Aalborg Universitet

HNA specificity and association to HLA-DRB1 and -DQB1 in patients with autoimmune neutropenia of early childhood

Kløve-Mogensen, Kirstine; Steffensen, Rudi; Nielsen, Hans Linde; Masmas, Tania Nicole; Glenthøj, Andreas Birkedal; Jensen, Christina Friis; Haunstrup, Thure Mors; Ratcliffe, Paul; Höglund, Petter; Hasle, Henrik; Nielsen, Kaspar René

[Link to publication from Aalborg University](#)

Full Length Article

HNA specificity and association to *HLA-DRB1* and *-DQB1* in patients with autoimmune neutropenia of early childhood

Kirstine Kløve-Mogensen [a,b,*], Rudi Steffensen [a], Hans Linde Nielsen [b,c], Tania Nicole Masmas [d], Andreas Glenthøj [e], Christina Friis Jensen [f], Thure Mors Haunstrup [a], Paul Ratcliffe [g], Petter Höglund [g], Henrik Hasle [h], Kaspar René Nielsen [a,b]

[a] *Department of Clinical Immunology, Aalborg University Hospital, Aalborg, Denmark*
[b] *Department of Clinical Medicine, Aalborg University, Aalborg, Denmark*
[c] *Department of Microbiology, Aalborg University Hospital, Aalborg, Denmark*
[d] *Pediatric Hematopoietic Stem Cell Transplantation and Immunodeficiency, Department of Pediatrics and Adolescent Medicine, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark*
[e] *Department of Hematology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark*
[f] *Department of Pediatrics, Aalborg University Hospital, Aalborg, Denmark*
[g] *Department of Medicine Huddinge, Karolinska Institute, Stockholm, Sweden*
[h] *Department of Pediatrics and Adolescent Medicine, Aarhus University Hospital, Aarhus, Denmark*

A B S T R A C T

Autoimmune neutropenia (AIN) of early childhood is caused by autoantibodies against antigens on the neutrophil membrane. Human leukocyte antigens (HLA) have previously been associated with AIN. This study investigated *HLA-DRB1* and *HLA-DQB1* alleles in 160 antibody positive patients and compared with 1000 controls. Increased risk was observed for *DRB1*10, DRB1*14, DRB1*16* and *DQB1*05*, and lower risk for *DRB1*04, DRB1*13* and *DQB1*03*. Haplotypes with higher risk included: *DRB1*10/DQB1*05, DRB1*14/DQB1*05* and *DRB1*16/DQB1*05*, while *DRB1*04/DQB1*03, DRB1*07/DQB1*02,* and *DRB1*13/DQB1*06* were associated with lower risk. Associated *HLA-DRB1* and *–DQB1* differed between patients positive for anti-HNA-1a-specific antibodies and patients positive for broad reactive anti-FcγRIIIb antibodies. *DRB1*01, DRB1*04* and *DQB1*03* was only associated for anti-HNA-1a positive, and *DRB1*10* was restricted to broad reactive anti-FcγRIIIb positive. Strong association between AIN and *HLA-DRB1* and *-DQB1* alleles and haplotypes suggested that they play a role in susceptibility or protection. Different associations regarding FcγRIIIb antibody specificities could indicate disease heterogeneity.

1. Introduction

In primary autoimmune neutropenia (AIN) in early childhood, antibodies recognize antigens of neutrophils (Human neutrophil antigens (HNA)), mostly located on immunoglobulin G (IgG) Fc receptor type 3b (FcγRIIIb (CD16b)), causing their peripheral destruction [1]. The antibodies are known to differ in specificity and the patients can in broad terms be divided into two groups, those with antibodies directed against a specific HNA variant (HNA-1a) and those with broad reacting FcγRIIIb antibodies, the biology behind this remains unknown. Among Danish AIN patients, anti-HNA-1a is the most common autoantibody, and the antibody is more common in cases with the *FCGR3B*01+,*02-,*03-* (HNA-1a) genotype [2]. AIN is usually benign and most patients are in complete remission after 2–3 years [3]. Information regarding the cause of this disease is limited due to the scarce data on the triggering etiology. Viral infections have been suggested as a possible trigger [4].

Presentation of antigens to the immune system relies on human leukocyte antigen (HLA) complex, the human version of the major histocompatibility complex (MHC). HLA genes are located as a family of gene clusters on chromosome 6p21.3 [5]. HLA genes are the most polymorphic genes in the human genome, and polymorphisms located within the beta-sheets floor and alpha-2 helix of HLA molecules result in various binding affinities [6–8]. HLA class II initiates immune responses by presenting them to CD4+ helper T cells, which facilitates cellular immune responses [5]. HLA-DR and -DQ molecules are involved in the presentation of antigens, including self-antigens, and the suppression of autoimmunity by regulatory T-cells (Tregs) [9]. Specific HLA types are known to be strongly associated with the risk of autoimmune diseases. The association is often disease-specific and may represent the superiority of these HLA proteins in binding antigens with autoantigenic potential [5]. Autoimmune disease associated alleles often differ from disease-non-promoting ones by only a few amino acids that are at the T

cell receptor (TCR) – HLA-II synapsis, explaining for the effect of even minor genetic changes. *HLA-DRB1* and *-DQB1* is amongst the most studied genetic factors associated with autoimmune diseases [10,11].

Association between the HLA-system and AIN was first described in 1991 in a German cohort (n=26), who showed association to serological determined DR2 (*HLA-DRB1*15* and **16*) and DQ1 (*HLA-DQB1*05* and **06*) [12]. The *HLA-DQB1*05:03* genotype have also previously been suggested for AIN in a small study from Taiwan (n=31) and confirmed by us in a Danish cohort (n=80), where we found association to *HLA-DRB1*14* as well [2,13]. To obtain better insight into the relationship between *HLA-DRB1* and *-DQB1* alleles and AIN, we expanded the cohort to 160 patients and compared it with a control group of thousand healthy Danish individuals. We further investigated the influence of FcγRIIIb antibody specificity on *HLA-DRB1* and *-DQB1* association, by separately comparing the two antibody groups, patients positive for antibodies directed specific against the HNA-1a epitope (n=81), and the remaining patients positive for FcγRIIIb antibodies (n=79), to the control group.

