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The prevalence of HPA-1a alloimmunization and the potential risk of FNAIT depend on both the *DRB3*01:01* allele and associated DR-DQ haplotypes

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Abstract

Alloimmunization against human platelet antigen (HPA)-1a during pregnancy can cause foetal/neonatal alloimmune thrombocytopenia (FNAIT) and severe bleeding in the foetus or newborn and likely depends on several factors. HPA-1a alloimmunization is associated with *DRB3*01:01,* which is associated with several DR-DQ haplotypes. However, it is not known to what extent these haplotypes contribute to the prevalence of HPA-1a alloimmunization. HPA-1a–alloimmunized women, identified in a prospective study, and random donors were typed for selected *DRB3*, *DRB4, DRB1*, *DQA1* and *DQB1* alleles to determine allele and DR-DQ haplotype frequencies. *DRB3*01:01* was carried by 94% HPA-1a–immunized women compared to 27% in the general population. In the first population, the DR3-DQ2 haplotype was overrepresented ($P < .003$). The prevalence of HPA-1a alloimmunization was estimated to be about twice as frequent with DR3-DQ2 compared to DR13-DQ6, together accounting for about 90% of *DRB3*01:01*–positive individuals. Further, we examined *DQB1*02* and *DRB4*01:01* alleles for their reported association with HPA-1a alloimmunization, in the context of DR-DQ haplotypes. Since ~ 80% of *DQB1*02* alleles are linked to the DR3-DQ2 haplotype, the association might be coincidental. However, the *DQB1*02:02*–associated DR7-DQ2 haplotype was also overrepresented in alloimmunized women, suggesting a role for this allele or haplotype in HPA-1a alloimmunization. As *DRB4*01:01* is predominantly associated with the DR7-DQ2 haplotype in HPA-1a–alloimmunized individuals, the reported association with FNAIT may be coincidental. Typing for DR-DQ haplotypes revealed important genetic associations with HPA-1a alloimmunization not evident from typing individual alleles, and the presence of different DRB3-associated DR-DQ haplotypes showed different prevalence of HPA-1a alloimmunization.

Maria Therese Ahlen and Gøril Heide contributed equally to this study.

1 | INTRODUCTION

FNAIT is a rare condition that can cause severe complications such as intracranial haemorrhage in the foetus or newborn due to the transfer of platelet-depleting alloantibodies from the mother to the foetus during pregnancy. The most common cause of FNAIT is maternal alloantibodies directed against HPA-1a on foetal platelets. This fetomaternal incompatibility is defined by a leucine/proline polymorphism at residue 33 in integrin β3.[1] About 2% of Caucasian women are homozygous for the HPA-1 variant with proline (HPA-1bb). Alloimmunization occurs in about 10% of these women,[2] and about one third of these will give birth to a child with FNAIT. The most severe complication is intracranial haemorrhage (ICH), which occurs in approximately 1 in 10,000 unselected pregnancies.[2] Several factors may in theory affect the natural history of FNAIT, as reviewed by Sachs and Santoso (2017),[3] including the influence of the maternal HLA class II genotype. The MHC class II allele *HLA-DRB3*01:01* is strongly associated with HPA-1a alloimmunization; more than 90% of immunized women carry this MHC allele,[4-6] which also shows a dose-dependent association to the severity of HPA-1a immunization.[7] This strong genetic association suggests that HPA-1a immunization is dependent on T cells restricted by the MHC class II molecule encoded by the *HLA-DRA/DRB3*01:01* alleles. There is support for this notion: HPA-1a– but not HPA-1b–derived peptides bind this molecule, and the allogeneic residue Leu33 serves to anchor the peptide.[8,9] Furthermore, HPA-1a–specific $CD4^+$ T cell clones have been isolated from alloimmunized women,[10-12] and these T cell clones are restricted by DRA/DRB3*01:01.[10] However, it is possible that there exist additional genetic factors that predispose for immunization. In that respect, both *DQB1*02* and *DRB4*01* have been shown to be associated with FNAIT.[13,14] However, these alleles and other genetic elements that may influence HPA-1a alloimmunization do not segregate entirely independently of each other. Rather, they are in linkage disequilibrium with other genes in conserved haplotypes. In this respect, the *DRB3*01:01* allele is known to be in linkage disequilibrium with several different DR-DQ haplotypes, and one of these contains a *DQB1*02* allele.[15,16]

In the present study, we aimed to examine the impact of *DRB3*01:01*–associated DR-DQ haplotypes on HPA-1a alloimmunization, and to determine the haplotype associations and relative importance in HPA-1a alloimmunization for *HLA-DQB1*02:01/*02:02* and *HLA-DRB4*01:01* alleles.

2 | METHODS

The study was approved by the Regional Committee for Medical Research Ethics, North Norway (approval no. P REK NORD 66/2005, 2009/1585 and 2012/1917). Blood samples were drawn from patients and healthy volunteers after written informed consent.

2.1 | Study groups

In total, 167 HPA-1a–immunized women were included in the study comprising two different populations: one group of HPA-1a–negative women who developed anti-HPA-1a IgG antibodies in connection with pregnancies, included from the Norwegian prospective FNAIT screening study (1995-2004) (prospective screening group, N = 123)[4] (More than 2/3 of these immunized women were included from the southern part of Norway), and one group of women referred to the Norwegian National Unit for Platelet Immunology (NNUPI) after giving birth to a child with severe thrombocytopenia and clinical signs of bleeding, also producing anti-HPA-1a IgG antibodies (retrospective group, N = 44). The criteria for inclusion of both subgroups were detectible levels of anti-HPA-1a antibodies.

