Unraveling the role of maternal anti-HLA class I antibodies in fetal and neonatal thrombocytopenia—Antibody specificity analysis using epitope data

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ABSTRACT

Anti-HLA class I antibodies have been suggested as a possible cause of fetal and neonatal alloimmune thrombocytopenia (FNAIT). The aim of this study was to characterize maternal anti-HLA class I alloantibodies in suspected cases of FNAIT.

The study population consisted of all nationwide referrals of neonates with suspected FNAIT to the National Unit for Platelet Immunology in Tromsø, Norway, during 1998–2009 (cases), and 250 unselected pregnancies originally included in a prospective study (controls). Inclusion criterion was a positive screening for maternal anti-HLA class I antibodies. Neonates with other identifiable causes of thrombocytopenia, including maternal anti-human platelet antigens (HPA) antibodies, were excluded. Ultimately, 50 cases with suspected FNAIT were compared with 60 controls.

The median neonatal platelet count nadir among cases was 24×10^9/L (range $4–98 \times 10^9$/L). Five children (10%) were reported to have intracranial hemorrhage. Maternal and neonatal HLA class I genotype was available for 33 mother/child pairs (66%). Immunization was not tied to any particular HLA class I antigen. Using epitope mapping, we could demonstrate that the maternal anti-HLA class I antibodies were specific towards mismatched paternally-inherited fetal epitopes, with little reactivity towards any third-party epitopes. Antibody reactivity patterns were similar to those found among controls, although the mean fluorescence intensities (MFI) among cases were significantly higher.

This study demonstrates the value of using data on HLA epitope expression, instead of HLA antigens, to examine alloimmune responses in connection with neonatal thrombocytopenia. Our findings support the idea that maternal anti-HLA class I antibodies are involved in FNAIT.

1. Introduction

Anti-HLA class I antibodies are detected in as many as 30–60% of all pregnant women, depending on parity and detection method (Morin-Papunen et al., 1984; Regan et al., 1991; King et al., 1996; Masson et al., 2013). Although the presence of anti-HLA class I antibodies can cause significant harm in other settings, such as platelet refractoriness following transfusion (Stanworth et al., 2015) and rejection of kidney allografts (Lee et al., 2002), their role during pregnancy remains uncertain (Lashley et al., 2013).

Maternal antibodies that react with incompatible fetal human platelet antigens (HPAs) can lead to fetal and neonatal alloimmune thrombocytopenia (FNAIT). Intracranial hemorrhage (ICH) in the fetus or newborn is the most feared complication of FNAIT. The diagnosis is most commonly suspected after detection of spontaneous bleedings, such as petechiae, or severe thrombocytopenia in an otherwise healthy

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term newborn. Although there are many causes of neonatal thrombocytopenia, FNAIT is the most common cause of severe thrombocytopenia in otherwise healthy term newborns (Burrows and Kelton 1993; Sainio et al., 2000).

In addition to HPAs, platelets also express HLA class I antigens. A strong presence of maternal anti-HLA class I alloantibodies is a common finding in suspected cases of FNAIT where no maternal anti-HPA antibodies can be detected. Based on these observations, it has long been hypothesized that anti-HLA class I antibodies can cause FNAIT (Saito et al., 2003; Moncharmont et al., 2004; Thude et al., 2006; Gramatges et al., 2009; Starcevic et al., 2010; Bonstein et al., 2015; Meler et al., 2016). Prospective studies on the role of maternal anti-HLA class I antibodies in connection with neonatal thrombocytopenia are few, involve small study populations, and the results have been inconclusive (Sharon and Amar 1981; Koyama et al., 1991; Panzer et al., 1995; King et al., 1996).

The aim of this study was to characterize maternal anti-HLA class I alloantibody responses in suspected cases of FNAIT, by studying referrals to the Norwegian National Unit for Platelet Immunology over an 11-year period and comparing these with an unselected population of pregnant women. We hypothesized that anti-HLA class I antibodies detected in cases of suspected FNAIT are specific towards paternal HLA antigens, and that certain fetal-maternal HLA class I mismatches are associated with fetal/neonatal thrombocytopenia.

2. Materials and methods

2.1. Study population

All cases of suspected FNAIT referred to the Norwegian National Unit for Platelet Immunology at the University Hospital of North Norway (UNN) in Tromsø, Norway, during the period January 1998 − April 2009 were considered for inclusion. We identified all pregnancies referred because of thrombocytopenia in the newborn, where maternal anti-HLA class I antibodies were detected. Cases with detectable anti-HPA antibodies or other identifiable causes of the thrombocytopenia (as further described under Results and in Fig. 1) were excluded. Maternal blood samples among the cases were collected postpartum, and stored as plasma. Maternal samples in the case population had a median sample time of six days postpartum. Only four of the samples were collected > 15 days postpartum. Sample time was missing for two cases.

Information regarding demographic characteristics, obstetric history, course and outcome of the pregnancy was obtained from medical records. All pregnancies were dated based on routine ultrasonography performed in the second trimester. Preeclampsia was diagnosed according to current ISSHP criteria (Tranquilli et al., 2014).

The controls were identified among an unselected population of pregnant women originally included in a prospective study investigating maternal-fetal haemodynamics and endothelial function at the University Hospital of Northern Norway during 2006–2010 (Flo et al., 2010, 2014), and were screened for anti-HLA class I antibodies during 22–24 weeks of gestation. Maternal blood samples were stored as plasma.

Information on transfusion history was not available for neither cases nor controls.

2.2. Definitions

Thrombocytopenia was defined as a platelet count $< 150 \times 10^9$/L, moderate thrombocytopenia as platelet count $50–149 \times 10^9$/L and severe thrombocytopenia as a platelet count $< 50 \times 10^9$/L.

Small for gestational age (SGA) was defined as birth weight less than the 10th percentile for gestational age based on singleton percentile curves (Skjaerven et al., 2000).

Infants born before 37^0 gestational weeks were defined as premature.