The Epstein-Barr virus (EBV) is associated with glandular fever, and the susceptibility seems to be HLA related [14]. EBV is also associated with a number of autoimmune diseases [15]. The HLA results from the present study inspired us to investigate if EBV is a possible triggering event in AIN. *HLA-DRB1*01:01* has been associated to infectious mononucleosis, an infection usually caused by EBV, so because of limited material, we focused on patients with this allele, and investigated samples taking at the time of diagnosis for post infection EBV antibodies [16].

2. Materials and methods

2.1. Study cohort

A total of 160 patients were included, all diagnosed with FcγRIIIb autoantibody positive AIN between 2004 and 2021 at the Department of Clinical Immunology, Aalborg University Hospital, Denmark, which is the national center for diagnostic AIN testing. The inclusion criteria were the presence of neutropenia, an absolute neutrophil count (ANC) not above 1.5×10^9 cell/L in two repeated tests, age under 5 years at the time of diagnosis, and the presence of anti-neutrophil antibodies in the flow cytometric indirect granulocyte immunofluorescence test (Flow-GIFT) as previously described [2]. Patients with initial negative antibody screening were repeated as suggested by Bux [3]. Patients with congenital neutropenia, neutropenia related to inborn syndromes, postinfection neutropenia, or hematological malignancies were excluded. Genetic material was available for all patients for genotyping for HLA. Serum from the time of diagnosis was available for investigation of Epstein Barr virus (EBV) IgG-specific antibodies in all patients. The control group consisted of 1000 randomly selected and anonymous, healthy adult Danish blood donors from the Aalborg University Hospital blood bank, Aalborg, Denmark. The high number of controls was used to provide certainty of the frequency of rare alleles. Both patients and controls consisted primarily of whites. Consent for study participation for all patients was obtained from legal guardians, and the study was approved by the local ethics committee (N-20170026).

2.2. DNA preparation

DNA was extracted from EDTA-stabilized whole blood or buccal swabs using the Maxwell 16 Blood DNA Kit or the Maxwell RSC Buccal Swab Kit on the Maxwell RSC instrument (Promega, US).

2.3. HLA genotyping

High-resolution *HLA-DRB1* and *HLA-DQB1* genotyping was performed by next-generation sequencing for 80 of the patients, which our group previously has described in [2]. Exons 2 and 3 were sequenced for *HLA-DRB1* and *-DQB1* genotypes, giving a resolution at the 2nd-field

level at Histogenetics [17] (New York, USA). Because of limited sample material, we decided to investigate the next 80 samples using the HLA-FluoGene DRDQ Kit on a Fluogene real-time platform according to the protocol from the supplier, giving a resolution of 1st-field level (inno-train Diagnostik GmbH, Germany). The control group was genotyped by next-generation sequencing where exons 2 and 3 were sequenced for *HLA-DRB1* and *-DQB1* alleles at Histogenetics [17] (New York, USA). Haplotypes were formed by *HLA-DRB1, -3, -4, -5 and HLA-DQB1* typing. Data for *DRB3, -4* and *-5* are not shown.

2.4. EBV serology

One hundred microliters of serum isolated from blood samples taken at the time of diagnosis and stored at -80 °C was investigated for IgG antibodies specific for EBV. A total of 22 patients carrying the *DRB1*01* allele were grouped by FcγRIIIb antibody specificity and age [16]. Analysis was performed on the VIDAS EBV Panel (BioMérieux, France). Due to a limited volume of serum only viral capsid antigen/early antigen (VCA/EA) IgG (Ref. 30 236) was analyzed. Test values below 0.10 were considered negative, between 0.10 and 0.20 inconclusive, and above 0.20 positive. Test for VCA IgM and the Epstein-Barr nuclear antigen (EBNA) IgG was not performed. We measured VCA/EA IgG antibodies as it indicates past infection more accurately than EBNA-IgG [18]. The patients were age-matched to minimize the effect of differences in exposure due to age and used serum from the first time they were diagnosed with AIN.

2.5. Statistics

Data analysis was conducted using the statistical program Stata (Version 17.0, StataCorp, College Station, Texas). The allele frequencies were calculated by direct counting [19]. The control group was confirmed to be in Hardy-Weinberg Equilibrium with SHEsisPlus software [20–22]. Odds ratios (OR) and 95% confidence intervals (CI) were calculated, and differences between groups were tested using Fischer's exact test. Bonferroni correction was used to adjust p-values in case of multiple statistical testing.

3. Results

3.1. Baseline characteristics

We included 160 patients diagnosed with AIN with a median age at diagnosis of 14.2 months (range 3–54 months). The sex distribution was 49% females and 51% males. All AIN patients were positive for anti-FcγRIIIb antibodies, and of these had 49% anti-HNA-1a-specific antibodies. The patients were both investigated as a combined group and as two individual groups divided by their antibody specificity. The two groups consisted of 81 patients which were anti-HNA-1a-positive, and the remaining 79 patients which will be referred to as anti-FcγRIIIb-positive. The control group consisted of 1000 healthy unrelated Danish blood donors.

