Tiller [h,j]

[a] Immunology Research Group, Department of Medical Biology, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø, Norway
[b] Department of Pathology, Center for Perinatal and Pregnancy-Related Pathology, Oslo University Hospital, Oslo, Norway
[c] Department of Hematological and Transfusion Immunology, Institute of Hematology and Transfusion Medicine, Warsaw, Poland
[e] Department of Pathology, Centre of Medical Postgraduate Education, Warsaw, Poland
[f] Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway
[g] Department of Fetal Medicine, Oslo University Hospital, Oslo, Norway
[h] Women's Health and Perinatology Research Group, Department of Clinical Medicine, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø, Norway
[i] Department of Obstetrics and Gynecology, Centre of Medical Postgraduate Education, Warsaw, Poland
[j] Department of Obstetrics and Gynecology, University Hospital of North Norway, Tromsø, Norway

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ABSTRACT

Introduction: Maternal alloimmunization against human platelet antigen (HPA)-1a has been implied to mediate both reduced birth weight and chronic placental inflammation. Fetal growth restriction is associated with different types of chronic inflammation in the placenta, mainly chronic histiocytic intervillositis and chronic villitis. The aim of this prospective study was to do a systematic examination of placentas from HPA-1a alloimmunized pregnancies, with focus on the histopathological and immunohistochemical diagnosis of variants of chronic inflammation.

Material and methods: In a Polish-Norwegian study, 48 placentas were examined. The histopathology of placentas from 27 HPA-1a immunized women was compared with 21 placentas from non-immunized HPA-1a negative women (controls). In the group of alloimmunized women, ten received antenatal intravenous immunoglobulin G (IVIg). Tissue sections from formalin fixed paraffin embedded placental tissue were stained with hematoxylin and eosin and microscopically examined with focus on various types of chronic placental inflammations.

Results: Chronic histiocytic intervillositis was observed in 40.7% of placentas from HPA-1a alloimmunized pregnancies, compared to none in the control group ($p = 0.001$). Chronic villitis of unknown etiology was more frequently found in the alloimmunized group, however this difference was not statistically significant. Maternal administration of IVIg did not seem to protect against chronic inflammatory lesions.

Discussion: Placentas with detectable maternal anti-HPA-1a antibodies are associated with highly increased risk of low-grade chronic histiocytic intervillositis.

1. Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is defined as the destruction of fetal platelets mediated by maternal anti-platelet antibodies crossing the placental barrier. Fetal-maternal incompatibility in human platelet antigens (HPAs) may lead to platelet alloimmunization. FNAIT is reported to occur in ~1/1000 live births [1], but the condition is significantly underdiagnosed [2]. A thrombocytopenic fetus or neonate (defined as a platelet count $< 150 \times 10^9$/L) is at increased risk of hemorrhage. The most serious complication is

Abbreviations: FNAIT, Fetal and neonatal alloimmune thrombocytopenia; HPA, Human platelet antigen; ICH, Intracranial hemorrhage; IVIg, Intravenous immunoglobulin G.

* Corresponding author. Immunology Research Group, Institute of Medical Biology, Faculty of Health Sciences, UiT, The Arctic University of Norway, Hansine Hansens veg 18, 9037, Tromsø, Norway.
E-mail address: Nora.h.nedberg@uit.no (N.H. Nedberg).

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intracranial hemorrhage (ICH) [3]. In most Western countries, maternal antenatal treatment with intravenous immunoglobulin G (IVIg treatment) is used to prevent severe FNAIT complications [4]. In Norway however, patients are treated more restrictively [5,6]. Whether nationwide antenatal screening programs to detect all HPA-1a negative women at risk of having a fetus/neonate with FNAIT should be implemented, is still under discussion [7–9]. An antibody-mediated prophylaxis to prevent HPA-1a alloimmunization is currently in clinical trials [10].

