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# High-resolution HLA genotyping identifies risk alleles in both class I and II for primary autoimmune neutropenia in early childhood in a Danish cohort

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HLA studies in patients with autoimmune neutropenia (AIN) have shown very consistent results for the association with HLA class II alleles at low resolution. This study aimed to examine the association of both HLA class I and class II at high resolution to clarify the contribution of risk alleles to the disease. A total of 107 AIN patients were genotyped for six loci of HLA class I (*HLA-A*, *-B* and *-C*) and class II (*HLA-DRB1*, *-DQB1*, and *-DPB1*) genes by a high-resolution (3-field, 6-digit) analysis and compared with HLA typing of 1000 healthy controls. Compared with the controls, the allele frequencies were significantly higher in AIN patients for *A\*02:17:01G*, *C\*01:02:01G*, *DRB1\*10:01:01G*, *DRB1\*14:01:01G*, *DRB1\*16:01:01G*, *DQB1\*05:02:01G*, and *DQB1\*05:03:01G* but lower significant for *C\*03:04:01G*, *DRB1\*04:01:01G*, *DRB1\*13:02:01G*, *DQB1\*03:02:01G*, and *DQB1\*06:04:01G*. Frequently associated two-locus haplotypes were found to be *DRB1\*10:01:01G-DQB1\*05:01:01G* and *DRB1\*16:01:01G-DQB1\*05:02:01G*, while the S2 (Q- or D-KRAA) shared epitope (SE) was associated with lower risk. A unique association with HLA alleles was observed between patients with specific anti-HNA-1a antibodies and broad-reacting anti-FcγRIIIb. Anti-HNA-1a

**Abbreviations:** AIN, autoimmune neutropenia; ANC, absolute neutrophil count; CI, confidence interval; EBV, Epstein Barr virus; FcγRIIIb, Fc-gamma receptor IIIb; Flow-GIFT, flow cytometric indirect granulocyte immunofluorescence test; HNA, human neutrophil antigens; HWE, Hardy-Weinberg equilibrium; IgG, immunoglobulin G; LD, linkage disequilibrium; OR, odds ratio; SE, shared epitope; SNP, single nucleotide polymorphism; Tregs, regulatory T cells.

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antibody-positive patients were associated with *C\*01:02:01G*, *DRB1\*01:01:01G*, *DRB1\*16:01:01G*, *DQB1\*05:01:01G*, *DQB1\*05:02:01G*, *DQB1\*06:04:01G*, and *DPB1\*10:01:01G*; the two-locus haplotypes *DRB1\*01:01:01G-DQB1\*05:01:01G* and *DRB1\*16:01:01G-DQB1\*05:02:01G*; and the S3P (Q- or R-RRAA) SE. Anti-FcγRIIIb antibody-positive patients were associated with the alleles *A\*02:17:01G*, *DRB1\*10:01:01G*, and *DQB1\*05:02:01G*; the haplotypes *DRB1\*10:01:01G-DQB1\*05:01:01G* and *DRB1\*11:01:02G-DQB1\*05:02:01G*; and the S3D (DRRAA) SE. The different associations regarding FcγRIIIb antibody specificities could indicate disease heterogeneity.

#### KEYWORDS

autoimmune neutropenia, genetics, haplotypes, HLA, immune-genetics

## 1 | INTRODUCTION

Genetic variations in HLA may be the result of evolutionary adaptations in response to environmental stress, such as climate and the prevalence of infectious diseases.<sup>1</sup> The molecular mechanisms connecting HLAs and autoimmune diseases are unclear, but antigen presentation and T-cell activation seem to be triggering in most autoimmune diseases.<sup>2,3</sup> Primary autoimmune neutropenia (AIN) in early childhood is a disease characterized by the presence of autoantibodies that recognize antigens of neutrophils (human neutrophil antigens [HNAs]), mostly located on immunoglobulin G (IgG) Fc gamma receptor type 3b (FcγRIIIb [CD16b]), causing their peripheral destruction.<sup>4</sup> Two main types of autoantibodies have been observed in AIN, antibodies directed against a specific HNA variant (HNA-1a), and broad reactive antibodies against FcγRIIIb. The clinical significance of autoantibody specificities remains unknown, but in several studies, we have reported different genetic backgrounds for the two groups.<sup>5,6</sup>

The cause of AIN is still unknown, and there is not enough data to propose a genetic or environmental cause. Viral infections have been suggested as a possible trigger especially in secondary AIN,<sup>7</sup> initiating the activation of autoreactive B cells and CD4+ T cells, but there are also indications of a general deficiency in peripheral self-tolerance mediated by an alteration in either the function or number of CD4+ Treg cells.<sup>8</sup> Activation of regulatory T cells (Tregs) is controlled by HLA, and certain genotypes protect or increase the risk of exposure to autoimmune diseases through Tregs. A connection between AIN and circulating Tregs has been shown by Nakamura et al.,<sup>9</sup> which supports HLA association and gives rise to further investigation.