3.2. HLA genotypes

The *HLA-DRB1* and *HLA-DQB1* alleles were determined for all 160 patients and 1000 controls. A significant increased risk for AIN was observed for *DRB1*10* (OR=5.83 (2.46–13.85)), *DRB1*14* (OR=3.76 (2.29–6.17)), *DRB1*16* (OR=7.52 (3.54–15.95)) and *DQB1*05* (OR=2.83 (2.17–3.70)). Looking only at the 81 anti-HNA-1a positive patients' alleles with an increased risk included *DRB1*01* (OR=2.63 (1.65–4.21)), *DRB1*14* (OR=4.33 (2.27–8.28)), *DRB1*16* (OR=8.32 (3.34–20.72)) and *DQB1*05* (OR=6.72 (4.06–11.10)). The 79 anti-FcγRIIIb positive patients had an increased risk for *DRB1*10* (OR=8.74 (3.29–23.23)), *DRB1*14* (OR=3.71 (1.88–7.37)), *DRB1*16* (OR=7.38 (2.86–19.08)) and *DQB1*05* (OR=2.87 (1.81–4.56)).

Table 1
Frequency of HLA-DRB1 alleles in AIN patients and controls.

HLA DRB1	Controls n=1000 (%)	All AIN patients n=160 (%)	OR (95% CI)	P-value[a]	Anti-HNA-1a antibodies n=81 (%)	OR (95% CI)	P-value[a]	Anti-FcγRIIIb antibodies n=79 (%)	OR (95% CI)	P-value[a]
DRB1*01	207 (10.4)	45 (14.1)	1.42 (1.00–2.00)	0.053	33 (20.4)	2.63 (1.65–4.21)	**0.0001**	12 (7.6)	0.69 (0.36–1.29)	0.309
DRB1*03	293 (14.7)	52 (16.3)	1.13 (0.82–1.57)	0.447	26 (16.0)	1.14 (0.70–1.85)	0.613	26 (16.5)	1.18 (0.73–1.93)	0.523
DRB1*04	328 (16.4)	26 (8.1)	0.45 (0.30–0.68)	**0.0001**	10 (6.2)	0.29 (0.15–0.57)	**0.0001**	16 (10.1)	0.52 (0.30–0.91)	0.024
DRB1*07	213 (10.7)	18 (5.6)	0.50 (0.30–0.82)	0.005	7 (4.3)	0.35 (0.16–0.77)	0.006	11 (7.0)	0.15 (0.31–1.15)	0.149
DRB1*08	58 (2.9)	14 (4.4)	1.53 (0.84–2.78)	0.164	6 (3.7)	1.30 (0.54–3.11)	0.470	8 (5.1)	1.83 (0.84–3.98)	0.139
DRB1*09	19 (1.0)	0 (0.0)	0.16 (0.01–2.63)	0.096	0 (0.0)	0.31 (0.02–5.16)	0.390	0 (0.0)	0.32 (0.02–5.29)	0.390
DRB1*10	11 (0.6)	10 (3.1)	5.83 (2.46–13.85)	**0.0002**	3 (1.9)	3.46 (0.95–12.65)	0.081	7 (4.4)	8.74 (3.29–23.23)	**0.0001**
DRB1*11	126 (6.3)	28 (8.8)	1.43 (0.93–2.19)	0.115	12 (7.4)	1.21 (0.64–2.29)	0.603	16 (10.1)	1.76 (0.99–3.15)	0.058
DRB1*12	41 (2.1)	4 (1.3)	0.60 (0.22–1.70)	0.510	0 (0.0)	0.14 (0.01–2.33)	0.067	4 (2.5)	1.25 (0.44–3.58)	0.565
DRB1*13	308 (15.4)	20 (6.3)	0.37 (0.23–0.59)	**< 0.00001**	8 (4.9)	0.25 (0.12–0.52)	**<0.001**	12 (7.6)	0.40 (0.21–0.75)	**0.003**
DRB1*14	46 (2.3)	26 (8.1)	3.76 (2.29–6.17)	**< 0.00001**	14 (8.6)	4.33 (2.27–8.28)	**0.0001**	12 (7.6)	3.71 (1.88–7.35)	**0.0006**
DRB1*15	337 (16.9)	62 (19.4)	1.19 (0.88–1.60)	0.265	35 (21.6)	1.50 (0.95–2.37)	0.089	27 (17.1)	1.02 (0.63–1.66)	1.000
DRB1*16	13 (0.7)	15 (4.7)	7.52 (3.54–15.95)	**< 0.00001**	8 (4.9)	8.32 (3.34–20.72)	**0.0001**	7 (4.4)	7.38 (2.86–19.08)	**0.0003**

[a] *P*-value using Fisher's exact test. Significance level after Bonferroni correction to α=0.05: $\alpha/13 = 0.004$

Table 2
Frequency of HLA-DQB1 alleles in AIN patients and controls.

HLA DQB1	Controls n=1000 (%)	All AIN patients n=160 (%)	OR (95% CI)	P-value[a]	Anti-HNA-1a antibodies n=81 (%)	OR (95% CI)	P-value[a]	Anti-FcγRIIIb antibodies n=79 (%)	OR (95% CI)	P-value[a]
DQB1*02	452 (22.6)	69 (21.6)	0.94 (0.71–1.25)	0.719	32 (19.8)	0.79 (0.50–1.26)	0.354	37 (23.4)	1.07 (0.67–1.69)	0.815
DQB1*03	590 (29.5)	64 (20.0)	0.60 (0.45–0.80)	**0.0004**	28 (17.3)	0.37 (0.23–0.59)	**<0.001**	36 (22.8)	0.58 (0.37–0.92)	0.024
DQB1*04	57 (2.9)	7 (2.2)	0.76 (0.34–1.69)	0.586	3 (1.9)	0.64 (0.19–2.08)	0.616	4 (2.5)	0.88 (0.32–2.50)	1.000
DQB1*05	273 (13.7)	99 (30.9)	2.83 (2.17–3.70)	**< 0.00001**	58 (35.8)	6.72 (4.06–11.10)	**<0.00001**	41 (25.9)	2.87 (1.81–4.56)	**<0.001**
DQB1*06	628 (31.4)	81 (25.3)	0.74 (0.57–0.97)	0.031	41 (25.3)	0.61 (0.39–0.96)	0.033	40 (25.3)	0.61 (0.38–0.96)	0.040