Besides bleeding complications, neonates from HPA alloimmunized pregnancies seem to be at increased risk of lower birth weight. We previously found that maternal anti-HPA-1a antibodies were strongly associated with lower birth weight, predominantly in boys [11], in line with other studies [12]. However, the effect of maternal anti-HPA-1a antibodies on fetal growth is not well understood. In general, fetal growth restriction (FGR) is mostly related to insufficient supply of nutrients and oxygen to the fetus, mediated by the placenta (placental insufficiency) [13]. Although the etiology of placental insufficiency is multifactorial, an associated pathological trait is chronic placental inflammation [14,15]. Chronic inflammatory lesions of the placenta are characterized by an infiltration of lymphocytes, plasma cells and/or histiocytic cells/macrophages in fetal membranes, the villous tree or in the decidua. One common inflammatory lesion is chronic villitis of unknown etiology, confining inflammatory cells to the fetal villous compartment. A more rare type is chronic histiocytic intervillositis affecting the maternal intervillous space, often with some degree of fibrin depositions. Without any proven infection, these inflammations are assumed to be caused by a maternal immune reaction against paternal/fetal antigens, analogous a host-versus-graft reaction [16]. An association between chronic placental inflammation and FNAIT has been suggested in previous studies [17–19]. In Caucasians, antibodies against HPA-1a are responsible for the majority of FNAIT-cases [20,21]. The integrin β3, carrying the HPA-1a antigen, pairs with integrin αIIb, forming the fibrinogen receptor (αIIbβ3) which is widely expressed on platelets. Integrin β3 is also expressed on other cell types and binds to integrin αV to form the vitronectin receptor (αVβ3), which is expressed on trophoblast [22–24] and endothelial cells [25]. A human monoclonal anti-HPA-1a antibody has been shown to bind placental-derived αVβ3 [26]. A pilot study using an *in vitro* model indicated that the same antibody partially inhibited adhesive and migratory capacity of the first trimester extravillous trophoblast cell line, HTR8/neo [27]. It is therefore plausible that maternal HPA-1a alloantibodies may play a role in the early pathophysiology of placental insufficiency including variants of unexplained chronic villous/intervillous inflammatory lesions.

The main aim of this study was to do a systematic histopathological examination of placentas obtained from HPA-1a alloimmunized and non-immunized HPA-1a negative singleton pregnancies with focus on low and high grade chronic villitis and chronic histiocytic intervillositis per newly established Amsterdam criteria. Furthermore, we aimed to assess the possible effect of antenatal treatment with IVIg on the incidence and/-or severity of such inflammatory lesions.

2. Material and methods

2.1. Study population

"PREVFNAIT – *Prevention of fetal/neonatal alloimmune thrombocytopenia"* was a large screening study conducted in Poland from September 2013 to March 2017. This project was done in collaboration with the Institute of Hematology and Transfusion Medicine (IHTM) in Poland and the Immunology Research Group at UiT The Arctic University of Norway. As part of PREVFNAIT, a research biobank was established. The biobank included blood samples from HPA-1a negative women, their newborns as well as the fathers. Details regarding the PREVFNAIT study are published by Dębska et al. [9]. Placental tissue samples from Polish and Norwegian women were added to the PREVFNAIT biobank from 2015 until April 2019. The Norwegian pregnancies were identified through clinical referrals to the Norwegian National Unit for Platelet Immunology (NNUPI) at the University Hospital of North Norway (UNN) in Tromsø. Comprehensive clinical data regarding pregnancy and outcome were registered as part of the study.

2.2. Data collection

Data on maternal age, parity, general health status, antenatal IVIg treatment, estimated date of delivery by ultrasound or last menstrual period, gestational age at delivery (GA), birth weight, sex of the newborn, platelet count at birth, signs of bleeding (petechiae, ICH) and neonatal outcome in cases of ICH were obtained from clinical records. Laboratory data included were: HPA-1 genotype (mother, father and neonate), detection status for anti-HPA-1a antibodies (yes/no), anti-HPA-1a antibody level (IU/mL) and detection of other anti-HPA specific antibodies (yes/no).

The macroscopic placental examination included placental weight, descriptions of the umbilical cord (insertion, length, number of vessels, knots) and description of membranes (color, thickness).

2.3. Inclusion and exclusion criteria

All HPA-1a negative women giving birth to a live born HPA-1a positive neonate were considered for inclusion, irrespective of maternal HPA-1 alloimmunization status (immunized or not-immunized). Twin pregnancies and HPA-1a negative neonates (thus compatible with the mother) were excluded. Women with other HPA antibodies were excluded from the control group as it is not known whether platelet immunization in general may affect the placenta.

2.4. Study design

The study was a systematic prospective study. Placentas included in the study were selected prospectively based on maternal HPA-1 alloimmunization status, and the placenta biobank was created solely for the purpose of this study.

2.5. Definitions

Immunized pregnancies comprised placentas obtained from mothers with anti-HPA-1a antibodies detected in at least one out of four plasma samples taken during pregnancy. HPA-1a negative women without detectable anti-HPA-1a antibodies during pregnancy were defined as non-immunized and the placentas were used as controls. If maternal anti-HPA-1a antibodies were detected only in a sample taken more than 48 h postpartum, and not during pregnancy, the pregnancy was included into the non-immunized group. The immunized group was further subdivided into treated and non-treated mothers. Mothers in the treated group received antenatal IVIg. These two groups were compared with a control group of placentas from non-immunized HPA-1a negative women.

Gestational age was based on ultrasound examination in the 2nd trimester of pregnancy. In cases where ultrasound data were missing (some of the Polish pregnancies), gestational age was calculated using last menstrual period: date of last menstrual period + 280 days (Naegele's formula) [28].