A background of genetic susceptibility, especially to class II HLA, has been confirmed for multiple ethnicities.

The first association between AIN and HLA was described in 1991 in a German cohort ( $n = 26$ ), which showed an association with serologically determined DR2 (*DRB1\*15* and *\*16*) and DQ1 (*DQB1\*05* and *\*06*).<sup>10</sup> Later, the *DQB1\*05:03* genotype was suggested for AIN in a small study from Taiwan ( $n = 31$ ) and confirmed by our group in a Danish cohort ( $n = 80$ ), where we also found an association with *DRB1\*14*.<sup>11,12</sup> In another study, we explored the relationship between *DRB1* and *DQB1* alleles and AIN, where we expanded the cohort to 160 patients and compared it with a control group of 1000 healthy Danish individuals.<sup>5</sup> In doing so, we found a higher risk associated with *DRB1\*10*, *DRB1\*14*, *DRB1\*16*, and *DQB1\*05* and a lower risk associated with *DRB1\*04*, *DRB1\*13*, and *DQB1\*03*. We also found that the associations with *DRB1* and *DQB1* alleles differed between patients positive for anti-HNA-1a-specific antibodies and patients positive for broadly reactive anti-FcγRIIIb antibodies. *DRB1\*01*, *DRB1\*04*, and *DQB1\*03* were only associated with anti-HNA-1a positivity, and *DRB1\*10* was restricted to broadly reactive anti-FcγRIIIb positivity.

Class I and II HLA alleles are important genetic risk factors for a variety of autoimmune diseases,<sup>13</sup> but prior to this study, only HLA class II alleles were investigated in regard to AIN. Study results for *DRB1* and *DQB1* have been concordant but with limited genotyping resolutions at the 1-field level. A study with higher resolution will not only provide more knowledge about subtypes and haplotypes but also make it possible to investigate the shared epitope (SE) concept in relation to AIN. SE's are based on amino acid motifs. For SE's of *DRB1*, this motif is at positions 70–74 and is considered the greatest heritable influence on susceptibility to rheumatoid arthritis (RA).<sup>14–16</sup> The SE hypothesis predicts that RA-associated *DRB1* molecules bind the same peptide(s) and thus facilitate the development of autoreactive T cells involved in

the pathogenesis of RA.<sup>17</sup> HLA-B SE are called Bw4 and Bw6 and are determined by amino acid residues 77 and 80–83, respectively, on the  $\alpha$ -1 helix.<sup>18,19</sup>

This study examined the association of six loci at the high resolution (3-field, 6 digit), *HLA-A*, *-B*, *-C*, *-DRB1*, *-DQB1*, and *-DPB1*, using third-generation sequencing technology to clarify their genetic contribution to AIN. The association between risk alleles and the specificity of autoantibodies, as well as two-locus haplotypes and SE's, was determined. The HLA gene pairs with the strongest linkage disequilibrium (LD) values were *B-C* and *DRB1-DQB1*, which are expected due to their chromosomal proximity.<sup>20</sup> Because of the high disease association with the *DRB1* and *DQB1* alleles, we chose to study this two-locus haplotype.

## 2 | MATERIALS AND METHODS

### 2.1 | Study cohort

The Department of Clinical Immunology, Aalborg University Hospital, is the national center for diagnostic AIN testing in Denmark and was the center for sample collection in this study. A total of 107 patients were included, all diagnosed with AIN and tested positive for Fc $\gamma$ RIIIb autoantibodies between 2004 and 2023. The inclusion criteria were the presence of neutropenia, an absolute neutrophil count not above  $1.5 \times 10^9$  cell/L in two repeated tests, age under 5 years at the time of diagnosis, and the presence of antineutrophil antibodies in the flow cytometric indirect granulocyte immunofluorescence test (Flow-GIFT) as previously described.<sup>12</sup> Patients with initial negative antibody screening underwent repeated tests as suggested by Bux.<sup>21</sup> Patients with congenital neutropenia, neutropenia related to inborn syndromes, postinfection neutropenia, or hematological malignancies were excluded. The 107 included patients belong to the same cohort as previously published studies investigating HLA association with AIN in Danish patients at low resolution.<sup>5,12</sup> The control group consisted of 1000 randomly selected, anonymous, healthy adult Danish blood donors from the Aalborg University Hospital blood bank, Aalborg, Denmark. A high number of controls was used to provide certainty of the frequency of rare alleles. Both, patients and controls, consisted primarily of White individuals.

