



Letter to the Editors-in-Chief

HL-A*11:01, -B*51:01, -DQB1*02:02 and -DRB1*07:01 are associated with inhibitor development in boys with severe haemophilia A receiving rFVIII prophylaxis in Poland

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Although haemophilia A is a model monogenic disease, there is a wide interpatient variability of basic clinical features such as bleeding phenotype, therapeutic FVIII coagulation factor pharmacokinetics or the generation of antibodies neutralizing FVIII ('inhibitor'). Apart from modifiable factors such as the mode of treatment, this is explained by the presence of a wide catalogue of causative *F8* defects that are not equally deleterious as well as the impact of normal population variation in other genes [1,2]. We previously provided a first larger description of the genetic epidemiology of severe haemophilia A in Poland. In that study we reported comprehensive analysis of 101 boys with severe haemophilia A in Poland and, apart from documenting recurrent disease-associated pathogenic variants, we reported 19 novel likely pathogenic variants expanding the catalogue of known *F8* gene mutations [3]. The impact of variation outside of *F8* on haemophilia phenotype has been studied for a long time, and currently there is a growing understanding that full genetic investigation in haemophilia, may aid most effective and potentially personalized treatment especially in view of the emerging therapeutic heterogeneity [1]. For example patients with predefined ultra-high inhibitor risk (such as those with large *F8* deletions) could potentially benefit from alternative modes of prophylaxis or treatment different than FVIII. Consistent with that, we performed a study of an association of MHC alleles with inhibitor (factor VIII inhibitory antibodies) development in boys with haemophilia using high-resolution HLA genotyping. There is a good biological rationale related to basic mechanism of antigen presentation for inhibitor-HLA dependence and there are number of reports on potential HLA-inhibitor associations [4]. Major reported HLA-inhibitor associations are summarized in Supplementary Table 1. The concordance of the studies so far is low and compelling data are lacking. This might be partly related to differences between ethnic groups, mode of treatment delivery or rFVIII brand. HLA genotyping play a significant diagnostic and/or prognostic role in several autoimmune diseases with the notable examples of coeliac disease or ankylosing spondylitis [5]. What is more, HLA variants are associated with the risk of immune-based adverse drug reactions similar to the phenotype studied here [6]. The associations reported so far in haemophilia are mainly limited to class II MHC alleles based on the assumption that class I alleles products are less relevant to

exogenous antigen presentation and thus immune response to *F8* protein [4]. This assumption may be questioned in view of the currently complex understating of immune response including antigen processing but also immunoregulation [7]. Here we performed high-resolution next-generation sequencing based genotyping of major MHC class I and II alleles, rather than limited the study to class I region only.

99 boys with severe haemophilia receiving FVIII prophylaxis were recruited via 14 centres coordinating factor VIII treatment in patients with haemophilia in Poland as described, including demographic data and listing of *F8* variants, in our previous publication [3]. The control group were anonymized HLA-genotyping data from healthy 651 males from Poland registered as potential bone marrow donors in our hospital (genotyped with the same technology and in the same laboratory as haemophilia patients). Appropriate ethical approval was obtained from the Local Ethical Board at the Medical University of Lodz (decision No RNN/247/16/KE).

All patients were treated with recombinant FVIII (octocog alpha-Advate, Kogenate, turoctocog alpha-NovoEight). The history of factor VIII inhibitor was positive for 26 patients, and negative for 73 patients. The description of *F8* genotyping is presented in our previous paper [3]. As described previously the frequency of inhibitor was, as expected highest in cases with large deletions followed by patients with recurrent intron 22 and 1 inversion, and nonsense or frameshift mutations [3]. For this study we defined high-risk genetics group of 76 patients (for inhibitor development) as presence deleterious *F8* variants (large deletions, intragenic inversions, nonsense and frameshift) and low-risk genetics group of 23 patients (missense variants) similar to for example SIPPET investigators [8], the comparison of the inhibitor frequency (24/76 in high-risk versus 2/23 in low-risk patients) between the groups reached statistical significance (Fisher exact test, $p = 0.032$).

High-resolution HLA-A, -B, -C, -DQB1 and -DRB1 genotyping was performed using a next-generation sequencing method on Illumina platform (Illumina, San Diego, CA, USA) using a semi-automated workflow by using MiaFora Flex5 typing kits (Immucor, Warren, NJ, USA). Results of sequencing were analysed by MiaFora NGS software (Immucor, Warren, NJ, USA). Data was considered sufficient whenever the coverage reached 40 and number of cReads exceeded 50,000.