The control group was made of 781 healthy random blood donors at the University Hospital of North Norway, referred to as the general population. No information regarding ethnicity was available for neither the random donors nor the immunized women. The Sami population is known to differ from the Norwegian population in several HLA types.[17] However, most individuals were expected to be Caucasians in both groups as all donors were recruited from the Norwegian population, and not from specific Sami areas of Norway. The frequency of *DQB1*02* in our control population also fits the frequency of *DQB1*02* in a group of about 8000 Norwegian donors at the bone marrow registry (32,6%), lending support to the notion that the control population in this study reflects the general population in Norway.

2.2 | DNA isolation

Cryopreserved genomic DNA was available from about 40% of the HPA-1a–alloimmunized women. In addition, DNA was isolated from blood samples from the rest of alloimmunized women and all randomly selected donors, using Blood Mini Kit (QIAGEN) and eluted in sterile H_2O. Short-time storage of DNA was carried out at −20ºC whereas −70ºC was used for long-time storage.

2.3 | Genotyping

Primer sequences, characteristics and cycling programs are listed in supplementary data (Tables S1 and S2). All primers were synthesized by Eurogentec (Belgium). All PCR were

performed in a total volume of 25μL, using 0.5U HotStarTaq (Qiagen) per reaction, and run on a GeneAmp9700 (Applied Biosystems). *HGH* was used as an internal DNA amplification control.[18] Genomic DNA from IHWG cell lines was used as controls for amplification, specificity and enzyme digestion: STEINLIN, DUCAF, EMJ, LZL, CEPH-1413, HAG, BER, WDV and EK (Table S3).

Restriction cutting of PCR products was performed with endonucleases (all from New England Biolabs) according to references (Table S2).

The risk of false-positive detection within each genotyping assay, for example amplification of infrequent alleles by group-specific primers, could be evaluated by conferring dbMHC (the MHC database) with updated sequences.

2.3.1 | Designation of HLA-DR-DQ haplotypes

HLA-DR-DQ haplotypes for each donor were designated based on known DR-DQ haplotypes in the Norwegian population,[15,17,19] based on typing of selected DR- and DQ alleles. To answer our main questions in this study, alleles known to be associated with *DRB3*01:01* and *DQB1*02* were specifically typed for (Table 1).

2.3.2 | Detection of *DRB3*01:01P*

Detection of *DRB3*01:01P* (http://hla.alleles.org/alleles/p_groups.html) was performed for all immunized women and random donors by PCR using sequence-specific primers for *DRB3*01:01/*03:01*,[20] combined with restriction fragment length polymorphism (RFLP) by the enzyme *Kpn*I, cutting amplified *DRB3*01:01,* but not *DRB3*03:01*. All *DRB3*01:01*–positive random donors (213) and immunized women (157) were included for further detection of HLA-DR and –DQ alleles.

Because the above-mentioned method of *DRB3*01:01* detection is both time- and resource demanding, we also developed a new *DRB3*01:01* typing assay. This assay was performed for almost all individuals as duplex assay for *DRB3*01:01* and *GAPDH* in a TaqMan RQ-PCR assay on genomic DNA: 10 to 100 ng DNA was used in 25 μL reactions, in parallel reactions, with TaqMan Fast Universal PCR Master Mix, run on Fast mode (95°C for 20 seconds followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds) on ABI Prism7900HT (Applied Biosystems).

Oligos for HLA-DRB3: HLA-DRB3 primers (1000nM) 5'- TCTTGGAGCTGCGTAAGTCTGA-3', 5'-TGTTCCAG GACTCGGCGA-3' and a specific HLA-DRB3*01:01 probe (150 nM) 5'-6-FAM-TCTPTCCAGGZACCG-BHQ-1-3' (P= G-LNA, Z=T-LNA nucleotides). For GAPDH as reference, primers (500nM) 5'-CCCCACACACATGCACT TACC-3', 5'-CCTAGTCCCAGGGCTTTGATT-3' and a probe (100nM) 5'-VIC-AAAGAGCTAGGAAGGACAGGC AACTTGGC-BHQ-1-3'. Samples from fully HLA typed cell lines were used as controls for method development: 2 copies of DRB3*01:01 (STEINLIN), 1 copy of DRB3*01:01 (D4BL4 in-house), as well as *DRB3*01:01*–negative, *DRB3*02:02/DRB3*03:01*–positive samples (DUCAF/EMJ).

2.3.3 | Detection of DQB1*02 and DQA1*05

Group-specific primers for *DQB1*02* and *DQA1*05* were multiplexed.[21,22] The primers for *DQA1*05* and *DQB1*02* theoretically amplify all alleles in the two different groups. To discriminate between the allele *DQB1*02:02* and other *DQB1*02 alleles,* typing for *DQB1*02* [23] was combined with digestion by the restriction endonuclease *Msc*I, cutting only the amplified product of *DQB1*02:02* in exon 3 of the DQB1 gene.

2.3.4 | Detection of DRB1*03:01, (*11:01/*11:02), *13:01, *13:02, *13:03, (*14:01)

Group-specific primers for two groups of alleles were used.[24] Primers for Group-1 amplified *DRB1*11:01*, **13:02*, and **13:03.* The restriction endonucleases, *Fok*I and *Sfa*NI, were

TABLE 1 Common HLA alleles and HLA DR-DQ haplotypes in the Norwegian population, specifically typed for in this study. Adapted from Spurkland et al, Harbo et a and Lande et al,[15,17,19] with modifications in DRB1*13[31]

Haplotype	*DRB1*	*DQA1*	*DQB1*	Additional DRB association
DR3-DQ2	****03:01***	****05:01***	****02:01***	***DRB3*01:01***
DR13(01)-DQ6	****13:01***	**01:03*	**06:03*	***DBR3*01:01***
DR13(03)-DQ3	****13:03***	**05:01*	**03:01*	***DRB3*01:01***
DR7-DQ2	****07:01***	**02:01*	****02:02***	***DRB4*01***
DR7-DQ3	****07:01***	**02:01*	**03:03*	***DRB4*01***

All alleles are written in italic. Alleles specifically typed for in this study are in bold.

used to distinguish between these three *DRB1* alleles. Primers for Group-2 amplified *DRB1*03:01*, **11:02*, **13:01*, and **14:01*. The restriction endonucleases *Fok*I, *Sfa*NI and *Kpn*I were used to distinguish between these alleles. For details, see Table S2.