All procedures performed in the study were in accordance with the ethical standard of the institution and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. It was approved by the North Denmark Region Committee on Health Research Ethics (Approval

number: N-20170026). Oral and written consent from the participants or their guardians was obtained according to the Danish Healthcare Act.

### 2.2 | DNA preparation

DNA was extracted from EDTA-stabilized whole blood using the Maxwell RSC Whole Blood DNA Kit on the Maxwell RSC instrument (Promega, USA).

### 2.3 | HLA genotyping

High-resolution (3-field, 6 digit) HLA genotyping of 11 HLA loci, *HLA-A*, *-B*, *-C*, *-DRB1*, *-DRB3*, *-DRB4*, *-DRB5*, *-DQA1*, *-DQB1*, *-DPA1*, and *-DPB1*, was performed on all 107 patients with NanoTYPE™ 24/11 kit v2 RUO HLA typing (Omixon Biocomputing Ltd., Budapest, HU). Sequencing was performed with a MinION 1 kb sequencer using third-generation sequencing technology from Oxford Nanopore Technologies (Oxford Nanopore, Oxford, UK). FastQ files were analyzed with NanoTYPER™ software version 1.2.0 (Omixon Biocomputing Ltd., Budapest, HU) and IPD-IMGT/HLA database version 3.52.<sup>22</sup> The control group was genotyped with high resolution (3-field, 6 digit) of six HLA loci, *HLA-A*, *-B*, *-C*, *-DRB1*, *-DQB1*, and *-DPB1*, by next-generation sequencing at Histogenetics (New York, USA). Data for *DRB3*, *DRB4*, *DRB5*, *DQA1*, and *DPA1* were therefore only available for the patients and were not compared. Ambiguities within the 3-field resolution are rather frequent when using next-generation sequencing, so to perform reliable comparison with third-generation sequencing data, analysis was performed with G-group resolution. Few of the rare alleles in the control group are only presented at four-digit resolution.

### 2.4 | Shared epitopes

HLA-B alleles were divided into two groups, Bw4 and Bw6, according to the IPD-IMGT/HLA database.<sup>22</sup> This classification of *DRB1* alleles, observed in our samples, was in accordance with the amino acid sequence at positions 70–74, as described by du Montcel et al.<sup>15</sup> *DRB1* alleles were divided into two groups (S and X alleles) according to the presence or absence of the RAA sequence at positions 72–74. The S alleles were subdivided into three categories depending on the amino acid at position 71. The S1 group had an alanine (A) or a glutamic acid (E) at position 71 (A- or E-RAA). S2 had a

lysine (K) at position 71 (K-RAA), and S3 had an arginine (R) in the sequence (R-RAA). S2 had either Q or D at position 70 (Q- or D-KRAA). S3 was subdivided according to the amino acid at position 70 into S3D (D-RRAA) and S3P (Q- or R-RRAA).

## 2.5 | Statistics

The HLA allele frequencies and two-locus haplotype frequencies were characterized by direct counting of the respective number of HLA alleles among healthy controls and AIN patients. Statistical analyses of the differences among patients and controls were determined by Fisher's exact test using Stata v.17 (StataCorp LLC, College Station, TX, USA). Two-tailed *p* values, odds ratios (ORs), and 95% CIs were obtained. SHEsis-Plus software was used to confirm if the control group was in Hardy-Weinberg equilibrium (HWE) and calculate LD.<sup>23–25</sup> SHEsisPlus software was also used to perform two-locus haplotype analysis on *DRB1* and *DQB1*, which are the most associated and closely linked loci.<sup>23–25</sup> Bonferroni correction was used to adjust *p* values in the case of multiple statistical testing; however, no adjustment for multiple comparisons was applied to the SE, as this was considered an exploratory study.

## 3 | RESULTS

### 3.1 | Demographic and clinical data

We included 107 patients diagnosed with AIN with a median age at diagnosis of 14.2 months (range, 3–54 months). All AIN patients were positive for anti-FcγRIIIb antibodies, and of these, 50.5% had anti-HNA-1a-specific antibodies. The patients were investigated both as a combined group and as two individual groups divided according to their antibody specificity. The two groups consisted of 54 patients who were anti-HNA-1a antibody positive and 53 patients who were anti-FcγRIIIb antibody positive. The control group consisted of 1000 healthy randomly selected and unrelated Danish blood donors. The control cohort was not in HWE for *A*, *B*, and *DPB1* but was in HWE for *C*, *DRB1*, and *DQB1* (Table S11). In this study, we present HLA alleles and two-locus haplotype frequencies, not as a population study but as a comparison to a disease cohort of the same origin. The control group consisted of randomly selected healthy individuals, but association with alleles not in HWE should be taken with caution.