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For the statistical analysis, we compared the carrier frequency of individual HLA alleles in the ‘cases’ and ‘controls’ based on the dominant model using the Fisher exact test (JMP V.11 software; SAS). The following comparisons were made: patients with inhibitor versus patients without inhibitor, patients with inhibitor versus healthy males, patients without inhibitor versus healthy males. With this approach the immune traits test for HLA-association were both the development and non-development (protection from) of inhibitor. The major comparison was inhibitor patients versus healthy controls as the comparison of patients with inhibitor versus patients without inhibitor is generally underpowered to show significant associations and haemophilia per se is not associated with HLA. The OR with a 95% CI (confidence intervals) was calculated with Fisher’s exact test (JMP V.11 software). Each allele was assessed as an independent variable and separate p values were calculated. Considering that distributions of HLA alleles are correlated, we empirically conducted a permutation test to assess multiple testing corrections with a previously described algorithm called PyHLA [9]. We set a two-step check for associations to be finally considered positive and reported as potentially true associations. First criterion was that the association is significant (Permutation test p value <0.05) in the major comparison i.e. of inhibitor patients versus healthy controls; and the second criterion is showing at least a consistent trend confirming the effect (Permutation test p value <0.1) in patients with inhibitor versus patients without inhibitor in the whole group or in patients with inhibitor versus patients without inhibitor the genetic high-risk inhibitor group.

We found no HLA associations for the ‘no inhibitor’ trait in either the whole population or in analysis limited to high-risk patients pointing to the lack of HLA-association with protection from inhibitor (all p values above 0.05, the exact statistics of this non-significant tests are not presented).

Table 1 presents the association results for HLA alleles that scored significant in at least one of the principal (i.e. inhibitor patients versus healthy males) analyses. Of these markers HLA A*11:01, B*51:01, DQB1*02:02 and DRB1*07:01 fulfil the requirement for a consistent trend in the between-group analysis of haemophilia patients. Although disappointingly, none of these markers is concordant with previous reports, in general the subsequent HLA-inhibitor studies published so far show no or minimal concordance, especially if unbiased ‘MHC-wide’

approaches and not predefined markers were used. Further, there is no overlap of these reported inhibitor-HLA associations with published associations between acquired haemophilia and HLA [10]. We argue our study is not superior to previous studies in terms of genotyping technology or numbers of individuals genotyped to uncover associations, also there is no trend in our cohort for previously reported markers that would justify extending the analysis. Among the markers we report, the association is most consistent for HLA A*11:01. Although in the past MHC class I alleles were usually not in focus of inhibitor association studies it must be noted that, probably most so far reported HLA–adverse drug reaction associations were also of class I MHC [9]. This might be because the modes of antigen presentation and its regulation are more complex than previously thought. In particular, it is now recognized that exogenous peptides may also be processed for MHC class I presentation [7]. This linkage might also be due to linkage with other variants in the HLA region vicinity. Moving to MHC class II genes, we believe the associations of DQB1*02:02 and DRB1*07:01 we report are of particular interest. This two markers demonstrate a high degree of linkage and exist in general as a DQB1*02:02-DRB1*07:01 haplotype. In our cohort this haplotype is associated with inhibitor development in the whole group <inhibitor patients versus healthy males, p = 0.016, OR 2.7656 (1.22–6.24) or versus non-inhibitor patients, p = 0.029, OR 3.18 (1.16–8.67)> and does not reach statistical significance in analysis limited to the genetic high-risk patient subgroup. Most interestingly, the only two genetic low-risk patients (F8 variants Pro540Ser and Glu1894Val) with inhibitor are the only two low-risk patients with the DQB1*02:02-DRB1*07:01 haplotype. This haplotype and/or DQB1*02:02 was previously associated with a relevant phenotype of a generation of anti-peptide drug antibodies (against L-asparaginase in leukemia patients) [7].

In conclusion, we did not reproduce previously reported HLA-FVIII inhibitor associations, which is a general fate of studies addressing this topic. We argue this is not because our study is underpowered or technologically compromised and Poland is a large and ethnically homogenous population, well suited for HLA-analysis association studies. Whether this low concordance is because there are variations dependent on the ethnic background and FVIII brand or spurious associations are reported remains an open issue. Based on current knowledge and despite decades of research, HLA-testing in stratifying the patients for inhibitor

Table 1

Permutation test significant p values (<0.05) are shown in **bold**, trend (<0.1) are shown in *italics*.