2.3.5 | Detection of *DRB1*07*

Detection of *DRB1*07* was done by group-specific PCR for *DRB1*07*.[25]

2.3.6 | Detection of *DRB4*01:01*

Two sets of primers and restriction endonucleases, for exon 2 and exon 3, were used. The first group-specific primers for *DRB4*01* in exon 2 amplify all *DRB4*01* alleles except *DRB4*01:05*.[26] Samples positive in the first PCR were further typed for exon 3, with primers specific for *DRB4*01:01, -*01:02, -*01:03, -*01:06, -*02:01,* and *-*03:01*.[27] The restriction endonuclease *Eae*I cuts *DRB4*01:01* and *DRB4*01:06*. Hence, samples with amplified products in both reactions and only restricted by the *Eae*I enzyme could be *DRB4*01:01* and/or *DRB4*01:06* positive.

2.3.7 | Confirmation of haplotypes and additional haplotype detection

To support the assigned *DRB3*01:01*–associated haplotype, for each immunized women and each individual in the control group, based on the results of PCR-RFLP genotyping and to uncover the additional haplotype, low-resolution typing for *DRB1* was performed by the Norwegian Bone Marrow Registry in Oslo, Norway; donors were typed for HLA-DRB1 by oligonucleotide probing defining HLA-DR1, DR2, DR3, DR4, DR7, DR8, DR9, DR10, DR11, DR12, DR13, DR14 and DR103 specificities. Due to too low amounts of DNA, some donors could not be low-resolution typed or typed for *DRB4*01:01*. However, all individuals included for analysis of the *DRB3*01:01*–associated haplotype were still sufficient typed to define the associated haplotype. Donors not typed for *DRB4*01:01* were excluded from the statistical analysis of *DRB4*01:01* or its associations. 'N' is given for each statistical analysis.

2.4 | Estimating the prevalence of HPA-1a alloimmunization with different *DRB3*01:01*–associated haplotypes

The prevalence of alloimmunization was estimated by combining data from the Norwegian FNAIT screening study with data from the general population (control population in the current study). The number of HPA-1a–negative women in the screening study who carried the various *DRB3*01:01*–associated DR-DQ haplotypes was estimated by applying the population frequencies of these haplotypes determined for the general population (control population in the current study). Patient samples of HPA-1a–alloimmunized women from the screening study were also examined for these haplotypes. The prevalence of HPA-1a alloimmunization was calculated as the per cent of HPA-1a–negative women who eventually became alloimmunized, for each DR-DQ haplotype. Not all HPA-1a–immunized women were included for HLA typing due to lack of DNA. The results from our typing of a proportion of immunized women were therefore adjusted to apply to the whole population of immunized women. Estimates are shown in Figure 1. Statistical analyses could not be used for estimated prevalence numbers. In support of the accuracy of these estimates, the binomial test for measured frequencies of these haplotypes showed that the HPA-1a–immunized individuals were significantly different from the general population.

2.5 | Genotyping of TNF-308, LTA252 and AGER-429 SNPs

TNF −308G>A (rs1800629), LTA 252A>G (rs909253) and AGER-429T>C (rs1800625) SNPs were determined with allelic discrimination assays. Primers and probes are listed in Table S4.

2.6 | Statistics

To compare allele and haplotype frequencies between two groups, the chi-square test was used. Differences between groups were considered significant if $P < .05$. As the number of a priori hypotheses was small and related to specific haplotypes, correction for multi-significance was not applied as recommended by Perneger TV.[28]

3 | RESULTS

3.1 | The DR3-DQ2 haplotype occurs at a higher frequency among HPA-1a–alloimmunized women than would be expected by random distribution

To identify the most common DR-DQ haplotypes in linkage disequilibrium with the HLA-DRB3*01:01 allele, we first identified *DRB3*01:01* positives among