2.6. Case selection

Placenta samples were collected from 59 Polish and Norwegian pregnancies where the mother was HPA-1a negative. Three twin pregnancies and seven HPA-1 compatible pregnancies were excluded. One pregnancy was excluded from the control group due to HPA-3a antibodies. In total, placentas from 48 singleton pregnancies were included for further analyses. Maternal anti-HPA-1a antibodies were detected in

27 women (immunized group), of which ten women received antenatal IVIg treatment. All immunized pregnancies were compared with a control group of 21 placentas from non-immunized HPA-1a negative women. Through PREVFNAIT, 29 women were identified prospectively, while 19 women were recruited in retrospect due to previous FNAIT history. One placenta from the control group was from a donor oocyte pregnancy.

2.7. HPA-1 antigen typing

HPA-1a antigen typing was determined by flow cytometry (FACS) [29] or by real-time polymerase chain reaction (PCR) [30], using DNA isolated from blood samples.

2.8. Detection and quantification of anti-HPA-1a antibodies

HPA-1a negative women participating in the PREVFNAIT study were screened for anti-HPA-1a antibodies at 16–20, 28, 32 and 40 weeks of gestation, as well as six weeks after delivery. For the Norwegian women, longitudinal repetitive measurements were taken roughly every 4th week, starting at 16–20 weeks of gestation. For assessment, the highest anti-HPA-1a antibody level measured during pregnancy was used. For pregnancies where the mother received antenatal IVIg treatment, we included the highest measurement taken before treatment commenced. Detection and quantification of anti-HPA-1a antibodies were performed using monoclonal antibody immobilization of platelet antigen (MAIPA) technique [31,32]. For two pregnancies, anti-HPA-1a antibodies were not detected in MAIPA. Due to a previous history of FNAIT, samples from these pregnancies were retested using PAK Lx (Immucor, Georgia, USA), a bead-based qualitative immunoassay. The test was performed on Luminex 200 (Luminex Corp., Austin, USA). In order not to exclude these weak antibody-responses from the analyses, we chose to give these cases an arbitrary very low value of 0.01 IU/ml.

2.9. Placenta sampling and examination methods

Placental tissue samples for histology were collected as a) transverse sections extending from the fetal/chorionic plate to the maternal/basal plate, b) transverse sections from the umbilical cord, c) membrane roll from the chorioamniotic membrane and d) additional sections from the basal plate. The sections were formalin fixed (10 % buffered formalin), routinely processed and paraffin embedded at UiT The Arctic University of Norway. The tissue blocks from the paraffin embedded placental tissue were serially cut in 3.5 μm thick section on Microm HM355S microtome (Leica Biostems, Wetzlar, Germany). Sections from all cases were automatically stained with Hematoxylin and Eosin (HE) (Sakura, Zoeterwoude, The Netherlands).

The HE and immunostained sections were scanned on a Pannoramic 250 slide scanner (3DHistech, Budapest, Hungary). The HE sections were analyzed independently by two investigators (GT and NHN) according to internationally agreed histological placental criteria (Amsterdam Workshop Consensus Group) [33,34].

Selected sections with extensive villitis and intervillositis were immunostained using a Ventana BenchMark ULTRA Autostainer System (Roche Diagnostics International AG, Rotkreuz, Switzerland), an automated immunostaining system based on the ABC avidin-biotin-peroxidase method. Optimal antigen retrieval, antibody concentrations and incubation times were pre-tested with positive and negative controls for the antibodies CD34 (endothelial marker), CD8 (marker for cytotoxic T-lymphocytes) and CD68 (marker for monocytic/histiocytic cells). One case from the immunized untreated group was selected for triple immunostaining using the same antibodies. Sequential staining was performed, with denaturation of the secondary antibody and neutralization of enzymes between each sequence. For the triple immunohistochemistry, optimal antigen retrieval, antibody concentrations and incubation times were pre-tested with positive and negative controls for the antibodies CD34 (endothelial marker, QBEn/10, mouse monoclonal, Ventana), CD8 (marker for cytotoxic T-lymphocytes, NLC-L-CD8-4B11, mouse monoclonal, Novocastra) and CD 68 (marker for monocytic/histiocytic cells, KP1, mouse monoclonal, Dako).

The immunohistochemistry was analyzed by two pathologists (GT and BR). CaseViewer (3DHistech, Budapest, Hungary) was used as software to visualize the scanned slides. GT was blinded to information concerning status on maternal immunization and antenatal treatment. The inter-observer correlation of the grading of inflammation was assessed by Cohen's kappa scores.

2.10. Histopathological evaluation

Histological criteria were discussed and agreed upon prior to the histological examination (Table 1). Each sample was analyzed by GT and NHN with re-discussion and consensus agreement in case of doubt. Inflammatory conditions included chronic chorioamnionitis, chronic villitis of unknown etiology, chronic histiocytic intervillositis and chronic deciduitis.