2.3. Laboratory analysis

All maternal samples from suspected cases of FNAIT were first screened for anti-HPA antibodies and anti-HLA class I antibodies using an in-house modified monoclonal antibody immobilisation of platelet antigen (MAIPA) technique (Kiefel et al., 1987; Killie et al., 2010). In MAIPA, samples were tested against paternal platelets when available. If paternal platelets were not available, the sample was tested against random donor platelets from at least four donors. To avoid false negative results due to the limited number of antigens on the random donor platelets, all samples that tested negative for anti-HLA class I antibodies against random donors were also tested with FlowPRA 1 Screening Test (One Lambda, Canoga Park, CA). This assay includes beads that are coated with most of the HLA class I antigens present in a
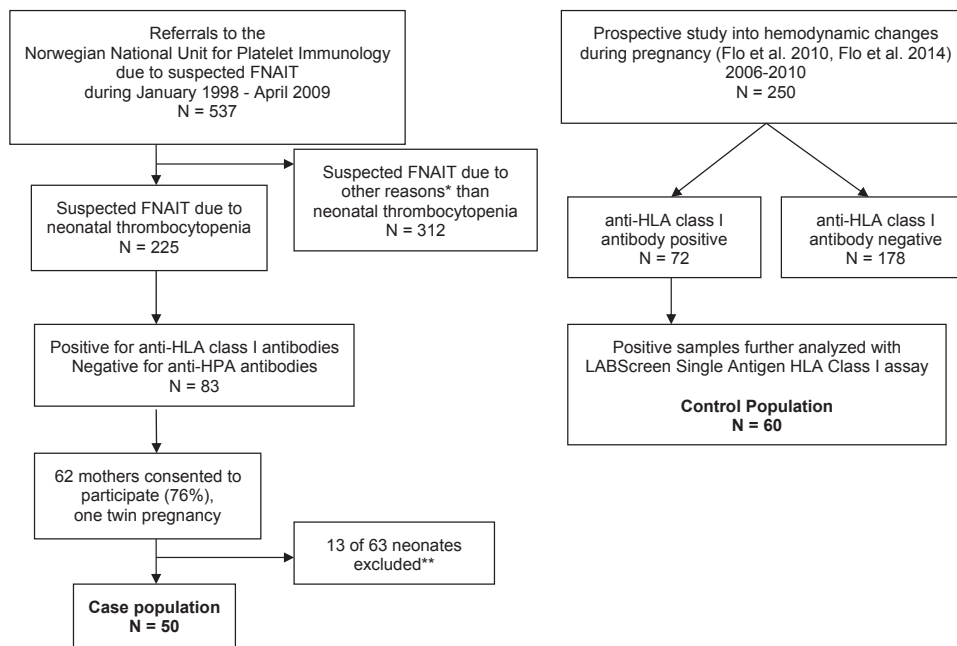

Fig. 1. Selection of Study Population.
*Neonatal thrombocytopenia in earlier pregnancy, low maternal platelet count, recurrent miscarriage, intrauterine fetal death, stillbirth and miscellaneous.
*Eight due to other possible reasons for neonatal thrombocytopenia (two congenital cytomegalovirus infections, one Jacobsen's syndrome, one maternal immune thrombocytopenic purpura, one neonatal hemochromatosis, one Noonan's syndrome, one Down's syndrome, one case of neonatal death 18 days after birth where autopsy showed underdeveloped bone marrow), and five cases where maternal sera were unavailable for antibody analysis.

Northern European population, and gives an overall mean fluorescence intensity (MFI) that reflects the presence or absence of anti-HLA class I antibodies targeting any of the HLA antigens present on the beads. Anti-human IgG antibodies were used to detect maternal antibodies bound to the antigen-coated beads.

After the primary identification of anti-HLA class I antibody-positive cases, all samples were re-tested with FlowPRA 1 Screening Test. All initial tests (MAIPA and FlowPRA 1 Screening Test) were performed at the Norwegian National Unit for Platelet Immunology at UNN, Tromsø, Norway. Maternal samples from the control group were screened with FlowPRA 1 Screening Test. Control samples were not screened for anti-HPA antibodies.

To determine anti-HLA class I antibody specificities, the samples from the suspected FNAIT cases and the antibody positive controls were further analyzed with LABScreen Single Antigen HLA Class I (One Lambda, Canoga Park, CA) assay. This assay not only detects the presence or absence of anti-HLA class I antibodies, like the FlowPRA I Screening Test, but also gives individual MFI responses for the binding of antibodies to specific HLA class I antigens coated on separate beads. Anti-human IgG antibodies were used to detect maternal antibodies bound to the antigen-coated beads. Data from the LabScreen Single Antigen assay was analyzed using HLA Fusion software (One Lambda, Canoga Park, CA) and HLAMatchmaker (http://www.epitopes.net).

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

Genotyping of maternal and neonatal HLA class I was done by in-house sequence-based typing validated and used in routine HLA typing, and analyzed using the Assign Software (Conexio Genomics, Fremantle, Australia). In cases where genotyping indicated two or more likely alleles, the most frequent allele according to data from the Norwegian Bone Marrow Donor Registry was chosen to represent the genotype in question for further analyses.

The LABScreen Single Antigen HLA Class I assay and the genotyping were performed at the Department of Immunology, Oslo University Hospital, Rikshospitalet, Norway.

Each of the beads in the LABScreen Single Antigen HLA Class I assay are coated with one specific HLA-A, HLA-B or HLA-C antigen, for example HLA-A*01:01. The whole assay includes all of the 97 most common antigens in the general population. Each of these antigens are comprised of multiple epitopes. By combining maternal and neonatal genotyping with the data from the HLA Epitope Registry and the MFI signals from the LABScreen Single Antigen HLA Class I assay, we could, in addition to the antigens, also determine the epitopes for the maternal antibodies.