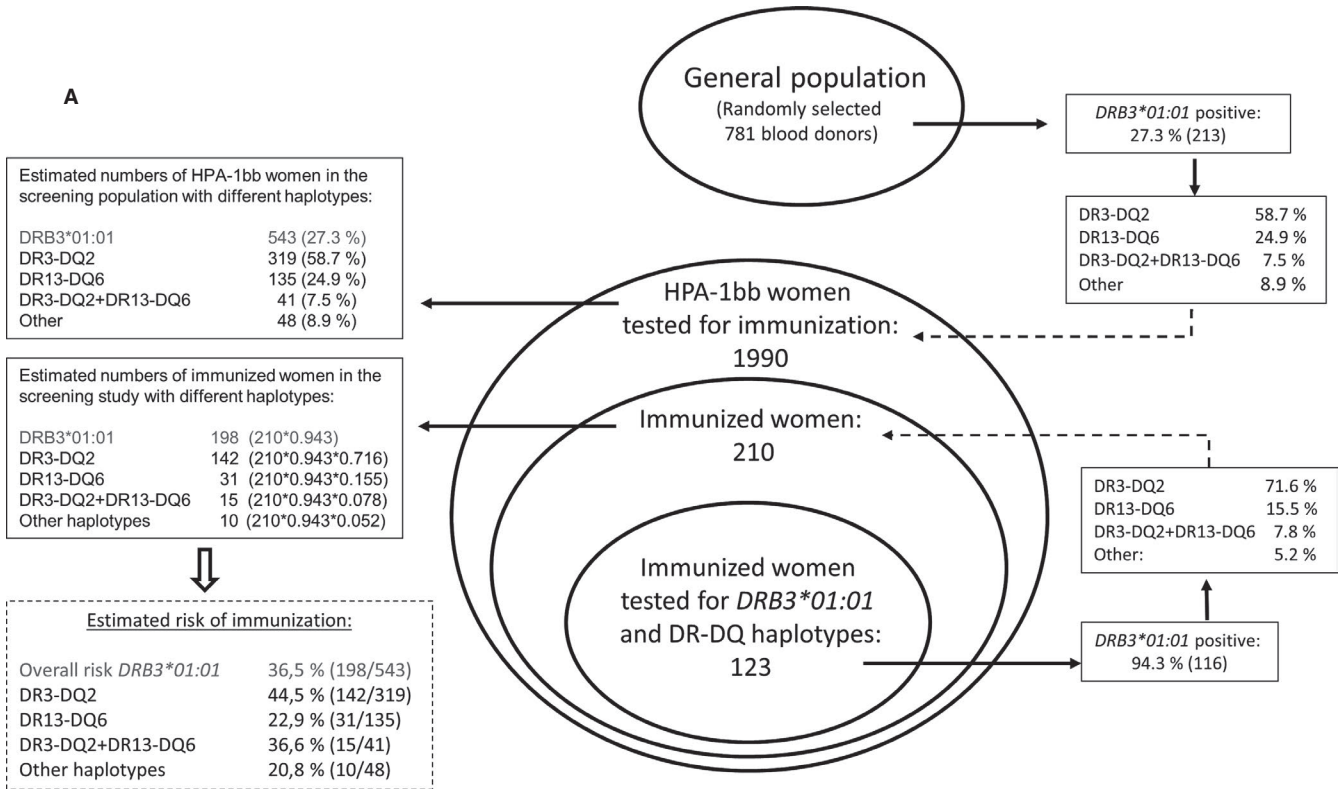

FIGURE 1 The estimated prevalence for HPA-1a alloimmunization is twice as high for women carrying DR3-DQ2 compared to DR13-DQ6. (A) The prevalence of HPA-1a alloimmunization for groups of women with different *DRB3*01:01*–associated DR-DQ haplotypes in a prospectively selected population was examined by combining data from the Norwegian screening study [4] and DR-DQ haplotype data generated in the current study. (B) Each bar represents individuals enrolled in the Norwegian screening study projected to carry one or two *DRB3*01:01*–associated DR-DQ haplotypes. The width of each bar represents the proportion of individuals with the indicated DR-DQ haplotype, projected from measured frequency of individuals carrying the different *DRB3*01:01*–associated DR-DQ haplotypes in the general population. The height of each bar represents the estimated prevalence of HPA-1a alloimmunization and is based on the actual number of HPA-1a–alloimmunized individuals determined to carry the specific haplotype. The overall prevalence of HPA-1a alloimmunization in *DRB3*01:01*–positive individuals is 36.5%. The prevalence of immunization with DR3-DQ2 (44.5%) is about twice as high as for DR13-DQ6 (22.9%). The accuracy of these estimates is supported from binomial test of statistical significance for measured frequencies of these haplotypes in the HPA-1a–immunized individuals against the general population: Frequency of HPA-1a–alloimmunized women with DR3-DQ2 (71,8%, n = 116) against the general population (58.7%): $Z = 2.67$, $P = .0038$. Frequency of HPA-1a–alloimmunized women with DR13-DQ6 (15.5%, n = 116) against the general population (24.9%). $Z = 2.35$, $P = .009$

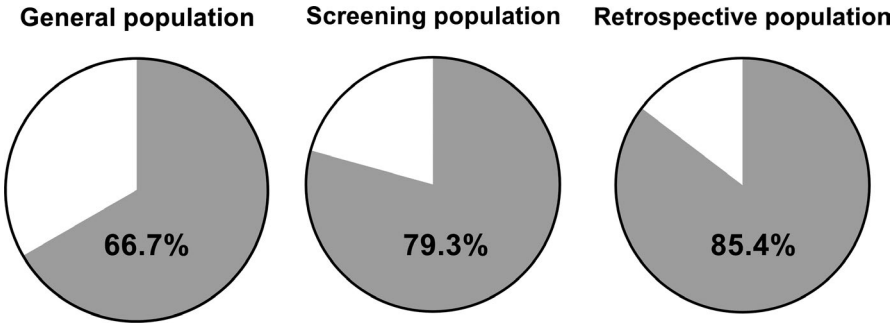

FIGURE 2 The frequency of the DR3-DQ2 haplotype in the general population and in HPA-1a–immunized women. Both in a population of HPA-1a–alloimmunized *DRB3*01:01*–positive women identified in a prospective screening study,[4] 'Screening population', and in a population consisting of women referred from the clinic following identification of suspected FNAIT cases, 'Retrospective population', the per cent individuals carrying the DR3-DQ2 haplotype were higher than in a general population of *DRB3*01:01*–positive individuals (screening: difference12.6%, $P = .016$. Retrospective: difference 18.7%, $P = .017$). The 'Retrospective population' is likely enriched in women who gave birth to severely thrombocytopenic neonates compared to the 'Screening population', in which roughly a third of affected neonates were severely thrombocytopenic

HPA-1a–immunized individuals (n = 167) (both prospectively and retrospectively selected) and in the general population (n = 781). The same populations were also typed for *HLA-DQB1*02* and *HLA-DRB4*01* alleles since these have also been reported to be associated with HPA-1a immunization.