[a] *P*-value using Fisher's exact test. Significance level after Bonferroni correction to α=0.05: $\alpha/5 = 0.01$

Significant lower risk for AIN for all 160 patients was observed for *DRB1*04* (OR=0.45 (0.30–0.68)), *DRB1*13* (OR=0.37 (0.23–0.59)) and *DQB1*03* (OR=0.60 (0.45–0.80)). For the anti-HNA-1a positive the same three HLA alleles was associated with a lower risk; *DRB1*04* (OR=0.29 (0.15–0.57)), *DRB1*13* (OR=0.25 (0.12–0.52)) and *DQB1*03* (OR=0.37 (0.23–0.59)), while only *DRB1*13* (OR=0.40 (0.21–0.75)) was significantly associated for the group of anti-FcγRIIIb positive patients. The distribution of all *HLA-DRB1* and *-DQB1* alleles is reported in Tables 1 and 2.

3.3. HLA haplotypes

The association was significantly different for six haplotypes of *HLA-DRB1* and *-DQB1* comparing the group of 160 AIN patients to the controls. A significant higher risk of AIN was observed for *DRB1*10/DQB1*05* (OR=5.83 (2.46–13.85)), *DRB1*14/DQB1*05* (OR=3.95 (2.37–6.58)) and *DRB1*16/DQB1*05* (OR=6.44 (2.77–14.97)). For the group of patients positive for anti-HNA-1a antibodies the HLA genotypes with a significantly higher risk was *DRB1*01/DQB1*05* (OR=2.72 (1.79–4.34)), *DRB1*14/DQB1*05* (OR=4.36 (2.23–8.51)) and *DRB1*16/DQB1*05* (OR=9.85 (3.82–25.26)), while the anti-FcγRIIIb positive patients had a higher risk with *DRB1*11/DQB1*05* (OR=91.55 (4.69–1788.69)) and *DRB1*14/DQB1*05* (OR=4.09 (2.05–8.13)).

A significant lower risk was observed for *DRB1*04/DQB1*03* (OR=0.40 (0.26–0.62)), *DRB1*07/DQB1*02* (OR=0.38 (0.20–0.74)) and *DRB1*13/DQB1*06* (OR=0.37 (0.23–0.59)) in the combined patient group. In the group of patients with specific anti-HNA-1a antibodies *DRB1*04/DQB1*03* (OR=0.29 (0.15–0.57)) and *DRB1*13/DQB1*06* (OR=0.23 (0.10–0.50)) was significantly associated to a lower risk of disease. None HNA genotypes were exclusively associated with a lower disease for the anti-FcγRIIIb positive patients. The distribution of all haplotypes is reported in Table 3.

The distribution of homo- and heterozygosity for the *HLA-DRB1/DQB1* haplotypes indicated a significant higher risk of AIN for heterozygous (OR=2.64 (1.32–5.30)) compared to homozygous (OR=0.38 (0.19–0.76)). Looking at the two antibody groups, this was only the case for the anti-HNA-1a positive, where heterozygosity was associated with higher risk (OR=25.7 (1.59–417.43)) and homozygosity with lower risk (OR=0.04 (0.00–0.63)). This is seen in Table 4.

3.4. EBV serological status

A total of 22 serum samples from age-matched patients carrying the *DRB1*01* allele were investigated for EBV IgG. Two samples were positive, 19 samples were negative, and one was inconclusive. There was no correlation between the positive samples and patients with a specific anti-HNA-1a antibody. Results of the EBV IgG investigation can be seen in Table 5.

4. Discussion

In this study, we investigated the association between AIN and *HLA-DRB1* and *-DQB1* genotypes and discovered an association between both disease and HNA antibody specificity. It is well established that there is an association between HLA antigens and autoimmune diseases, but the molecular mechanism is unclear, even though antigen presentation and T-cell activation seems to be the triggering event in most autoimmune diseases [9,11]. The mechanism and triggering event may differ in diseases characterized by primary T-cell mediated organ destruction and antibody mediated autoimmune diseases as AIN, but this seems not to be clear. The genetic susceptibility to immune cytopenia has only been studied in more detail in immune thrombocytopenia (ITP), and these data suggest a genetic association in the HLA-II area, a T-cell driven pathogenesis resulting in autoantibody productions, and the possibility of a viral trigger [23–25]. In the case of AIN, there is not yet enough data to propose a genetic or environmental cause. A possible event trigger-

Table 3

Frequency of HLA-DRB1 and -DQB1 haplotypes in AIN patients and controls.