2.4. Epitope analyses

Epitope data was retrieved from HLAMatchmaker (http://www.e-pitopes.net) and the HLA Epitope Registry (http://www.epregis-try.com.br/index/databases/database/ABC/) in February 2016. This data was used to determine which epitopes on the paternally-inherited HLA antigens that were the most probable causes of immunization, i.e. neonatal epitopes that were inherited from the father and not shared by the mother. In order to be a likely cause of immunization the epitope also had to be labeled as "antibody reactive" (confirmed or provisional) in the HLA Epitope Registry (Duquesnoy et al., 2013). Paternally-inherited epitopes found to be the most probable cause of immunization are from here on referred to as "mismatched epitopes".

2.5. Ethics

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

2.6. Statistics

Normality of data distribution was tested using Kolmogorov-Smirnov test. Depending on distribution of the data, independent samples *t*-test or Mann-Whitney *U* test was used to compare means for continuous variables. Variance of continuous variables between groups was tested using One-Way ANOVA with Bonferroni post-hoc test. The Fisher's exact test was used to compare frequencies for categorical variables. When testing correlation between normally and not normally distributed data we report Pearson's correlation coefficient or Spearman's correlation coefficient, respectively. A *P*-value of < 0.05 was considered significant, and 95% confidence intervals (CI) are reported where appropriate.

All statistical data were analyzed using SPSS software (Version 23.0 SPSS Inc., Chicago, IL, USA). Figures and tables were produced using Microsoft PowerPoint, Microsoft Excel, Adobe Photoshop and Plotly (plot.ly).

3. Results

3.1. Study population characteristics

The Norwegian National Unit for Platelet Immunology at UNN received 537 referrals of suspected FNAIT during the study period. 225 of these 537 referrals (42%) were due to neonatal thrombocytopenia. In 83 of these 225 cases (37%), the maternal sample was found to be positive for anti-HLA class I antibodies only, and negative for anti-HPA antibodies.

Of the 82 mothers initially included, 62 consented to participate. There was one twin pregnancy. Thirteen cases were excluded from analysis: Eight due to other possible reasons for neonatal thrombocytopenia (two congenital cytomegalovirus infections, one Jacobsen's syndrome, one maternal immune thrombocytopenic purpura, one neonatal hemochromatosis, one Noonan's syndrome, one Down's syndrome, one case of neonatal death 18 days after birth where autopsy showed underdeveloped bone marrow), and five cases where maternal sera were unavailable for antibody analysis. Thus, data on mother and child from 50 pregnancies with suspected FNAIT were included for further analysis. Selection of this case group is presented as a flow chart in Fig. 1. DNA from mother and child for genotyping was available for 33 (66%) of the 50 mother/child pairs.

The control group consisted of 250 pregnancies (Flo et al., 2010, 2014), where 72 (29%) tested positive for maternal anti-HLA class I antibodies. Based on sample availability, 60 of these 72 positive cases were further analyzed with LABScreen Single Antigen HLA Class I assay (from here on referred to as controls). Platelet counts were obtained from 45 randomly selected neonates in the control group, of which none were thrombocytopenic. DNA from mother and child was only available for 5 mother child/pairs.

3.2. Clinical characteristics

The median neonatal platelet count nadir was 24×10^9/L (range 4–98×10^9/L) in the case group. Most of the newborns (42/47) had severe thrombocytopenia. Five children (10%) had moderate thrombocytopenia. Three children were reported as *thrombocytopenic* in the medical record, but no platelet count was available. Thirteen neonates (26%) had recorded skin bleedings.

Mean gestational age at birth was 38^3 weeks, with nine children being born prematurely (range 28^4-36^6 weeks, median 34^0 weeks). The prematurely born children did not have a significantly lower platelet count nadir than the children born at term (mean difference = 9.5×10^9/L, P = 0.169).

Maternal and neonatal characteristics for the case group are presented in Table 1.

Five neonates (10%) in the case group were diagnosed with an

Table 1
Maternal and Neonatal Characteristics.

	Cases	Controls
	(n = 50)	(n = 60)
Maternal age, mean years (SD)	30.9 (5.2), 1 missing	30.4 (5.0), 0 missing
Nulliparity, n (%)	21 (45), 3 missing	11 (18), 0 missing
Preeclampsia, n (%)	6 (13), 2 missing	1 (2), 0 missing
Delivery by caesarean section, n (%)	24 (50), 2 missing	7 (12), 3 missing
Gestational age at delivery, mean weeks^{days}(SD)	$38^3(3^1)$, 2 missing	39^5 (2^3), 0 missing
Birth weight, mean grams (SD)	3005 (1039), 2 missing	3517 (601), 0 missing
SGA*, n (%)	23 (48), 2 missing	6 (10), 0 missing
Nadir platelet count, median (SD)	24×10^9/L (19), 3 missing*	Not available
Skin bleedings, n (%)	13 (26), 22 missing	Not available
ICH**, n (%)	5 (10), 24 missing	Not availbale

(*) Small for gestational age
(**) Intracranial hemorrhage

*In three cases the neonates were reported as *thrombocytopenic* in patient records, but the precise platelet count could not be obtained.

intracranial hemorrhage. Neonatal platelet count nadirs in this sub-population ranged from 4 to 34×10^9/L. All neonates were born alive, but one died during the neonatal period, and the surviving children were all reported to have severe neurological sequelae. The intracranial bleedings described are miscellaneous in locations and extent. A detailed description of the pregnancies complicated by ICH in the new-born is shown in Supplementary Table S1.

Comments regarding the high frequency of preeclampsia and SGA, as well as detailed clinical characteristics of the controls, can be found in the recent JRI paper by Dahl et al. (Dahl et al., 2016). Maternal and neonatal characteristics of the 60 controls analyzed with the LABScreen Single Antigen HLA Class I assay are also included in Table 1.