As expected, in total, 94% (157) of the HPA-1a–immunized women carried the *DRB3*01:01* allele, compared to only 27.3% (213) of the general population ($P < .0001$; Table 2); notably near similar frequency of *DRB3*01:01* was found in the general population in other countries.[29]

Almost all immunized women and random donors were also typed by the TaqMan assay for detection of *DRB3*01:01,* and the results were in concordance with the old assay for typing of *DRB3*01:01*.

In total, 82.0% of the HPA-1a–immunized women carried a *DQB1*02* allele, compared to only 31,0% in a random selection of the general population ($P < .0001$) (Table 2).

Only 10.5% of HPA-1a–immunized women carried the *DRB4*01:01* allele, not significantly different from 6.9% in a random selection of the general population ($P = .23$) (Table 2).

Next, in a detection regime to identify *DRB3*01:01*–associated DR-DQ haplotypes, *DRB3*01:01*–positive individuals were further genotyped for selected alleles (as outlined in 'Methods'). *DRB3*01:01* is known to be associated with DRB1*03, DRB1*11, DRB1*12, DRB1*13 and DRB1*14 alleles.[29] However, some of the combinations are very rare in Caucasians.[29] DR alleles known to be associated with *DRB3*01:01* were successfully identified in 98.6% of random donors, and all but one of the immunized women. For the remaining random donors and immunized woman, we did not have enough DNA to perform low-resolution typing for DRB1; the typing results were therefore uncertain.

A comparison of the *DRB3*01:01*–associated DR-DQ haplotypes (Table 3) showed that there were two dominating haplotypes, DR3-DQ2 and DR13-DQ6, together accounting for > 90% of the immunized women. Furthermore, individuals with DR3-DQ2 were overrepresented among HPA-1a–immunized women (80.9%), compared to the general population (66.7%; $P < .003$). In the remaining few per cent of immunized women, *DRB3*01:01* was associated with less frequent haplotypes.

3.2 | DR3-DQ2 is overrepresented in both the retrospective and the screening groups

The HPA-1a–alloimmunized women in the study comprised of women identified in connection with a prospective screening study described previously [4] and women referred from the

TABLE 2 Frequency of individuals carrying *DRB3*01:01*, *DQB1*02* and *DRB4*01:01* alleles in HPA-1a–alloimmunized women and in the general Norwegian population

Allele	HPA-1a–alloimmunized women	General population	
*DRB3*01:01*	94.0[a] (N = 167)	27.3 (N = 781)	$P < .0001$
*DQB1*02*	82.0 (N = 167)	31.0 (N = 200)	$P < .0001$
*DRB4*01:01*	10.5 (N = 153)	6.9 (N = 204)	$P = .23$

Note: Number of individuals typed for the indicated allele in each population.

[a]Per cent of individuals in each population that typed positive for the indicated allele.

TABLE 3 Frequencies of the two most common *DRB3*01:01*–associated DR-DQ haplotypes in HPA1a–alloimmunized women and in the general population of Northern Norway

Haplotype	*DRB3*01:01*–positive HPA-1a–immunized women(n = 157)	*DRB3*01:01*–positive General population(n = 213)	
*DRB1*03:01-DQA1*05:01-DQB1*02:01* (DR3-DQ2)	80.9 (127)	66.7 (142)	$P < .003$
*DRB1*13:01-DQA1*01:03-DQB1*06:03* (DR13-DQ6)	20.4 (32)	32.9 (70)	$P < .008$

clinic following a pregnancy where FNAIT was diagnosed. Both groups showed overrepresentation of DR3-DQ2, 79.3% in the prospective group and 85.4% in the retrospective group (Figure 2), compared to 66.7% in the general population (diff 12,6, $P = .016$; diff 18,7, $P = .017$). Population frequencies of *DRB3*01:01*–associated DR-DQ haplotypes in each of these groups are shown in Figure S1.

3.3 | More women become HPA-1a alloimmunized when DRB3*01:01 is associated with the DR3-DQ2 haplotype

Since the proportion of those who have the DR3-DQ2 haplotype is higher in the immunized population in this study compared to the control population, we wanted to determine the prevalence of HPA-1a alloimmunization among *DRB3*01:01*–positive individuals having this or other DR-DQ haplotypes, that is the proportion of women with different *DRB3*01:01*–associated DR-DQ haplotypes who have become alloimmunized after one or several incompatible pregnancies. In lack of a control group of non-immunized HPA-1a–negative women, which would have been our preferred control group, the prevalence of immunization among women with different haplotypes had to be estimated. For this estimation, we used data from the Norwegian FNAIT screening study, in which more than 100.000 women were screened for HPA-1a negativity and HPA-1a alloimmunization in connection with pregnancy. Since DR-DQ haplotypes were not determined for subjects in the screening study, we estimated the proportion of the women carrying the different *DRB3*01:01*–associated DR-DQ haplotypes by applying the frequency of each of the *DRB3*01:01*–associated DR-DQ haplotypes determined for the control population in the current study. The prevalence of alloimmunization was then calculated from the number of HPA-1a–alloimmunized women with a particular *DRB3*01:01*–associated DR-DQ haplotype (typed in the current study) and the estimated number of HPA-1a–negative women in the screening study who carried the same haplotype (Figure 1A). The prevalence of alloimmunized HPA-1a–negative, *DRB3*01:01*–positive, DR3-DQ2–positive women was 44.5%, that for DR13-DQ6 haplotype is only 22.9% (Figure 1A,B). Hence, in HPA-1a–negative *DRB3*01:01*–positive women, we estimate that about twice as many will become HPA-1a–alloimmunized during or after one or multiple HPA-1a–incompatible pregnancies when the *DRB3*01:01* allele is associated with the DR3-DQ2 haplotype, compared to those with DR13-DQ6. The same approach was used to calculate the prevalence of alloimmunized HPA-1a–negative, *DRB3*01:01*–positive women: 36.5%.