Haplotype DRB1/DQB1	Controls n=1000 (%)	All AIN patients n=160 (%)	OR (95% CI)	P-value[a]	Anti-HNA-1a antibodies n=81 (%)	OR (95% CI)	P-value[a]	Anti-FcγRIIIb antibodies n=79 (%)	OR (95% CI)	P-value[a]
*01/*03	5 (0.3)	0 (0.0)	0.57 (0.03–10.26)	1.000	0 (0.0)	1.11 (0.06–20.26)	1.000	0 (0.0)	1.14 (0.06–20.77)	1.000
*01/*05	202 (10.1)	45 (14.1)	1.46 (1.03–2.06)	0.040	33 (20.4)	2.72 (1.79–4.34)	**0.0001**	12 (7.6)	0.71 (0.38–1.33)	0.378
*03/*02	292 (14.6)	52 (16.3)	1.13 (0.82–1.57)	0.446	24 (14.8)	1.02 (0.62–1.68)	1.000	28 (17.7)	1.33 (0.82–2.15)	0.251
*04/*03	326 (16.3)	23 (7.2)	0.40 (0.26–0.62)	**< 0.00001**	10 (6.2)	0.29 (0.15–0.57)	**0.0001**	13 (8.2)	0.41 (0.22–0.75)	0.0024
*07/*02	155 (7.8)	10 (3.1)	0.38 (0.20–0.74)	**0.0015**	4 (2.5)	0.14 (0.05–0.39)	0.0082	6 (3.8)	0.45 (0.19–1.05)	0.070
*07/*03	58 (2.9)	8 (2.5)	0.86 (0.41–1.82)	0.856	4 (2.5)	0.84 (0.30–2.39)	1.000	4 (2.5)	0.87 (0.31–2.45)	1.000
*08/*04	55 (2.8)	12 (3.8)	1.38 (0.73–2.60)	0.366	6 (3.7)	1.37 (0.57–3.30)	0.451	6 (3.8)	1.41 (0.59–3.39)	0.443
*09/*03	18 (0.9)	0 (0.0)	0.17 (0.01–2.87)	0.156	0 (0.0)	0.33 (0.02–5.46)	0.390	0 (0.0)	0.33 (0.02–5.59)	0.636
*10/*05	11 (0.6)	10 (3.1)	5.83 (2.46–13.85)	**0.0002**	3 (1.9)	3.46 (0.95–12.65)	0.081	7 (4.4)	8.84 (3.29–23.23)	**0.0001**
*11/*03	124 (6.2)	23 (7.2)	1.17 (0.74–1.86)	0.536	12 (7.4)	1.23 (0.65–2.33)	0.489	11 (7.0)	1.14 (0.59–2.22)	0.723
*11/*05	0 (0.0)	3 (0.9)	44.11 (2.27–855.93)	0.0026	0 (0.0)	NA	NA	3 (1.9)	91.55 (4.69–1788.66)	**0.0004**
*12/*03	40 (2.0)	4 (1.3)	0.62 (0.22–1.75)	0.507	0 (0.0)	0.15 (0.01–2.39)	0.067	4 (2.5)	1.28 (0.45–3.67)	0.557
*13/*03	11 (0.6)	0 (0.0)	0.27 (0.02–4.59)	0.379	0 (0.0)	0.53 (0.03–9.04)	1.000	0 (0.0)	0.54 (0.03–9.27)	1.000
*13/*05	4 (0.2)	0 (0.0)	0.69 (0.04–12.89)	1.000	0 (0.0)	1.36 (0.07–25.46)	1.000	0 (0.0)	1.39 (0.07–26.10)	1.000
*13/*06	293 (14.7)	19 (5.9)	0.37 (0.23–0.59)	**< 0.00001**	7 (4.3)	0.23 (0.10–0.50)	**0.000**	12 (7.6)	0.43 (0.23–0.81)	0.0063
*14/*03	4 (0.2)	0 (0.0)	0.69 (0.04–12.89)	1.000	0 (0.0)	1.36 (0.07–25.46)	1.000	0 (0.0)	1.39 (0.07–26.10)	1.000
*14/*05	42 (2.1)	25 (7.8)	3.95 (2.37–6.58)	**< 0.00001**	13 (8.0)	4.36 (2.23–8.51)	**0.0001**	12 (7.6)	4.09 (2.05–8.13)	**0.0003**
*15/*05	3 (0.2)	3 (0.9)	6.30 (1.27–31.35)	0.038	1 (0.6)	4.15 (0.43–40.40)	0.268	2 (1.3)	8.63 (1.42–52.44)	0.0458
*15/*06	334 (16.7)	58 (18.1)	1.10 (0.81–1.50)	0.521	34 (21.0)	1.44 (0.91–2.29)	0.143	24 (15.2)	0.87 (0.53–1.43)	0.622
*16/*05	11 (0.6)	11 (3.4)	6.44 (2.77–14.97)	**0.0001**	8 (4.9)	9.85 (3.82–25.26)	**0.000**	3 (1.9)	3.55 (0.97–12.99)	0.077
*16/*06	2 (0.1)	3 (0.9)	9.45 (1.57–56.80)	0.021	1 (0.6)	6.24 (0.56–69.54)	0.209	2 (1.3)	12.96 (1.80–93.3)	0.0288
Other	10 (0.5)	11 (3.4)			2 (0.2)			9 (5.7)		

[a] *P*-value using Fisher's exact test. Significance level after Bonferroni correction to $\alpha=0.05$: $\alpha/22 = 0.0023$.

Table 4

Homozygosity of HLA-DRB1/DQB1 haplotypes in AIN patients and controls.

	Controls n=1000 (%)	AIN patients n=160 (%)	OR (95%CI)	P-value[a]	Anti-HNA-1a antibodies n=81 (%)	OR (95% CI)	P-value[a]	Anti-FcγRIIIb antibodies n=79 (%)	OR (95% CI)	P-value
Homozygots	136 (13.6)	9 (5.6)	0.38 (0.19–0.76)	**0.003**	0 (0.0)	0.04 (0.00–0.63)	**0.000**	9 (11.4)	0.82 (0.40–1.67)	0.732
Heterozygots	864 (86.4)	151 (94.4)	2.64 (1.32–5.30)	**0.003**	81 (100.0)	25.7 (1.59–417.43)	**0.000**	70 (88.6)	1.22 (0.60–2.51)	0.732

n= number of cases

Table 5

EBV IgG serum investigation on 22[†] HLA- DRB1*01 carriers.