3.3. HLA class I allele frequencies

Maternal and neonatal DNA was available for genotyping in 33 of the 50 mother/child pairs (66%) in the case group, and 5 mother/child pairs in the control group. HLA-A, HLA-B and HLA-C allele frequencies of mother and child in the case group are presented in Supplementary Table S2. These allele frequencies were compared with background population data from the Norwegian Bone Marrow Donor Registry (NBMDR) (Nordang et al., 2013): There was a significantly higher frequency of HLA-A*68 (P = 0.028) and HLA-B*08 (P = 0.036) among mothers in the case group. No neonatal allele had a frequency that significantly differed from the background population. P-values were not adjusted for multiple comparisons.

All neonates inherited at least one HLA-A or HLA-B allele from the father that was not shared by the mother. 79% of mother/child pairs were mismatched for HLA-A, and 97% for HLA-B.

Table 2 lists all mismatched alleles in the case group, and their relative proportions. In general, the mismatched alleles in our study group were not rare alleles in the background population, although HLA-A*31 and HLA-B*27 constituted a relatively large proportion of mismatched alleles (12% and 15% of cases, respectively) compared to their frequency in the background population (allele frequencies of 4% and 7%, respectively). However, sample sizes were discrepant (n = 33 for cases versus n = 929 in the background population) and the possibility of selection bias cannot be excluded.

For the five mother/child pairs from the control population where we had DNA available for genotyping, the mismatched alleles were as follows: First control A*26:01 and B*40:02. Second control A*11:01 and B*51:01. Third control B*40:01. Fourth control A*03:01. The fifth control did not have any mismatched HLA-A or −B alleles.

Table 2
Overview of Mismatched Alleles in the Case Population.

HLA Allele	% of Cases, (n)
A*02	30 (10)
B*07	18 (6)
A*01	18 (6)
B*40	15 (5)
B*27	15 (5)
B*08	15 (5)
A*31	12 (4)
A*03	9 (3)
B*51	6 (2)
B*15	6 (2)
B*18	6 (2)
B*35	6 (2)
B*57	3 (1)
B*50	3 (1)
B*56	3 (1)
A*32	3 (1)
A*24	3 (1)
A*25	3 (1)

3.4. Sera from both case and control samples demonstrated broad reactivity against multiple antigens

Results from the LABScreen Single Antigen HLA Class I assay showed that both case and control samples demonstrated broad reactivity against multiple antigens. There were more samples that had one or more beads with MFI > 10 000 among the cases (96%) than among the controls (52%, p < 0.001).

There were higher MFI levels against HLA-B than HLA-A for the case population as a whole, with HLA-B beads having a median MFI of 3893 while HLA-A beads had a median MFI of 838. The median MFI among controls was 282 for HLA-A beads, and 564 for HLA-B beads. This shows that antibody levels were higher among cases compared to controls, and indicates that antibody level may be more important than antibody specificity in relation to neonatal alloimmune thrombocytopenia.

An overview of the results from the LABScreen Single Antigen HLA Class I assay for both cases and controls is presented in Fig. 2.

3.5. Broad reactivity patterns explained by presence of paternal epitopes on multiple beads

All maternal case samples, except one, were strongly reactive towards at least one paternal antigen, and none were reactive towards self-antigens. These broad reactivity patterns persisted after dilution assays were performed in ten maternal samples (data not shown). Due to the wide reactivity patterns, further analyses considering the epitope expression on each bead were performed to determine whether the observed anti-HLA class I antibody responses were paternally specific or not, and to evaluate cross-reactivity. Fig. 3 shows that the majority of all maternal anti-HLA class I antibody reactivity was directed against mismatched paternal epitopes, regardless of which antigen/bead that expressed the epitopes in the LABScreen Single Antigen HLA Class I assay. The heatmap also shows a strong degree of reactivity towards beads with paternal antigens, and no reactivity towards beads with maternal antigens. There was a clear drop in MFI between beads in the LABScreen Single Antigen HLA Class I assay that had at least one mismatched paternal epitope compared with beads that had none (mean MFI difference = 7450, p < 0.001). An increasing number of mismatched epitopes per bead was associated with increasing MFI (r = 0.228, p < 0.001) (Fig. 4).