3.4 | The DR7-DQ2 haplotype and DRB4*01:01 are overrepresented and DR15-DQ6 is underrepresented in DRB3*01:01–positive HPA-1a–alloimmunized women

Both haplotypes were successfully determined, by low-resolution DRB1 typing combined with typing for specific alleles, in 197 of 213 (92%) individuals in the *DRB3*01:01*–positive general population group and in 139 of 157 (88%) of HPA-1a–immunized *DRB3*01:01*–positive women.

Although all individuals in this study carry the *DRB3*01:01* allele on one or both chromosomes, *HLA-DQB1*02* is also strongly associated with HPA-1a alloimmunization.[13] *HLA-DQB1*02* is present in two different DR-DQ haplotypes. The *HLA-DQB1*02:01* allele is in linkage disequilibrium with *DRB3*01:01* in the DR3-DQ2 haplotype, while *HLA-DQB1*02:02* is present in the DR7-DQ2 haplotype, which segregates independent of *DRB3*01:01*. Furthermore, a negative association with HPA-1a alloimmunization has been reported for *HLA-DRB1*15:01*.[30] This allele is present in DR15-DQ6, which also segregates independent of *HLA-DRB3*01:01*. To determine the relative influence of these two DR-DQ haplotypes on HPA-1a alloimmunization, we independently typed for DR7-DQ2 and DR15-DQ6 on the second, non–*DRB3*01:01*-associated chromosome in each individual.

The DR7-DQ2 haplotype was seen more frequently among the HPA-1a–immunized women (11.7%) compared to the general population (5.4%; $P = .03$) (Figure S2A). This suggests that there is a weak association of the DR7-DQ2 haplotype in *DRB3*01:01*–positive HPA-1a–immunized women. However, there were too few individuals included in the study to conclude upon a potential dose effect of DQ2 on immunization. Also, DR15-DQ6 is underrepresented in HPA-1a–immunized women ($P = .01$) (Figure S2B).

To also determine whether the *DRB4*01:01* allele is associated with HPA-1a alloimmunization, we examined its presence in *DRB3*01:01*–positive immunized women and

random blood donors. When comparing these two groups, the frequency of *DRB4*01:01* is higher in immunized women, 9.2% (Table S5), than in the random population (2.9%; $P = .01$). *DRB4*01* is known to be associated with DRB1*04, DRB1*07 and DRB1*09.[29]

All individuals carrying the *DRB4*01:01* allele, both in the group of immunized women and in the general population, also carried either the DR7-DQ2 (all *DRB4*01:01*–positive individuals except one) or the DR7-DQ3 haplotype (one immunized individual) (data not shown).

3.5 | An inflammation-associated single nucleotide polymorphism occurs at a higher frequency in alloimmunized compared to general population DR3-DQ2–positive individuals

Since DR3-DQ2 was found to be more associated with HPA-1a immunization, we wanted to examine whether this haplotype is associated with known factors that may increase the chance of immunization. Therefore, most individuals with a detected DR3-DQ2 haplotype were also typed for the Conserved Extended Haplotype (CEH) markers *TNF-308A/ LTA252G/ AGER-429C* by in-house allele discrimination assays (117 immunized women and 145 random donors were tested). Ninety per cent and 86% carried these markers, among immunized women and general population, respectively (data not shown). In addition, we observed that 29.4% of the immunized women with the DR3-DQ2 haplotype in the retrospective group were homozygous for the *TNF-308A* marker, a more frequent occurrence compared to both the prospective group (10.9%; $P < .025$) and the control group (7.0%; $P < .0008$).

4 | DISCUSSION

The association of *HLA-DRB3*01:01* with HPA-1a alloimmunization is well documented. In the present study, we show that also DR-DQ haplotype association significantly influences the prevalence of HPA-1a alloimmunization; women carrying DR3-DQ2 are estimated to be about twice as likely to become HPA-1a alloimmunized compared to those carrying DR13-DQ6, even though both of these haplotypes are associated with the *DRB3*01:01* allele. One third of HPA-1a–negative, *DRB3*01:01*–positive pregnant women with an HPA-1a–positive child were immunized; the estimated prevalence is, however, 44.5% if the *DRB3*01:01* allele is linked to the DR3-DQ2 haplotype.

The frequency of different HLA-DR-DQ haplotypes in the Norwegian population is known from typing by the Norwegian Bone Marrow Registry.[15,17,19] We have focused on the most common haplotypes. The *DRB3*01:01* allele is associated with several of these HLA-DR-DQ haplotypes,[15,29,31,32] and DR3-DQ2 and DR13-DQ6 represent the major two of these.[29] The increased risk of alloimmunization with DR3-DQ2 suggests that other genetic elements that