	AIN patients with anti-HNA-1a antibodies n=11		AIN patients with anti-FcγRIIIb antibodies n=10	
Age	Pos (%)	Neg (%)	Pos (%)	Neg (%)
<8 month	0 (0.0%)	2 (18.2%)	0 (0.0%)	3 (30.0%)
9–12 month	0 (0.0%)	3 (27.3%)	0 (0.0%)	2 (20.0%)
12–15 month	0 (0.0%)	4 (36.4%)	1 (10.0%)	2 (20.0%)
15–30 month	0 (0.0%)	2 (18.2%)	1 (10.0%)	1 (10.0%)

n= number of cases

[†] One sample was inconclusive

ing AIN is viral infection [4], initiating the activation of autoreactive B cells and CD4+ T cells, but research also points to a general deficiency in peripheral self-tolerance, mediated by an alteration in either the function or number of CD4+ Tregs cells [26]. Activation of Tregs is HLA restricted, and certain HLA genotypes protect or increase susceptibility to autoimmune diseases through Tregs cells. A connection between AIN and circulating Tregs cells has been shown by Nakamura et al. [27], which supports HLA association and gives rise to further investigation. Three former studies have investigated an association between the HLA system and AIN. In 1991, Bux et al. [12] investigated 26 German children diagnosed with AIN using serological HLA typing methods and found an association with the HLA-DR2 (DRB1*15 and DRB1*16) and with the HLA-DQ1 (DQB1*05 and DQB1*06) phenotypes. However, this was not reproduced in a later study by Wang et al. [13], who genotyped 31 Taiwanese AIN patients and found a strong association with the HLA-DQB1*05:03 genotype. In an earlier study, we reproduced the

HLA-DQB1*05 signal in a Danish Caucasian cohort with 80 patients and also found an association for HLA-DRB1*14 [2].

We expanded the number of patients from our former publication to 160, establishing the largest study to date to investigate the association between HLA and AIN [2]. This also made it possible for us to compare our findings with HNA antibody specificity. Informed by our earlier findings, we focused on HLA-DRB1 and -DQB1, because of their previously associations to autoimmune diseases. In addition to supporting the association of DRB1*14, when expanding the study group, we also found a disease association for DRB1*10 and DRB1*16 and a protective association for DRB1*04 and DRB1*13. Furthermore, we provide support for the role of DQB1*05 as a risk allele, while the DQB1*03 allele shows protection against AIN. Moreover, we found associations for several DRB1/DQB1 haplotypes. Most of them, DRB1*04/DQB1*03, DRB1*10/DQB1*05, DRB1*13/DQB1*06, DRB1*14/DQB1*05 and DRB1*16/DQB1*05, are related to the associa-

tion observed for the individual HLA alleles, but we also see a protective association for *DRB1*07/DQB1*02*.

DRB1*04 and *DRB1*13* are known to be protective against rheumatoid arthritis [28], and *DRB1*04* has also been found to be protective against sarcoidosis [28]. *DQB1*03* has shown protection against bullous pemphigoid [28]. A small study from 1996 showed a higher risk of autoantibodies and autoimmune hemolytic anemia for individuals carrying *DQB1*05* [29], supporting a key role in relation to immune cytopenia.

AIN is characterized by antibodies directed against epitopes on the FcγRIIIb receptor. However, the AIN autoantibodies group in specificity in broad terms, into anti-HNA-1a and anti-FcγRIIIb antibodies. Whether these groups differ clinically is largely unknown. Among Danish AIN patients, anti-HNA-1a antibodies is slightly the most common FcγRIIIb autoantibody [2], and when isolated comparison of HLA-alleles for patients with specific anti-HNA-1a antibodies with the control group, and patients with anti-FcγRIIIb antibodies with the control group, we found a significant difference in the distribution of disease associated *HLA-DRB1* and *-DQB1* genotypes. *DRB1*01,* which were not significantly associated to AIN in the combined group, is a risk factor for the group of anti-HNA-1a positive. *DRB1*04* was significant in the combined group, but the association seems to only come from the anti-HNA-1a positive and not the anti-FcγRIIIb positive, and the same is the case for *DQB1*03*. The opposite is the case for *DRB1*10* where the association found in the combined group appears to only come from the anti-FcγRIIIb positive.

HLA-DRB1*01:01 has been shown to be overrepresented in individuals who develop infectious mononucleosis compared to individuals with asymptomatic EBV infection [16]. *HLA-DRB1*01:01* carriers might be more permissive to EBV entry, resulting in a more enhanced proliferation during the early stage of infection and effective stimulation of an immune response. Ramagopalan et al. showed a significant reduction in EBV copy number in individuals with *HLA-DRB1*01:01*, suggesting that immune control of viral replication is more effective in these individuals [30].

Based on this hypothesis, we investigated 22 patients carrying the *DRB1*01* allele for their EBV serological status. Patients positive for a specific anti-HNA-1a antibody were compared to patients with anti-FcγRIIIb antibodies. EBV IgG testing resulted in two positive test results, 19 negative results and one inconclusive result Table 5. There was no correlation between EBV IgG and antibody specificity. The two patients who tested positive were both more than 12 months old at the time of their AIN diagnosis.

HLA-DRB1*01:01 has also been shown to be a potent stimulator of CD8+ and CD4+ T-cell responses after mumps vaccination [31]. In Denmark, children are vaccinated the first time against mumps at 15 months of age, and the mean age of our patient group was 14 months. A relationship between vaccines and autoimmune diseases is a subject of debate, and there is no evidence to confirm a relationship [32].

The significant number of HLA associations reported in this study could suggest multiple causes. This is supported by the finding of a significant difference in the distribution of associated *HLA-DRB1* and *–DQB1* between the patients who have anti-HNA-1a antibodies and patients with anti-FcγRIIIb antibodies, indicating disease heterogeneity and the need for further studies including clinical data on the included patients.

We do see an interesting difference in the distribution of homo- and heterozygous regarding HLA, indicating that the risk of AIN is higher if you have a broader repertoire of molecular receptors on the surface of your cells.