### 3.2 | HLA typing and linkage analysis

High-resolution analysis of HLA alleles in 1000 healthy controls and 107 AIN patients was performed and analyzed at G-group resolution. Associated class I and II alleles are reported in Table 1, and all detected HLA alleles are reported in Tables S1–S6. Linkage analysis revealed that the pairs *B-C* ( $D' = 0.88$ ) and *DRB1-DQB1* ( $D' = 0.93$ ) had the highest LD values (Figure S1). Two-locus haplotype frequencies of *DRB1-DQB1* were determined in cases and controls. Associated two-locus haplotypes are reported in Table 2, and all haplotypes with frequencies above 1% are reported in Tables S7–S9. SE's in HLA-B and -DRB1 proteins were determined based on frequencies of the alleles in the two loci, and comparisons between the healthy controls and AIN patients can be seen in Tables 3 and S10.

### 3.3 | Risk HLA alleles and HLA class II two-locus haplotypes in AIN patients

An association with a higher risk of AIN was found for two class I alleles: *A\*02:17:01G* ( $p = 0.0009$ , OR = NA [not found in controls]) and *C\*01:02:01G* ( $p = 0.0001$ , OR = 3.19 [1.73–5.63]) (Table 1). For HLA class II alleles, a higher risk was found for three *DRB1* alleles, *DRB1\*10:01:01G* ( $p = 0.0009$ , OR = 6.11 [1.98–17.46]), *DRB1\*14:01:01G* ( $p = 0.0008$ , OR = 3.34 [1.60–6.53]), and *DRB1\*16:01:01G* ( $p < 0.0001$ , OR = 7.73 [2.61–21.98]), and three *DQB1* alleles, *DQB1\*05:02:01G* ( $p < 0.0001$ , OR = 7.37 [3.13–16.83]) and *DQB1\*05:03:01G* ( $p = 0.0001$ , OR = 3.77 [1.94–6.99]) (Table 1). No association was observed between *DPB1* alleles and a higher risk of AIN (Table 1). The risk-associated two-locus haplotypes found in the AIN patients were *DRB1\*10:01:01G-DQB1\*05:01:01G* ( $p = 0.0008$ , OR = 6.11 [2.35–15.94]) and *DRB1\*16:01:01G-DQB1\*05:02:01G* ( $p = 0.0001$ , OR = 7.73 [3.02–19.80]) (Table 2). No association was observed between a higher risk and SE's in HLA-B or -DRB1 proteins (Tables 3 and S10). An overview of the risk-associated alleles, two-locus haplotypes, and SE's is presented in Table 4.

### 3.4 | Protective HLA alleles and HLA class II two-locus haplotypes in AIN patients

Protection against AIN was not observed for any *HLA-A* or *-B* alleles but was observed for *C\*03:04:01G* ( $p = 0.0003$ , OR = 0.35 [0.17–0.65]) (Table 1). A protective association of HLA class II alleles for AIN was found



**TABLE 1** Significant alleles in HLA class I and II among all AIN patients, anti-HNA-1a antibody and anti-FcγRIIIB antibody positive.