DNA was available for five mother/child pairs among the controls. One of these had a reactivity pattern similar to that described for most of the cases, with high MFI against the mismatched paternal antigens, as well as any other antigen that also expressed a mismatched paternal
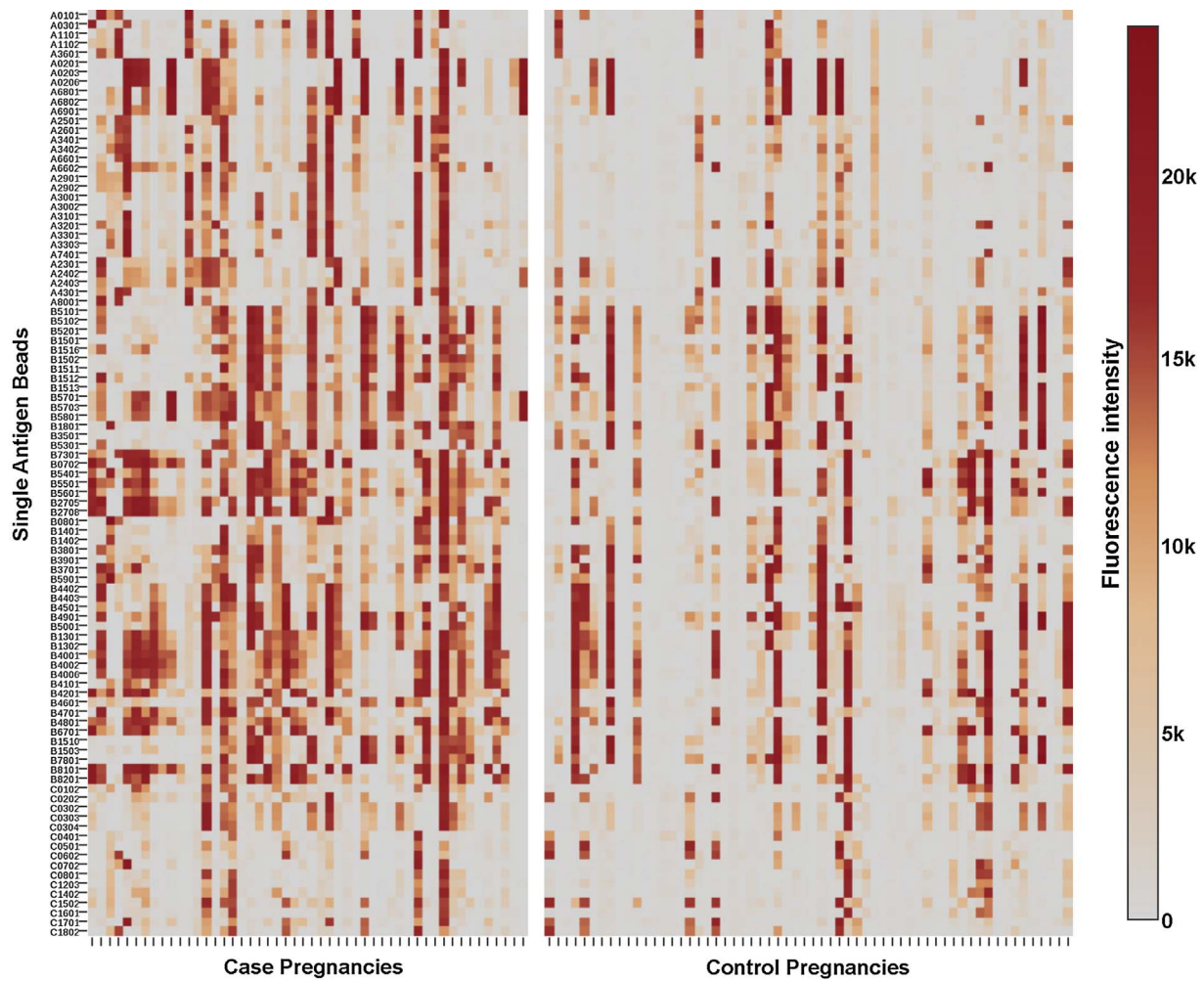
Fig. 2. Antibody Levels and Specificities: Maternal Anti-HLA Class I Antibodies in Pregnancies with Neonatal Thrombocytopenia (Cases) and Normal Pregnancies (Controls) Detected Using LABScreen Single Antigen HLA Class I Beads.

epitope. One appeared strongly reactive, but not against antigens expressed by the present child. The remaining three controls appeared to be only borderline positive, or even negative (Fig. 3).

3.6. Immunizing antigens and clinical outcome

Fetal/paternal-specific MFI was higher in multiparous mothers compared with nulliparous mothers (mean difference = 4494, P = 0.029, Fig. 5b). All of the cases with antibody reactivity patterns that remained unresolved after the described sorting process (cases number 1, 2, 3, 11, 13, 16 and 21) were from pregnancies where the mother was multiparous (data missing for one case). This could indicate that the maternal immunization occurred during a previous pregnancy, potentially against paternal epitopes not inherited by the current child.

There was a tendency towards an inverse correlation between fetal/paternal-specific MFI and neonatal platelet count nadir (r = −0.318, P = 0.081, Fig. 5a). Also, neonates with a reported ICH had a tendency towards higher MFI compared to neonates without a reported ICH (mean difference = 5755, P = 0.187, Fig. 5b).

Interestingly, in the five cases where HLA-B*27 was identified as the immunizing paternal allele, the neonatal platelet count nadir was significantly lower than the rest of the newborns in the study group (mean difference = 15×10^9/L, P = 0.003, Fig. 5c).
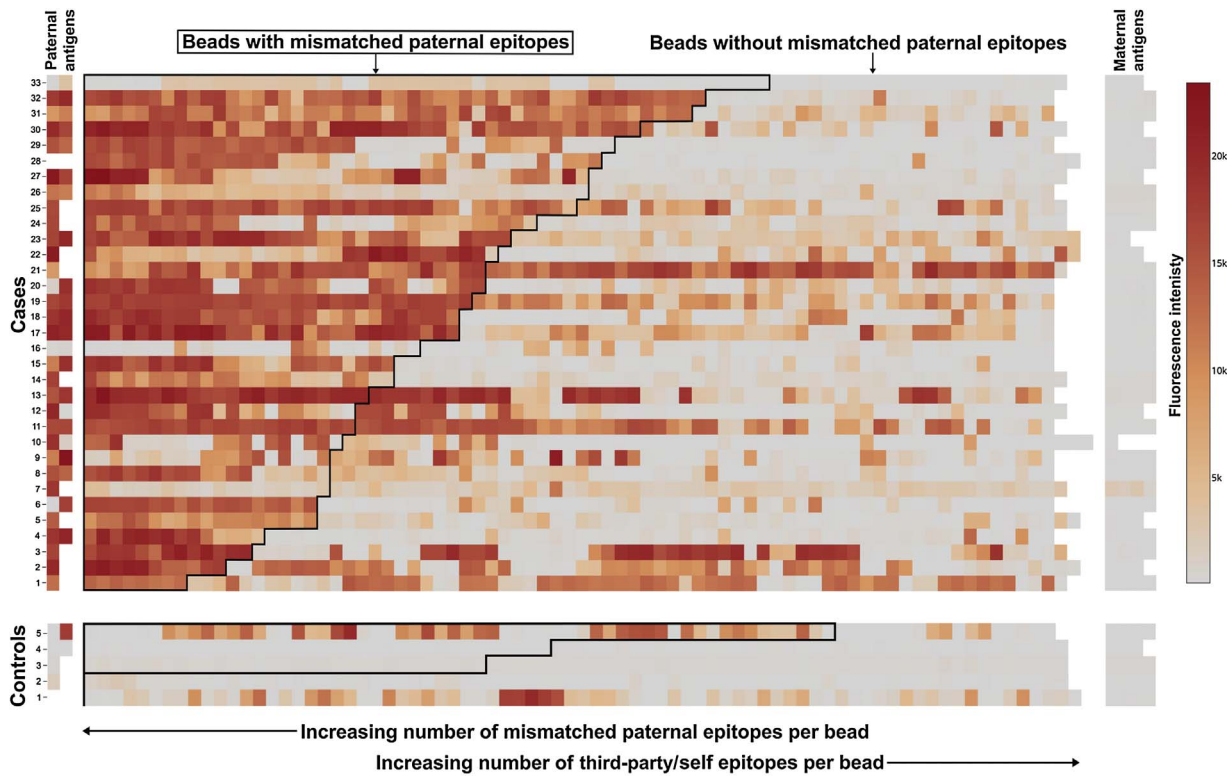
4. Discussion