Along with differences in methodology and techniques by which HLA genotypes were identified, other limits to our findings include population heterogeneity and diagnostic bias due to the mild course of disease. The relatively low number of patients are divided into a large number of HLA alleles and haplotypes, this is even more evident when we divide the patients into two groups based on antibody specificity, and it is impossible to avoid some bias, but for a rare disease this will always be

an unavoidable limitation. Combined with the low amount of available material on these children, that is also an argument for, why this study only consists of 1st-field level data. For future studies with a larger cohort, it could definitely be interesting to investigate HLA associations with higher resolution.

Our patient group is selected based on strict criteria, and our findings can only be expected to regard this restricted part of patients suffering from neutropenia. In the future it will be interesting to be able to link antibody specificity and HLA types to clinical outcome.

It is not known whether the HLA associations are related to AIN or if it is indirectly associated via a risk of acquiring other diseases, such as infections. Even though this is the largest study to date exploring the association between HLA and AIN, it is still limited by a small number of patients. It does, however, replicate the associations we found in a smaller study cohort, even with a new and larger control group.

Interestingly, despite using new techniques and a larger study group, we were able to replicate all the earlier reported associations for AIN; HLA-DR2 (*HLA-DRB1*16*) in a German study and *HLA-DQB1*05* in a Taiwanese study, indicating that AIN patients have some genetic similarities across different ethnicities [13,33]. The significant number of HLA associations found in our study might to some extend be explained by the signal from the *HLA-DQB1*05* allele, who is involved in multiple haplotypes with the associated *HLA-DRB1* alleles. *HLA-DQB1*05* was found in both the German (*HLA-DRB1*16* is in haplotype with *HLA-DQB1*05*) and the Taiwanese study, and combined with our results, it could suggest a connection between *HLA-DQB1*05* and AIN unaffected by ethnicities.

Our results suggest that *HLA-DRB1* and *-DQB1* alleles and haplotypes might play a role in susceptibility or protection against AIN. There is evidence of disease heterogeneity with different associations for several *HLA-DRB1* and *-DQB1* genotypes in regard to the different FcγRIIIb antibody specificities detected in Danish patients with AIN.

Funding

Author contributions

KKM, KRN, RS and PH were involved in all aspects of the study conception, design, analysis, interpretation, and report generation. TNM, HH, AG, TMH, HLN, CFJ and PR were involved in data acquisition, study design and report drafting. All authors critically revised the manuscript, read and approved the final manuscript.

Ethical statement

Consent for study participation for all patients was obtained from legal guardians, and the study was approved by the local ethics committee (N-20,170,026).

Declaration of Competing Interest

All authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

References

[1] B.K. Flesch, A. Reil, Molecular Genetics of the Human Neutrophil Antigens, Transfus. Med. Hemotherapy Off. Organ Der Dtsch. Gesellschaft Fur Transfusionsmedizin Und Immunhamatologie. 45 (2018) 300–309, doi:10.1159/000491031.

[2] K.R. Nielsen, S.R. Bojsen, T.N. Masmas, A.-L. Fjordside, J. Baech, T.M. Haunstrup, R. Steffensen, Association between human leukocyte antigens (HLAs) and human neutrophil antigens (HNAs) and autoimmune neutropenia of infancy in Danish patients, Pediatr. Allergy Immunol. 32 (2021) 756–761, doi:10.1111/pai.13450.

[3] J. Bux, G. Behrens, G. Jaeger, K. Welte, Diagnosis and clinical course of autoimmune neutropenia in infancy: analysis of 240 cases, Blood 91 (1998) 181–186.

[4] I. Callejas Caballero, M. Illán Ramos, A. Berzosa Sánchez, E. Anguita, J.T. Ramos Amador, Autoimmune neutropenia associated with influenza virus infection in childhood: a case report, BMC Infect. Dis. 21 (2021) 1–5, doi:10.1186/s12879-021-06506-9.

[5] E. Wang, S. Adams, D.F. Stroncek, F.M. Marincola, Human Leukocyte Antigen and Human Neutrophil Antigen Systems, 7th Ed, Elsevier Inc., 2018, doi:10.1016/B978-0-323-35762-3.00113-X.

[6] K. Falk, O. Rötzschke, S. Stevanović, G. Jung, H.G. Rammensee, Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules, Nature 351 (1991) 290–296, doi:10.1038/351290a0.

[7] J. Ruppert, J. Sidney, E. Celis, R.T. Kubo, H.M. Grey, A. Sette, Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules, Cell 74 (1993) 929–937, doi:10.1016/0092-8674(93)90472-3.

[8] S. Trabace, HLA and disease associations, J Headache Pain 1 (2000) 109–113, doi:10.1201/9780429268755-13.

[9] S. Tsai, P. Santamaria, MHC Class II polymorphisms, autoreactive T-cells, and autoimmunity, Front. Immunol. (2013) 4, doi:10.3389/fimmu.2013.00321.

[10] J.A. Gebe, E. Swanson, W.W. Kwok, HLA class II peptide-binding and autoimmunity, Tissue Antigens 59 (2002) 78–87, doi:10.1034/j.1399-0039.2002.590202.x.

[11] C.A. Dendrou, J. Petersen, J. Rossjohn, L. Fugger, HLA variation and disease, Nat. Rev. Immunol. 18 (2018) 325–339, doi:10.1038/nri.2017.143.

[12] J. Bux, G. Mueller-Eckhardt, C. Mueller-Eckhardt, Autoimmunization against the neutrophil-specific NA1 antigen is associated with HLA-DR2, Hum. Immunol. 30 (1991) 18–21, doi:10.1016/0198-8859(91)90065-h.