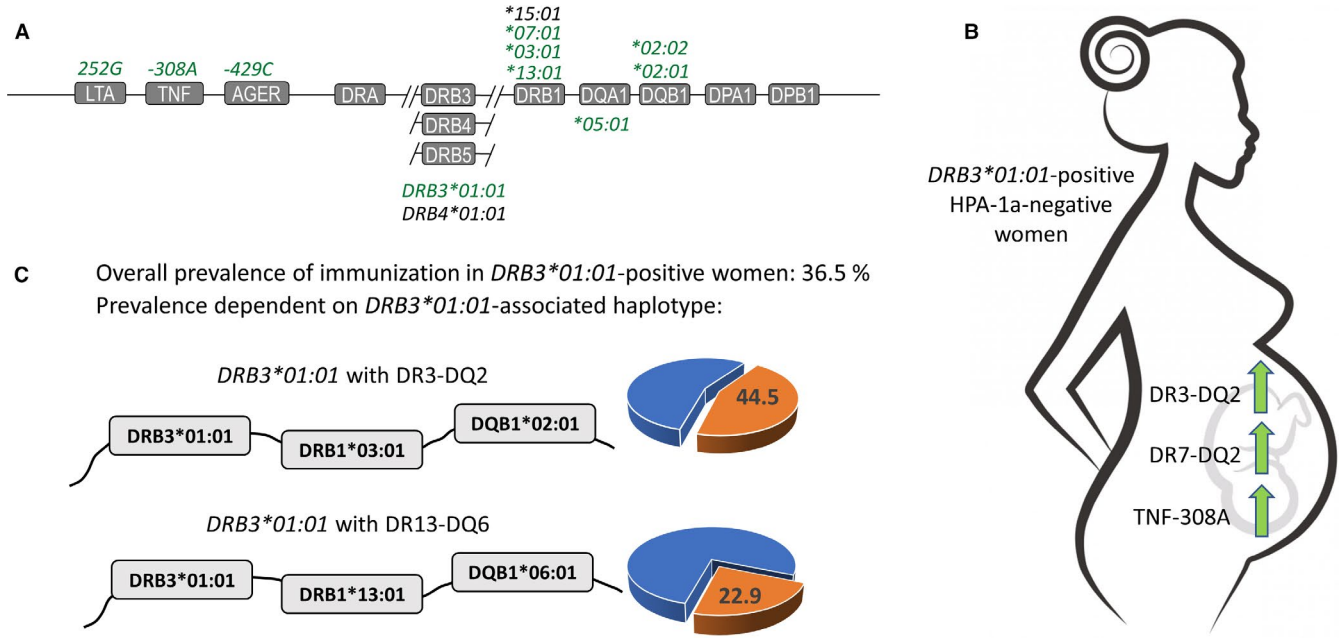

FIGURE 3 HLA-DR-DQ haplotype association with HPA-1a alloimmunization. (A) Alleles and SNPs that were specifically examined in this study in relation to HPA-1a alloimmunization. Those positively associated with HPA-1a alloimmunization are marked in green. (B) Haplotypes and SNPs positively associated with HPA-1a alloimmunization in HPA-1a–negative *DRB3*01:01*–positive women. C. Prevalence of HPA-1a alloimmunization in HPA-1a–negative women carrying *DRB3*01:01* with DR3-DQ2 or with DR13-DQ6

somehow increase the chance of HPA-1a alloimmunization are associated with this haplotype.

In addition to *DRB3*01:01*, the *DQB1*02* alleles are also strongly associated with HPA-1a alloimmunization.[13,30] As shown herein, this association can largely be accounted for by its genetic linkage to the DR3-DQ2 haplotype; in the present study, 80.9% of *DRB3*01:01*–positive women carried the DR3-DQ2 haplotype. This linkage was even stronger (85.4%) when only retrospective cases were considered, which is similar to that analysed in a previous study.[13] Therefore, the reported DQ2 association with alloimmunization may be coincidental. Alternatively, it is possible that *DQB1*02* may represent a genetic element in the DR3-DQ2 haplotype that contributes to increased risk of alloimmunization with HPA-1a. In support of the latter, DQ2 is associated with alloimmunization also by a second allele not linked to DR3-DQ2. This second allele, *DQB1*02:02*, is present in the DR7-DQ2 haplotype,[33] which is the only other DR-DQ haplotype including DQ2 besides DR3-DQ2.[15]

The *DQB1*02:02* allele and, thus, the DR7-DQ2 haplotype are present at a higher frequency in HPA-1a–alloimmunized *DRB3*01:01*–positive women compared to in *DRB3*01:01*–positive individuals in the general population. Unlike the *DRB3*01:01* allele, which has been functionally associated with alloimmunization, there is no evidence for a similar function associated with DQ2 alleles. Still, the possibility that such cells exist in HPA-1a–immunized women cannot be ruled out.

Taken together, there is still no direct evidence for a functional role of *DQB1*02* in HPA-1a alloimmunization. Whether it is coincidental that both haplotypes found to be associated with HPA-1a immunization and FNAIT contain a *DQB1*02* allele remains to be determined.

The *DRB4*01:01* allele was also suggested by Loewenthal et al[14] to be important for HPA-1a immunization, by acting in synergy with the *DRB3*01:01* allele to cause more serious immunization in women positive for both these alleles.[14] The opposite was found, however, by L'abbé et al,[13] where *DRB4*01* seemed to be more frequent in random donors than in immunized women. Wienzek-Lischka et al,[34] Delbos et al [35] and Sainio et al[36] subsequently examined the role of this allele and did not find it to be overrepresented in HPA-1a–immunized women. Wienzek-Lischka et al also found that the combination of *DRB3*01:01* and *DRB4*01:01/03* did not enhance immunization compared to only carrying the *DRB3*01:01* allele alone.[34] Although earlier reports[14,30,34] did not distinguish between *DRB4*01:01* and *DRB4*01:03*, because there is no difference in exon 2 of these alleles and they phenotypically are the same, we chose to only focus on *DRB4*01:01*. We were more curious about the different DRB1 allele associations of *DRB4*01:01* than the potential function of the molecule it codes for.

In this study, the *DRB4*01:01* allele was relatively rare, but more frequent in already *DRB3*01:01*–positive immunized women compared to the general population. For all women, except one, carrying this allele, it was associated with the DR7-DQ2 haplotype. Taken together, this points to a role for the DR7-DQ2 haplotype, and thereby may be for *DQB1*02:02*, rather than for the *DRB4*01:01 allele*, in HPA-1a immunization.