The possible causative role of maternal anti-HLA class I antibodies

in FNAIT has long been unresolved. In this study we have shown that the maternal anti-HLA class I antibodies in suspected cases of FNAIT are specific towards paternally-inherited fetal epitopes. Antibody level, rather than specificity, may be important for risk of neonatal thrombocytopenia. Our results emphasize the importance of HLA class I epitope data as a platform for investigating alloimmune responses during pregnancy.

The retrospective design of the study implies a possible selection bias. We excluded cases with other apparent causes of thrombocytopenia. However, some conditions, such as non-confirmed infections or genetic syndromes diagnosed later in life, could not be ruled out. A recent report on a similar population of suspected FNAIT due to anti-HLA class I antibodies in Sweden, concluded that there were other possible causes for the thrombocytopenia in most of their cases (Refsum et al., 2016), such as congenital infections, genetic syndromes, maternal ITP or complications related to the delivery.

The high frequency of preeclampsia (12%) and SGA neonates (48%) among the case group implies a possible bias, as both conditions are reported to be associated with neonatal thrombocytopenia (Christensen et al., 2015; Kalagiri et al., 2016). However, neonates born to preeclamptic mothers and neonates who were SGA did not have a lower platelet count compared to the rest of the case neonates, and the correlation between antibodies and birth weight described previously for this population was independent of preeclampsia status (Dahl et al., 2016). Therefore we chose not to exclude preeclamptic pregnancies or pregnancies where the child was SGA from our study population. The unknown cause-effect relationship is also an argument in itself not to
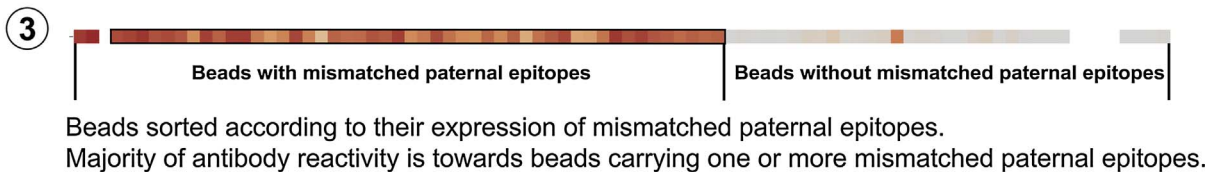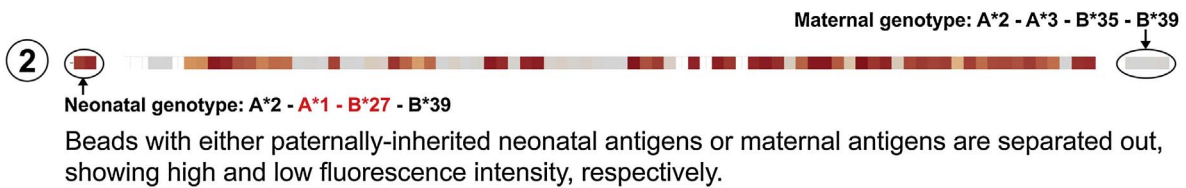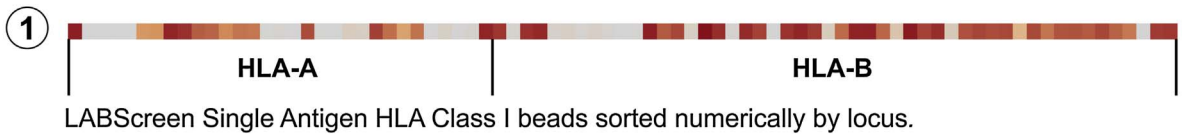
Sorting Process:

① LABScreen Single Antigen HLA Class I beads sorted numerically by locus.

Maternal genotype: A*2 - A*3 - B*35 - B*39

② Neonatal genotype: A*2 - A*1 - B*27 - B*39

Beads with either paternally-inherited neonatal antigens or maternal antigens are separated out, showing high and low fluorescence intensity, respectively.

③ Beads sorted according to their expression of mismatched paternal epitopes. Majority of antibody reactivity is towards beads carrying one or more mismatched paternal epitopes.

Fig. 3. Maternal Anti-HLA Class I Antibody Specificities in Pregnancies with Neonatal Thrombocytopenia and Five Control Pregnancies: LABScreen Single Antigen HLA Class I Beads Sorted by Expression of Mismatched Paternal Epitopes.

exclude high-risk pregnancies.

The presence of antibodies could also be the result of undisclosed factors. For instance, none of the participants were screened for previous transfusions. Repeated blood transfusions may lead to production of anti-HLA class I antibodies, and lack of such data in our study represents a potential bias. However, this is unlikely in a population of young and mostly healthy women of fertile age. Inflammation has also been shown to increase the number of specificities and level of anti-HLA

class I antibodies (Locke et al., 2009). It is therefore possible that the observed difference in antibody levels between cases and controls may be secondary to, for example, an increased inflammatory state among the cases. Still, the mere presence of antibodies may have harmful effects on the pregnancy.