[13] L.-Y. Wang, C.-L. Wang, C.-C. Chu, H.-L. Lee, H.-T. Ho, D.-C. Liang, H.-C. Liu, M. Lin, Primary autoimmune neutropenia in children in Taiwan, Transfusion 49 (2009) 1003–1006, doi:10.1111/j.1537-2995.2008.02084.x.

[14] Q. Li, J.I. Cohen, Epstein-Barr virus and the human leukocyte antigen complex, Curr. Clin. Microbiol. Reports. 6 (2019) 175–181, doi:10.1007/s40588-019-00120-9.

[15] G. Houen, N.H. Trier, Epstein-Barr virus and systemic autoimmune diseases, Front. Immunol. (2021) 11, doi:10.3389/fimmu.2020.587380.

[16] L.S. Young, A.B. Rickinson, Epstein-Barr virus: 40 years on, Nat. Rev. Cancer. 4 (2004) 757–768, doi:10.1038/nrc1452.

[17] N. Cereb, H.R. Kim, J. Ryu, S.Y. Yang, Advances in DNA sequencing technologies for high resolution HLA typing, Hum. Immunol. 76 (2015) 923–927, doi:10.1016/j.humimm.2015.09.015.

[18] M.K. Smatti, D.W. Al-Sadeq, N.H. Ali, G. Pintus, H. Abou-Saleh, G.K. Nasrallah, Epstein–Barr virus epidemiology, serology, and genetic variability of LMP-1 oncogene among healthy population: an update, Front. Oncol. 8 (2018), doi:10.3389/fonc.2018.00211.

[19] Y. Yao, L. Shi, J. Yu, S. Liu, Y. Tao, L. Shi, Distribution of killer-cell immunoglobulin-like receptor genes and combinations of their human leucocyte antigen ligands in 11 ethnic populations in China, Cells 8 (2019) 711, doi:10.3390/cells8070711.

[20] J. Shen, Z. Li, J. Chen, Z. Song, Z. Zhou, Y. Shi, SHEsisPlus, a toolset for genetic studies on polyploid species, Sci. Rep. 6 (2016) 24095, doi:10.1038/srep24095.

[21] Z. Li, Z. Zhang, Z. He, W. Tang, T. Li, Z. Zeng, L. He, Y. Shi, A partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers: update of the SHEsis (http://analysis.bio-x.cn), Cell Res 19 (2009) 519–523, doi:10.1038/cr.2009.33.

[22] Y. Yong, L. He, SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci, Cell Res 15 (2005) 97–98, doi:10.1038/sj.cr.7290272.

[23] M. Kuwana, J. Kaburaki, J.P. Pandey, M. Murata, Y. Kawakami, H. Inoko, Y. Ikeda, HLA class II alleles in Japanese patients with immune thrombocytopenic purpura. Associations with anti-platelet glycoprotein autoantibodies and responses to splenectomy, Tissue Antigens 56 (2000) 337–343, doi:10.1034/j.1399-0039.2000.560405.x.

[24] S.M.S. Pezeshki, M.T. Jalali, A. Amin Asnafi, K. Jaseb, N. Saki, HLA-B5, 7, 8, 27, and 51 antigens and immune thrombocytopenic purpura, J. Pediatr. Hematol. Oncol. 42 (2020) e32–e37, doi:10.1097/MPH.0000000000001543.

[25] A. Amin Asnafi, M.T. Jalali, S.M.S. Pezeshki, K. Jaseb, N. Saki, The association between human leukocyte antigens and ITP, TTP, and HIT, J. Pediatr. Hematol. Oncol. 41 (2019) 81–86, doi:10.1097/MPH.0000000000001381.

[26] C.M. Costantino, C.M. Baecher-Allan, D.A. Hafler, Human regulatory T cells and autoimmunity, Eur. J. Immunol. 38 (2008) 921–924, doi:10.1002/eji.200738104.

[27] K. Nakamura, M. Miki, Y. Mizoguchi, S. Karakawa, T. Sato, M. Kobayashi, Deficiency of regulatory T cells in children with autoimmune neutropenia, Br. J. Haematol. 145 (2009) 642–647, doi:10.1111/j.1365-2141.2009.07662.x.

[28] B. Liu, Y. Shao, R. Fu, Current research status of HLA in immune-related diseases, Immunity, Inflamm. Dis. 9 (2021) 340–350, doi:10.1002/iid3.416.

[29] J. Wang-Rodriguez, A. Rearden, Reduced frequency of HLA-DQ6 in individuals with a positive direct antiglobulin test, Transfusion 36 (1996) 979–984, doi:10.1046/j.1537-2995.1996.36111297091742.x.

[30] S.V. Ramagopalan, U.C. Meier, M. Conacher, G.C. Ebers, G. Giovannoni, D.H. Crawford, K.A. McAulay, Role of the HLA system in the association between multiple sclerosis and infectious mononucleosis, Arch. Neurol. 68 (2011) 469–472, doi:10.1001/archneurol.2011.48.

[31] I.G. Ovsyannikova, R.M. Jacobson, N. Dhiman, R.A. Vierkant, V.S. Pankratz, G.A. Poland, Human leukocyte antigen and cytokine receptor gene polymorphisms associated with heterogeneous immune responses to mumps viral vaccine, Pediatrics 121 (2008) e1091–e1099, doi:10.1542/peds.2007-1575.

[32] É. Toussirot, M. Bereau, Vaccination and induction of autoimmune diseases, Inflamm. Allergy Drug Targets. 14 (2015) 94–98, doi:10.2174/1871528114666160105113046.

[33] J. Bux, K. Kissel, K. Nowak, U. Spengel, C. Mueller-Eckhardt, Autoimmune neutropenia: clinical and laboratory studies in 143 patients, Ann. Hematol. 63 (1991) 249–252, doi:10.1007/BF01698373.