2.11. Statistical analysis

All statistical analyses were performed using SPSS (Version 25.0 SPSS, SPSS Inc., Chicago, IL, USA). Quantitative data were described by their mean and standard deviation if the data were normally distributed. If the data were skewed, we used median and range. Qualitative data were described by their frequency and percentages. An independent sample *t*-test was used to compare means for continuous dependent variables between the groups. If the data were skewed, we used Mann-Whitney *U* test (Non-parametric test). For assessing the relationship between non-continuous variables, Fisher's exact test was used. Adjusted birth weights were calculated through a linear regression model adjusting for gestational age at delivery. The level of significance was set at $p < 0.05$. Statistical analysis included the evaluation of inter-observer agreement using kappa statistics (Cohen's k) with possible

Table 1
Morphological/histopathological criteria [33–38].

Placental lesions	Classification	Microscopic findings
Chronic Chorioamnionitis	**Definition**	Infiltration of lymphocytes in chorioamniotic membranes or chorionic plate.
	Low grade	Patchy inflammation or ≥3 foci
	High grade	Diffuse inflammation
Villitis of unknown etiology	**Definition**	Lymphocytic infiltration in the placental villi with stroma destruction[a]
	Low grade	<10 villi pr. focus, either focal (2–3 foci) (observed in one location) or multifocal in more than 3 foci (observed in more than one location)
	High grade	>10 villi pr. focus affected, patchy with more than one focus and diffuse with more than 5 % affected villi
Chronic histiocytic intervillositis	**Definition**	Infiltration of lymphocytes and histiocytes/macrophages in the intervillous space
	Low grade	5–50% of the intervillous space affected
	High grade	>50% of the intervillous space affected
Chronic Deciduitis	**Definition**	Presence of plasma cells in the decidua and/or heavy infiltration of lymphocytes
	Low grade	<50 lymphocytes/high power field (HPF) are present
	Moderate grade	>50 lymphocytes are present multifocal with few confluent areas
	High grade	Several confluent areas with diffuse inflammation are visible

[a] Chronic villitis in anchoring villi were not included [34].

values ranging between 0 (indicating no agreement) and 1 (indicating complete agreement). Interpretations of agreement were used according to Landis and Koch [39].

2.12. Ethical considerations

The PREVFNAIT study was approved by The Bioethical Committee at the Institute of Hematology and Transfusion Medicine (IHTM), Warsaw, Poland (Approval no: 38/2013). The PREVFNAIT biobank established in Norway was approved by the Regional Committee for Medical Research Ethics, North Norway (2014/83). Norwegian placental biopsies were added to the PREVFNAIT biobank in 2015 for the purpose of this study and the current study was approved by the Regional Committee for Medical Research Ethics, North Norway (REK Nord 2015/2192). Written informed consent was obtained from all participants.

3. Results

Placentas from 48 singleton pregnancies were included in the study. Clinical characteristics comparing immunized pregnancies (both treated and untreated) and controls are shown in Table 2. There were significantly more nulliparous women among the controls compared to the immunized group ($p = 0.008$). The median gestational age at delivery was lower among the immunized pregnancies ($p < 0.01$). This is as expected since women with anti-HPA-1a antibodies are often delivered by caesarean section 1-2 weeks prior to term [20]. Adjusted median birth weight was lower in neonates from the immunized group compared with controls ($p < 0.01$). Two neonates from the immunized

Table 2
Clinical characteristics of 48 pregnancies comparing immunized pregnancies (both non-treated and IVIg-treated) with controls.

Clinical and laboratory data	Non-treated immunized pregnancies, n = 17 (A)	IVIg-treated immunized pregnancies, n = 10 (B)	Immunized pregnancies (all) n = 27 (C)	Non-immunized controls n = 21 (D)	p-value
Maternal age, mean ± *SD*	32.2 ± 6.5	35.4 ± 3.7	33.4 ± 5.4	33.1 ± 5.4	AD = 0.637 BD = 0.227 CD = 0.859
Nulliparous women, n (%)	7 (41.2)	0	7 (25.9)	14 (66.7)	AD = 0.190 BD = 0.000 CD = 0.008
Smoking, n (%)	0	0	0	0	
C-section, n (%)	16 (94.1)	6 (60.0)	22 (81.5)	12 (57.1)	AD = 0.012 BD = 1.0 CD = 0.109
Gestational age in weeks, median (range)	37.7 (35.7–40.0)	37.6 (25.1–40.3)	37.7 (25.1–40.3)	39.4 (38.0–40.9)	AD= <0.01 BD= <0.01 CD= <0.01
Adjusted birth weight*, median (range)	3142.4 (2789.0–3524.9)	3114.6 (994.2–3557.1)	3142.4 (994.2–3557.1)	3427.1 (3177.0–3682.4)	AD= <0.01 BD= <0.01 CD= <0.01
Placental weight, mean ± *SD* Missing, n (%)	565.9 ± 95.1 10 (58.8)	494.6 ± 142.3 3 (30.0)	530.2 ±122.0 13 (48.1)	569.7 ± 70.1 8 (38.1)	AD = 0.919 BD = 0.127 CD = 0.318
Sex of the fetus, boys n (%)	7 (41.2)	7 (70)	14 (51.9)	10 (47.6)	AD = 0.752 BD = 0.280 CD = 1.0
Lowest (nadir) neonatal platelet count, median (range)	114.0 (7–291)	131.5 (39–242)	114 (7–291)	263 (111**-372)	AD= <0.01 BD= <0.01 CD= <0.01
ICH, n (%)	1 (5.9)	1 (10.0)	2 (7.4)	0	AD = 0.447 BD = 0.323 CD = 0.497