To evaluate whether the difference in time point of sample collection between cases and controls could have interfered with results, we compared samples from seven control pregnancies where maternal
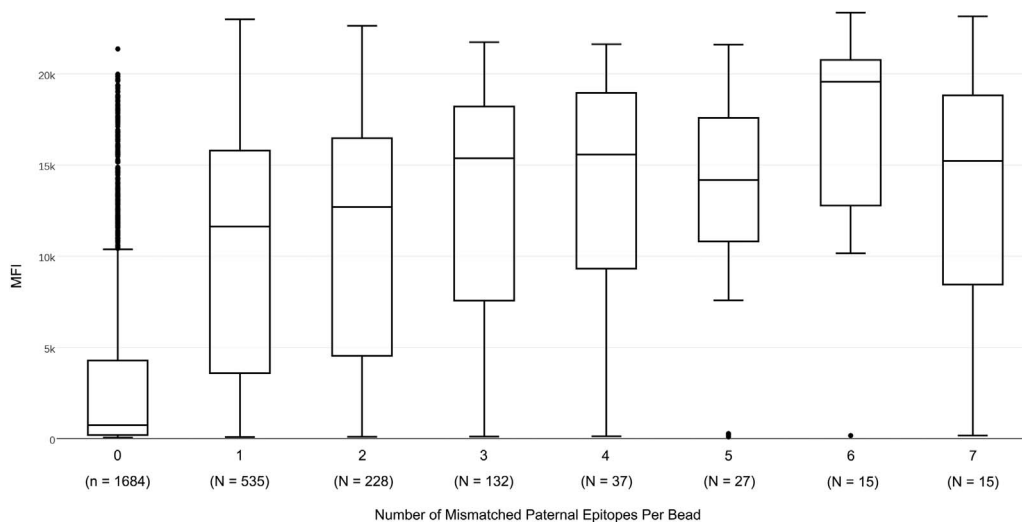
Fig. 4. MFI for Beads in the LABScreen Single Antigen HLA Class I Assay Sorted According to Number of Mismatched Paternal Epitopes Expressed on the Coated Antigen/Bead.

samples were available from both 22–24 weeks and postpartum. Samples taken at week 22–24 had a MFI that was on average 66% higher than MFI of the postpartum samples (Mean 1.66, 95% CI 0.94-2.38), indicating that anti-HLA class 1 antibody level tends to fall after delivery (Dahl et al., 2016). Therefore, the differences between cases and control were likely to have been even larger if blood samples were taken at similar time points for both groups.

Detection of broad reactivity patterns in solid phase assays, such as the Luminex assays, is a common occurrence. By including data on HLA epitope expression it was possible to separate third-party reactivity from reactivity towards the antigen in question. Epitope-focused analysis of pregnancy-induced anti-HLA class I antibodies have been published by others (Dankers et al., 2004; Duquesnoy et al., 2005; Honger et al., 2013; Lashley et al., 2014; Duquesnoy et al., 2015; Geneugelijk et al., 2015; Resse et al., 2015; Duquesnoy et al., 2016; Meuleman et al., 2016), and generally demonstrate that maternal anti-HLA class I antibodies are fetal-specific also in normal pregnancies. This is, however, the first time HLA class I epitope-analysis has been performed in suspected cases of FNAIT, with a demonstration that the antibodies detected were almost exclusively fetal/paternal-specific in the majority of cases. This is in accordance with what we would expect, since pregnancy was likely the only exposure to allogenic HLA. We only had fetal or maternal DNA available for a limited number of controls, and it was therefore not possible to make a complete comparative analysis.

There were two maternal alleles in our case population that had a significantly different frequency compared with the background population, but given the relatively low size of the study population these findings are likely incidental. In summary, the observed HLA immunization was not tied to any particular HLA antigen. We did, however, find that in pregnancies where HLA-B*27 was the most probable cause of maternal immunization, the neonates had a significantly lower platelet count nadir compared with the rest of the case group. HLA-B*27 is well known to be associated with inflammatory diseases (Hammer et al., 1990; Braun et al., 1998), and anti-HLA-B*27 antibodies have been suggested as a possible cause of neonatal thrombocytopenia (Thude et al., 2006). Whether immunization towards certain HLA antigens is associated with clinical outcome needs to be addressed in a larger prospective study.

Although there were similar antibody reactivity patterns for cases and controls, there was a significantly stronger antibody response among cases. Given the high frequency of nulliparous mothers among the cases, this is somewhat surprising. We also found that there was a tendency towards the fetal/paternal-specific antibody level being inversely correlated with the nadir neonatal platelet count, and that the cases with ICH had higher antibody levels. This could be related to an increased inflammatory state among the cases (Locke et al., 2009), but

may also indicate that the antibody *level* plays a role in the pathophysiology of neonatal thrombocytopenia. It is in our opinion imperative that future studies of anti-HLA class I antibodies in connection with pregnancy complications include epitope expression analyses as well as quantification of antibody levels. Further, our data provides a good basis and documentation for using epitope mapping when designing an algorithm to search for platelet donors in multi-immunized patients with platelet transfusion refractoriness. We are already working on such an algorithm and will start testing in some regional blood banks.

We found that the response towards beads coated with HLA-C antigens were generally much lower than that towards beads coated with either HLA-A or HLA-B, and that the stronger responses appeared to be directed against epitopes that were also expressed on HLA-A or HLA-B beads with similar MFI signals. The reactivity patterns of apparent anti-HLA-C antibodies were also not necessarily consistent with the expression of mismatched paternal epitopes, in the same manner that anti-HLA-A or −B antibodies were. We therefore concluded that immunization towards paternal HLA-C antigens are negligible compared to HLA-A and HLA-B, and that the observed HLA-C reactivity in many cases might be the result of immunizations originally targeting HLA-A or HLA-B antigens that share epitopes with a given HLA-C antigen. This is consistent with HLA-C only being expressed at a low level on most normal cells (Apps et al., 2015) as well as platelets (Mueller-Eckhardt et al., 1980). These assumptions were, however, not further tested in this study.