Allele	Carrier frequency ‘cases’	Carrier frequency ‘controls’	OR (with 95% confidence intervals)	p value	p value permutation test (PyHLA)
All patients with inhibitor (n = 26) vs. healthy males (n = 651)					
A*11:01	0.1667	0.0664	2.81 (1.21–6.55)	0.0231	0.0094
B*51:01	0.1154	0.0476	2.61 (1.07–6.36)	0.0422	0.0307
C*07:01	0.0400	0.1302	0.28 (0.07–1.16)	0.0785	0.0390
DQB1*02:02	0.2000	0.0943	2.40 (1.17–4.93)	0.0250	0.0051
DRB1*07:01	0.2500	0.1446	1.97 (1.03–3.77)	0.0456	0.0211
All patients with inhibitor (n = 26) vs. all non-inhibitor (n = 73)					
A*11:01	0.1667	0.0714	2.60 (0.90–7.49)	0.1240	<i>0.0607</i>
B*51:01	0.1154	0.0580	2.12 (0.70–6.44)	0.2135	0.1427
C*07:01	0.0400	0.0769	0.50 (0.11–2.37)	0.5148	0.3646
DQB1*02:02	0.2000	0.0758	3.05 (1.19–7.86)	0.0304	0.0125
DRB1*07:01	0.2500	0.1301	2.23 (1.10–4.92)	0.0506	0.0211
Genetic high-risk patients with inhibitor (n = 24) vs. healthy males (n = 651)					
A*11:01	0.1842	0.0655	3.22 (1.39–7.43)	0.0119	0.0038
B*51:01	0.1042	0.0476	2.33 (0.89–6.10)	0.0850	<i>0.0534</i>
C*07:01	0.0217	0.1328	0.15 (0.02–1.06)	0.0244	0.0094
DQB1*02:02	0.1739	0.0982	1.93 (0.89–4.20)	0.1280	<i>0.0689</i>
DRB1*07:01	0.2292	0.1452	1.75 (0.89–3.46)	0.1451	<i>0.0854</i>
Genetic high-risk patients with inhibitor (n = 24) vs. genetic high-risk patients non-inhibitor (n = 52)					
A*11:01	0.1842	0.0854	2.42 (0.78–7.48)	0.1339	<i>0.0562</i>
B*51:01	0.1042	0.0217	5.23 (0.98–28.1)	0.0466	0.0222
C*07:01	0.0217	0.0465	0.46 (0.05–4.20)	0.6576	0.3479
DQB1*02:02	0.1739	0.1000	1.89 (0.68–5.29)	0.2741	0.1711
DRB1*07:01	0.2292	0.1327	1.94 (0.79–4.73)	0.1579	<i>0.0720</i>

risk has little practical utility. We propose a further validation of the DQB1*02:02-DRB1*07:01 haplotype.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.thromres.2021.03.027>.

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CRediT authorship contribution statement

Szymon Janczar recruited study subjects, provided clinical data, analysed data and wrote the manuscript. Maciej Borowiec and Wojciech Mlynarski analysed data and wrote the manuscript. Katarzyna Bąbol-Pokora and Bogusław Tytoniuk performed the laboratory studies and analysed data. Ming Songy and You-qiang Song performed the HLA-associations statistical analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Number of patients	HLA associations	Haemophilia severity	Author [Reference]
49	No significant linkage for class II	mild	Bril [1]
100	No strong correlation	severe /moderate	Chuansumrit [2]
447	HLA-DRB1*13	severe	David [3]
122	HLA-C*16, and -DRB1*14	severe	De Barros [4]
176	HLA-DQA1*01:02	severe	Hay [5]
101	HLA-DRB1*01:01	severe	Hosseini [6]
100	Various, subgroup dependent	severe	Kim [7]
57	HLA-DRB1*15	not available	Nathalang [8]
46	HLA-A*24	not available	Ohta [9]
71	No strong correlation	severe	Oldenburg [10]
260	HLA-DRB1*15, DQB1*0602	severe	Pavlova [11]
52	Various HLA-DRB1 and -DQB1 alleles	severe	Pergantou [12]
119	HLA-DRB1*02	severe	Pinto [13]

Table S1. The summary of previous studies in HLA-inhibitor associations.

50	Various class I and II alleles	not available	Wieland [14]
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