*Adjusted for gestational age.
**Two women in the control group gave birth to children with neonatal thrombocytopenia. The first child had a platelet count of 265×10^9/L at birth, later measured to 118×10^9/L. The other child had a platelet count of 111×10^9/L.

pregnancies were diagnosed with ICH and none among the non-immunized. The first neonate was a boy born with birthweight 1050 g in week 25 and thrombocytopenia at birth (50×10^9/L). Due to a previous severe obstetric history of FNAIT and stillbirths, this mother was treated with IVIg already from week 15. The second neonate was a girl born in week 36 with a neonatal platelet count of 56×10^9/L and weighed 2900 g. This mother had no previous history of FNAIT, and she did not receive any treatment in the current pregnancy.

Antenatal IVIg is usually offered to women whose neonates were affected by FNAIT in a previous pregnancy. All women receiving antenatal IVIg-treatment were thus multipara, compared to 41.2% nullipara among non-treated immunized pregnancies ($p = 0.026$) (supplementary Table 1). There was a trend that IVIg-treated women had higher median anti-HPA-1a antibody level (10.0 IU/ml, range 0.01–178.7) compared to non-treated women (5.1 IU/ml, range 0.01–158.9, $p = 0.874$). Almost a third of the HPA-1a immunized women 8/27 (29.6%) had other anti-HPA antibodies (seven with anti-HPA-3a antibodies and one with HPA-15a antibodies).

The histopathological findings are listed in Table 3. Overall, chronic inflammatory lesions were observed in 74.1% of placentas from the immunized group (with and without treatment), a significantly higher portion compared to the control group ($p = 0.019$). Chronic histiocytic intervillositis was detected in 40.7% of placentas from immunized pregnancies, all low grade, compared to none in the control group ($p = 0.001$). Notably, both pregnancies with fetal/neonatal ICH had chronic histiocytic intervillositis. Villitis of unknown etiology was observed more often in the immunized group (37.0%) compared to controls (14.3%), although the association was not statistically different ($p = 0.107$). One case from the immunized untreated group was defined as high grade, the remaining cases were all low grade. There were no significant differences between the immunized group and controls for chronic chorioamnionitis ($p = 0.186$) and chronic deciduitis ($p = 1.0$).

Antenatal IVIg treatment did not influence the frequency of villitis of unknown etiology or chronic histiocytic intervillositis among immunized pregnancies (supplementary Table 1). Of note, chronic deciduitis was observed more than twice as frequent in placentas from treated pregnancies compared to untreated pregnancies, although the association was not statistically significant. Chronic histiocytic intervillositis correlated with a non-significant higher maternal antibody level (19.1 IU/ml) compared to immunized pregnancies without this lesion (4.2 IU/ml), while the neonatal platelet count was lower (112 vs 141.5 $\times 10^9$/L, supplementary Table 2).

The overall interobserver agreement between GT and NHN was 0.67, indicating substantial agreement. The Kappa values were 0 for chronic chorioamnionitis ($n = 2$), 0.789 for villitis of unknown etiology, 0.94 for chronic histiocytic intervillositis and 0.953 for chronic deciduitis.

Triple-staining on a selected case from the immunized untreated group with high grade chronic villitis and low grade chronic histiocytic intervillositis, revealed destruction of fetal villous capillaries in areas with high grade villitis. Immunohistochemical analysis revealed gradual destruction of the CD34 positive villous capillary endothelium, ending up with avascular villi. The inflammatory infiltrate in the villi and the intervillositis was dominated by CD68 positive monocytes/histiocytes and CD8 positive T-cells.

Table 3
Chronic inflammatory lesions from HPA-1a immunized pregnancies (treated and non-treated) and non-immunized controls.