The relatively high proportion (45%) of nulliparous mothers in our study population is also noteworthy, since the occurrence of detectable anti-HLA class I antibodies is typically correlated with number of pregnancies (Densmore et al., 1999; Powers et al., 2008; Triulzi et al., 2009).

We have recently described significant associations between presence of maternal anti-HLA class I antibodies and perinatal complications in the setting of neonatal thrombocytopenia, including reduced birth weight, reduced placental weight, and the neonate being SGA (Dahl et al., 2016), indicating that the antibodies may affect perinatal outcome. The frequency of ICH (10%) in our case population is similar to the reported incidence of ICH caused by HPA-1a alloimmunization in retrospective populations (Mueller-Eckhardt et al., 1989; Ghevaert et al., 2007; Kjeldsen-Kragh et al., 2007). These findings indicate a need for increased clinical awareness.

The traditional diagnostic criteria of FNAIT are: 1. Fetal/neonatal thrombocytopenia, 2. demonstration of HPA incompatibility between mother and child, and 3. detection of maternal antibodies that target the given mismatched antigen. In this study of neonatal thrombocytopenia, we have demonstrated HLA class I incompatibility between mother and child, and shown that the maternal anti-HLA class I

a) Fetal/Paternal-Specific MFI versus Neonatal Platelet Count Nadir Among Cases
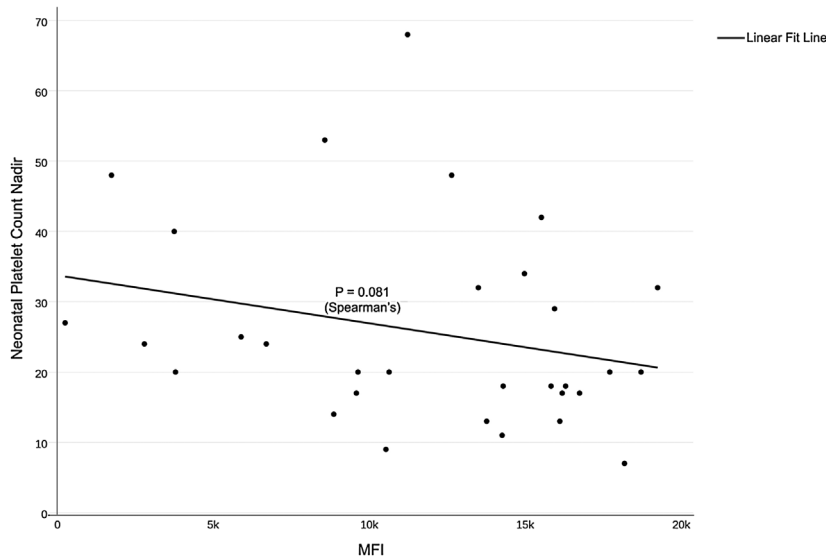
b) Fetal/Paternal-Specific MFI for Sub-Groups of the Case Population
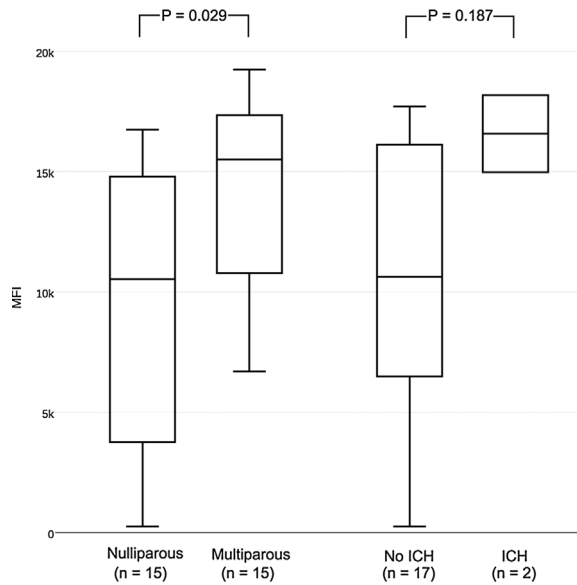
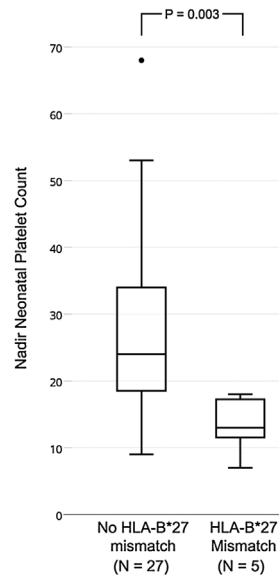c) Neonatal Platelet Count Nadirs for Cases With and Without Fetalmaternal HLA-B*27 Mismatch

Fig. 5. Fetal/Paternal-Specific MFI and Clinical Outcome.
Fetal/paternal-specific MFI could be determined in 32 of 33 genotyped cases.
a) 1 missing platelet count.
b) 2 missing parity status, 13 missing ICH status.
c) 1 missing platelet count.

antibodies were specific towards fetal/paternal epitopes, with little reactivity against any third-party or self. Therefore, our findings are principally consistent with the diagnostic criteria for traditional FNAIT. However, this does not necessarily imply causation, which was beyond the scope of this study.

5. Conclusions

In pregnancies where the newborn had thrombocytopenia at birth and where maternal anti-HLA class I antibodies was the suspected cause due to absence of anti-HPA antibodies, we found that the anti-HLA class I antibodies were specific toward fetal/paternal HLA antigens. The reactivity of anti-HLA class I antibodies among mothers whose newborns had thrombocytopenia was significantly higher compared to controls. Immunization was not tied to any particular antigen. Our results support the idea that maternal anti-HLA class I antibodies may contribute to neonatal thrombocytopenia.

Conflicts of interest

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jri.2017.06.003.

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