A major question addressed in this study is whether particular DR-DQ haplotypes are associated with increased prevalence of HPA-1a alloimmunization. This was determined by using prospective data from the Norwegian FNAIT screening study [4] and DR-DQ haplotype frequencies determined for the control population (random Norwegian blood donors that have the *DRB3*01:01* allele) in the current study. The measured allele frequency of *DRB3*01:01* in the Norwegian population in the current study was 14.3% (781 random donors with a total of 1562 alleles, 224 positively typed DRB3*01:01 alleles), close to the allele frequency (14.9%) measured in a large European Caucasian population in the United States,[29] lending support to the validity of our control population. We show that the prevalence of HPA-1a alloimmunization in the group of *DRB3*01:01*–positive women carrying DR3-DQ2 is twice as high as for women carrying the DR13-DQ6 haplotype. In support of these findings, both Sainio et al[36] and Wienzek-Lischka et al[34] found that both *DR3-DRB3*01:01* and *DR13-DRB3*01:01* are overrepresented in immunized women compared to controls and that *DR3-DRB3*01:01* is found more often than *DR13-DRB3*01:01*. Both these studies are based on retrospective data, showing a *DRB3*01:01* frequency of 100% and 98%, respectively. Benefiting from prospective data, however, we show a lower frequency of this allele. Most important, by comparing already *DRB3*01:01*–positive individuals we show here that *DRB1*13:01* is overrepresented only by association with *DRB3*01:01* and that the prevalence of immunization in the group of *DRB3*01:01*–positive women carrying DR13-DQ6 actually is lower than the prevalence of immunization in other *DRB3*01:01*–positive women.

The DR3-DQ2 haplotype has been studied for years, due to its association with a number of immune-mediated diseases, and especially autoimmune disorders.[37-40] In Caucasians, the DR3-DQ2 haplotype is commonly a part of the conserved extended haplotype AH 8.1, (HLA-A1 - B8 - DRB3*01:01 - DRB1*03:01 - DQB1*02:01). In addition to the antigen-presenting role of the separate HLA class II variants encoded within the haplotype, the non-MHC coding alleles embedded have also been studied, with *C4A, TNF, LTA* and *AGER* as candidate markers.[39,41] There are several reports of constitutively higher systemic levels of TNFα in healthy individuals with AH 8.1; however, evidence for direct influence of the 8.1 AH marker (TNF-308A) on TNFα levels is ambiguous.[42] Our finding that almost all DR3-DQ2–positive women also

carry this 8.1 AH marker and that the frequency of homozygous TNF-308A is higher in immunized women suggests that it may contribute to increase the likelihood of HPA-1a immunization.

Associations between HLA alleles and diseases have been known for about 50 years. *DRB3*01:01* has also been shown to be associated with several diseases, in addition to HPA-1a immunization; for example, *DRB3*01:01* is increased in patients with Grave's disease in Jamaicans[43] and has been associated with sarcoidosis.[44] Whether the association between *DRB3*01:01* and several different autoimmune diseases is coincidental because of the close linkage to other alleles in the AH8.1 haplotype is, however, uncertain. In addition to this, other HLA alleles and haplotypes have also been associated with different diseases. One such well-known association is the linkage between *DQB1*02* and celiac disease, where the disease also is shown to be more frequent with a double dose of DQB1*02.[45]

In contrast to the positively associated alleles, the *DRB1*15:01* allele has previously been reported as negatively associated with FNAIT, suggesting a regulatory or suppressive role of this allele.[30] This negative association was also found in the current study, although we here suggest that the negative association can be accounted for by the presence of this allele in an underrepresented haplotype: DR15-DQ6. It is possible that the negative association with FNAIT could be due to other elements present in this haplotype and not necessarily the *DRB1*15:01* allele itself.

As the methods for HLA typing that were applied were published some years ago and do not reflect the current standard of HLA typing, it cannot completely be ruled out that new HLA alleles could have been amplified by the primers used for HLA typing. Alignments of all DRB3 alleles known to this date, with primers in the current study for detection of *DRB3*01:01,* show that the primer pairs would also have amplified, among others, DRB3*02:06, *02:08, *02:21, *02:44, and *02:56. Common for all these alleles, however, is that they have only been found once by one lab and only one of them is confirmed. In addition, the prevalence of these alleles in different ethnic populations is not known. Thus, if one or two new HLA alleles had erroneously been amplified, it is unlikely that this would have influenced our main results.

The main results of this study are summarized in Figure 3. In summary, this study represents one of few studies in which MHC allele associations with HPA-1a immunization have taken into consideration the strong linkage disequilibrium that exist between specific MHC alleles. More importantly, and based on data from a prospective screening study, our results indicate that the haplotype which is associated with the *DRB3*01:01* allele has a considerable impact on the chance of HPA-1a alloimmunization, likely due to yet not identified associated genetic elements. The haplotypes and alleles identified herein point to genetic elements that will be investigated for greater insight into the immune response that results in FNAIT. Potentially, these may guide a more accurate identification of pregnancies most at risk of FNAIT, in a clinical setting.

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CONFLICT OF INTEREST

AH, BS and J.K-K. are three of the founders and owners of Prophylix AS, which has been developing a hyperimmune anti-HPA-1a IgG for the prevention of foetal and neonatal alloimmune thrombocytopenia. J.K-K. is a consultant for Rallybio IPA, LLC, which recently acquired the assets of Prophylix AS.

AUTHORS' CONTRIBUTIONS

Contribution**:** MTA and TBS planned the study. MTA and GH conducted the study. MTA, GH and TBS wrote the manuscript. AH, JKK and BS reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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