Chronic inflammation	HPA-1a immunized pregnancies n = 27	Non-immunized controls n = 21	p-value
Chorioamnionitis, n (%)	0	2 (9.5)	0.186
Villitis of unknown etiology, n (%)	10 (37.0)	3 (14.3)	0.107
Histiocytic intervillositis, n (%)	11 (40.7)	0	0.001
Deciduitis, n (%)	10 (37.0)	7 (33.3)	1.0
At least one type of chronic inflammation, n (%)	20 (74.1)	8 (38.1)	0.019

4. Discussion

This is the first prospective study of chronic placental inflammatory lesions in relation to maternal platelet alloimmunization. We found a strong association between anti-platelet antibodies towards the HPA-1a antigen in maternal plasma and chronic histiocytic intervillositis in the placenta. Although not significant, villitis of unknown etiology was found twice as often in cases of maternal HPA-1a alloimmunization as compared to non-immunized mothers. This supports the hypothesis that an alloimmune response may play a role in the pathophysiology of unexplained chronic villous/intervillous inflammatory lesions [40,41].

The concept that chronic inflammation in the placenta is linked to maternal platelet alloimmunization and FNAIT is not novel. Althaus *et al.* analyzed placentas from 14 FNAIT-affected pregnancies and reported that chronic villitis was frequently manifested in placentas from untreated FNAIT pregnancies compared to the IVIg-treated group [18]. A case report from Tchakarov, described one FNAIT pregnancy with massive chronic intervillositis [19]. Moreover, a recent retrospective cohort study reported an association between FNAIT and chronic chorioamnionitis, basal chronic villitis and chronic intervillositis [17]. However, these placentas were identified retrospectively based on clinical indications other than FNAIT. Additionally, several of the cases included were siblings or dichorionic twins. In our study, placentas were prospectively included based on maternal HPA-1a status alone. Furthermore, our control group was carefully selected from HPA-1a negative women.

Although the exact mechanism of IVIg remains incompletely understood, antenatal IVIg treatment is widely used in pregnant HPA-1a negative women to prevent fetal/neonatal ICH [42,43]. Murine models have shown that IVIg ameliorates vascular damage induced by platelet antibodies [44]. The possible effect of IVIg treatment with regards to placental inflammation has not been systematically studied. However, Altheus *et al.* reported that the seven women who received IVIg treatment displayed no signs of chronic villitis, while it was frequently observed in six untreated pregnancies [18]. In our study, antenatal treatment with high-dose immunoglobulins did not seem to have a protective effect against chronic placental inflammations. In fact, placentas from treated pregnancies had a higher frequency of chronic deciduitis compared to untreated pregnancies (not significant). Noteworthy, in the 10 pregnancies where antenatal IVIg was given, all mothers had an obstetric history of FNAIT, suggesting a possible selection bias. Women with a prior history of FNAIT in their offspring will in general have more severe HPA-1a alloimmunization compared to any HPA-1a negative pregnant woman identified through screening. Assuming a correlation between the level of HPA-1a alloantibodies and the severity of chronic placental inflammations, we could expect a higher incidence of placental lesions in the treated group. Yet, there were no major differences between the immunized treated pregnancies versus non-treated immunized pregnancies regarding the frequency of chronic inflammatory lesions. Whether the lack of any significant difference in placenta lesions is explained by IVIg treatment, or whether IVIg had no effect at all, cannot be deciphered. The possible clinical effect of antenatal IVIg on placental function needs to be studied further. The large number of chronic deciduitis seen in our sample population may have been over-diagnosed as we looked for either plasma cells and/or extensive amounts of lymphocytes in the decidua. Due to the normal abundance of T-cells in the decidua, chronic deciduitis may be poorly reproducible unless plasma cells are seen [45].

Chronic histiocytic intervillositis is a rare placental lesion (0.17% of pregnancies) [48], first described in 1987 by Labarrere and Mullen [49]. The observed frequency of this lesion in more than 40 % among the HPA-1a alloimmunized mothers in this study is therefore remarkably high. Chronic histiocytic intervillositis is clinically important because of

its associations with adverse pregnancy outcomes: recurrent miscarriages, fetal growth restriction or intrauterine fetal death (IUFD) [14,50–52]. Chronic histiocytic intervillositis also has a high recurrence rate in subsequent pregnancies [53]. All the placentas with chronic histiocytic intervillositis in the current study were of low grade and ended in a live born neonate. This is in line with previous observations, where pregnancy loss is associated mainly with high grade chronic histiocytic intervillositis [44]. Two of the women in the IVIg-treated group of immunized pregnancies had a previous obstetric history of IUFD. In fact, one of these women had two previous pregnancies ending with IUFD, where the current child was born with ICH and the placenta showed low-grade chronic histiocytic intervillositis. We do not have histopathological results from the IUFD pregnancies, as these pregnancies happened before this study. It is tempting to speculate whether a more severe chronic histiocytic intervillositis combined with platelet alloimmunization may have contributed to the devastating outcome of the previous pregnancies. The other placenta showed chronic villitis. Immunohistochemical staining of a single placenta revealed gradual destruction of fetal villous capillaries in areas with high grade villitis, ending up with avascular villi. The destruction of the capillary endothelium seemed to be linked to CD8-reactivity, as villi with CD8 positive T-cells were seen in villi with fragmented capillary endothelium, ending up in avascular villi. Villi without T-cells had intact capillary endothelium. Fetal endothelial cells being actively damaged in FNAIT has been hypothesized by Altheus *et al.* [18], but this is the first time it is shown with triple immunohistochemistry staining (Fig. 1). The inflammatory infiltrate in the villi and the intervillous space was dominated by CD68 positive monocytes/histiocytes and CD8 positive T-cells. The infiltrating T-lymphocytes in chronic villitis of unknown etiology have been demonstrated to be of maternal origin [54]. The molecular mechanism of how these maternal cytotoxic T-cells enter the fetal compartment however, is still not clear [16]. Accumulating evidence suggests that chronic villous/intervillous inflammatory lesions are caused by a maternal immune reaction against paternal/fetal antigens, analogous a host-versus-graft reaction. Since the fetus has inherited half of its genes from the father, it is considered to be a semi-allograft in the mother. The effector mechanism for rejection in antibody-mediated allograft damage involves complement activation [16]. C4d, a breakdown product of the classic complement cascade, has been detected on villous syncytiotrophoblasts and endothelial cells of placentas with villitis of unknown etiology [55]. Interestingly, a strong villous C4d staining was also demonstrated on a placenta with chronic histiocytic intervillositis lacking villous inflammation [55]. Gene expression profiles have also shown that genes involved in graft-versus-host disease pathways are significantly altered in placentas with chronic villitis of unknown etiology compared to unaffected controls [56].

Human leukocyte antigens (HLA) are important predictors of transplant rejection [57]. A study by Lee *et al.* demonstrated that mothers with chronic chorioamnionitis and villitis of unknown etiology had significantly higher anti-HLA class I seropositivity than those without these lesions [58]. In our study, we did not look at HLA-antibodies, nor other detectable HPA antibodies. As nearly 30% of the alloimmunized women also had other detectable HPA antibodies, we cannot rule out the possibility that these antibodies may also have an effect. The possible link between maternal anti-HLA class I antibodies and FNAIT has been extensively studied, but a definite causative relationship is not yet established [46,59–61]. Future studies addressing maternal anti-HLA class I antibodies in connection with both placental inflammations as well as neonatal thrombocytopenia may aid to clarify these issues.

Understanding the immunological mechanisms behind cell migration into the chorionic villi and fetal endothelium is crucial when aiming to prevent and treat pregnancies at risk of placenta-related complications such as chronic villous/intervillous inflammatory lesions of (yet) unknown etiology. Of clinical interest would be to assess whether mothers having pregnancy complications related to these inflammations should be examined for platelet alloimmunization. Future studies may clarify whether chronic placental inflammation and FNAIT truly are distinct entities, or perhaps originate from similar pathophysiologic mechanisms. An interesting question is whether it is the alloantibodies that induces inflammation or perhaps the other way around.

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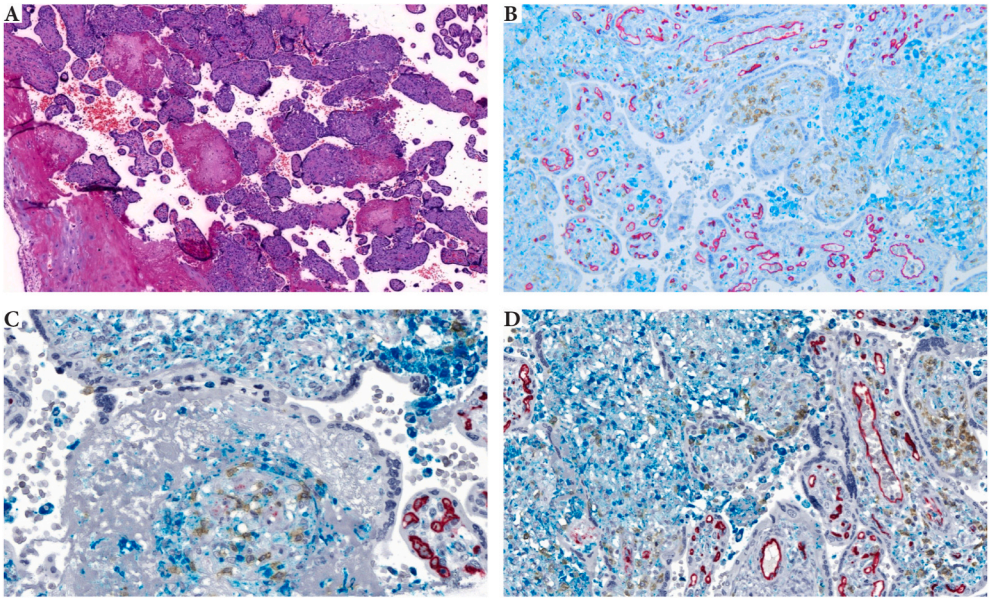

Fig. 1. Placental villi from the immunized untreated group, with lymphocytic infiltration demonstrating combined chronic histiocytic intervillositis and chronic villitis. **A**: A Hematoxylin-Eosin stained placental tissue with high grade chronic villitis (5x). **B**: Closer view on the same histological section, immunostained with a combination of CD 34 (red stained fetal endothelium), CD 8 (brown stained T8-lymphocytes) and CD68 (turquoise stained monocytes/macrophages) with contrast staining using hematoxylin (blue nuclei and greyish stroma) (10x). The picture shows villi with intact villous capillaries, in contrast to villi with only fragments of CD34 positive cells and brown CD8 positive T-cells. Avascular villi without intact capillary endothelium were dominated by brown CD8 positive T-cells and turquoise CD68 positive monocytes/histiocytes. The CD68 positive cells dominated in the intervillous space as well as inside the fetal compartment, together with a few brown CD8 cells. **C:** Closer view on one villos showing CD8 positive T-cells in the core of the villi, surrounded by CD68 positive monocytes/histiocytes and a few red spots indicating fragmented fetal endothelium (41.1x). **D**: Villi dominated by CD68 positive monocytes/histiocytes showed more fetal endothelial damage compared to villi with many CD8 positive T-cells (27x).

Authors' contributions

HT, AH and EB conceived the project and planned the overall study protocol. NHN prepared and processed all placental samples and together with GT, analyzed the HE stained placental slides. The immunohistochemistry was analyzed by BR and GT. NHN and HT wrote the manuscript. MN supervised in preparing and processing placental samples. VS collected placenta samples from patients recruited from Oslo University Hospital and analyzed the data. KG, EB, MD and MU recruited, diagnosed and followed all Polish PREVNAIT participants. EP performed the macroscopic evaluation of placentas and took placental biopsies from all Polish placentas. All authors contributed to analyses and interpretation of data, critically revised the manuscript and approved the final version.

Declarations of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: AH is one of the founders and owners of Prophylix Pharma AS, which has been developing a prophylaxis for the prevention of FNAIT. Prophylix Pharma did not finance or influence the study. The company has been sold to RallyBio. The other authors declare that they have no competing interests.

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Supplementary

Table 1
Chronic inflammatory lesions from non-treated and IVIg-treated HPA-1a immunized pregnancies

Chronic inflammation	Non-treated immunized pregnancies, n = 17	IVIg-treated immunized pregnancies, n = 10	p-value
Chorioamnionitis, n (%)	0	0	–
Villitis of unknown etiology, n (%)	7 (41.2)	3 (30.0)	0.692
Histiocytic intervillositis, n (%)	7 (41.2)	4 (40.0)	1.0
Deciduitis, n (%)	4 (23.5)	6 (60.0)	0.101
At least one type of chronic inflammation, n (%)	12 (70.6)	8 (80.0)	0.678

Table 2
Clinical and laboratory findings for HPA-1a immunized pregnancies with and without chronic histiocytic intervillositis

All immunized cases	Immunized pregnancies with chronic histiocytic intervillositis, n = 11	Immunized pregnancies without chronic histiocytic intervillositis, n = 16	P-value
Maternal age, mean ± *SD*	34.7 ± 6.1	32.4 ± 5.5	0.315
Nulliparous women, n (%)	2 (18,2)	5 (31.3)	0.662
Highest Anti-HPA-1a antibody level during pregnancy and before IVIg, IU/ml median (range)	19.1 (0–158,85)	4.2 (0.01–178.71)	0.421
Other HPA antibodies detected during pregnancy, n (%)	3 (27,3)	5 (31.3)	1.0
IVIg, n (%)	4 (36,4)	6 (37.5)	1.0
C-section, n (%)	10 (90,9)	12 (75.0)	0.618
Gestational age in weeks, median (range)	36,9 (25,1-40,3)	37,9 (32,7–40,0)	0.099
Birth weight, median (range)	3060,0 (1050–3870)	3042.5 (1890–3714)	0.928
Sex of the fetus, boys, n (%)	6 (54,5)	8 (50.0)	1.0
Neonatal platelet count, nadir, median (range)	112 (21–253)	141.5 (7–291)	0.645
Neonatal thrombocytopenia, n (%)	6 (54,5)	8 (50.0)	1.0
Previous FNAIT history, n (%)	8 (72,7)	8 (50)	0.427

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