Risk	Locus	Allele	Controls n = 1000 (%)		All AIN patients n = 107 (%)		Anti-HNA-1a antibodies n = 54 (%)		Anti-FcγRIIIB antibodies n = 53 (%)						
			n	(%)	n	(%)	OR (95% CI)	p-value <sup>a</sup>	OR (95% CI)	p-value <sup>a</sup>	OR (95% CI)	p-value <sup>a</sup>			
Class I	A	02:17:01G	0 (0.0)		3 (1.4)	NA	0 (0.0)	0.0009	NA	3 (2.8)	NA	0.0001			
	C	01:02:01G	56 (2.8)		18 (8.4)	3.19 (1.73–5.63)	12 (11.1)	0.0001	4.34 (2.04–8.52)	0.0001	6 (5.7)	2.08 (0.72–4.98)	0.127		
Class II	DRB1	01:01:01G	184 (9.2)		28 (13.1)	1.49 (0.93–2.29)	22 (20.4)	0.086	2.52 (1.47–4.19)	0.0006	6 (5.7)	0.59 (0.21–1.36)	0.294		
	DRB1	10:01:01G	11 (0.6)		7 (3.3)	6.11 (1.98–17.46)	1 (0.9)	0.0009	1.69 (0.04–11.82)	0.469	6 (5.7)	10.85 (3.22–32.64)	0.0001		
	DRB1	14:01:01G	38 (1.9)		13 (6.1)	3.34 (1.60–6.53)	6 (5.56)	0.0008	3.04 (1.02–7.46)	0.023	7 (0.9)	3.58 (1.31–8.37)	0.007		
	DRB1	16:01:01G	10 (0.5)		8 (3.7)	7.73 (2.61–21.98)	5 (4.6)	0.0001	9.66 (2.54–31.56)	0.0006	3 (2.8)	5.80 (1.01–22.92)	0.025		
	DQB1	05:01:01G	215 (10.8)		37 (17.3)	1.74 (1.15–2.56)	24 (22.2)	0.006	2.37 (1.41–3.87)	0.0008	13 (12.3)	1.16 (0.59–2.13)	0.630		
	DQB1	05:02:01G	16 (0.8)		12 (5.6)	7.37 (3.13–16.83)	6 (5.6)	<0.0001	7.29 (2.28–20.10)	0.0006	6 (5.7)	7.44 (2.33–20.51)	0.0005		
	DQB1	05:03:01G	42 (2.1)		16 (7.5)	3.77 (1.94–6.99)	8 (7.4)	0.0001	3.73 (1.47–8.31)	0.0032	8 (7.6)	3.81 (1.50–8.49)	0.0029		
	DPB1	10:01:01G	19 (1.0)		8 (3.7)	4.05 (1.51–9.82)	6 (5.6)	0.003	6.13 (1.96–16.38)	0.001	2 (1.9)	2.01 (0.22–8.49)	0.286		
	Protective	Class I	C	03:04:01G	267 (13.4)	11 (5.1)	0.35 (0.17–0.65)	0.0003	6 (5.6)	0.0003	0.38 (0.14–0.87)	0.018	5 (4.7)	0.32 (0.10–0.79)	0.007
			Class II	DRB1	04:01:01G	219 (11.0)	8 (3.7)	0.32 (0.13–0.65)	0.0003	3 (2.8)	0.0003	0.23 (0.05–0.71)	0.004	5 (4.7)	0.40 (0.13–0.99)
DRB1			13:02:01G	134 (6.7)	2 (0.9)	0.13 (0.02–0.49)	0.0001	0 (0.0)	0.0001	NA	0.0016	2 (1.9)	0.27 (0.03–1.01)	0.064	
DQB1			03:02:01G	214 (10.7)	9 (4.2)	0.37 (0.16–0.72)	0.002	4 (3.7)	0.002	0.32 (0.09–0.80)	0.015	5 (4.7)	0.41 (0.13–1.01)	0.049	
DQB1	06:04:01G	123 (6.2)	1 (0.5)	0.07 (0.00–0.41)	0.0001	0 (0.0)	0.0001	NA	0.002	1 (0.9)	0.14 (0.00–0.84)	0.019			

Note: NA = not applicable (only observed in one group).

Abbreviations: AIN, autoimmune neutropenia; CI, confidence interval; HNA, human neutrophil antigens; OR, odds ratio.

<sup>a</sup>p-value using Fisher's exact test. Significance level after Bonferroni correction  $\alpha = 0.05$ : HLA-A  $p = \alpha/36 = 0.001$ , HLA-B  $p = \alpha/58 = 0.0009$ , HLA-C  $p = \alpha/32 = 0.002$ , HLA-DRB1  $p = \alpha/45 = 0.001$ , HLA-DQB1  $p = \alpha/18 = 0.003$ , HLA-DPB1  $p = \alpha/28 = 0.002$ . Significant p values are highlighted with bold.

for two *DRB1* alleles, *DRB1\*04:01:01G* ( $p = 0.0003$ , OR = 0.32 [0.13–0.65]) and *DRB1\*13:02:01G* ( $p = 0.0001$ , OR = 0.13 [0.02–0.49]), and two *DQB1* alleles, *DQB1\*03:02:01G* ( $p = 0.002$ , OR = 0.37 [0.16–0.73]) and *DQB1\*06:04:01G* ( $p = 0.0001$ , OR = 0.07 [0.00–0.41]) (Table 1). No association was observed for *DPB1* alleles or any *DRB1-DQB1* haplotypes (Tables 1 and 2). The S2 (Q- or D-KRAA) SE for the *DRB1* protein was found to be protective against AIN ( $p = 0.0016$ , OR = 0.38 [0.18–0.73]) (Table 3). Analysis of HLA-Bw epitopes showed no significant association with AIN (Table S10). An overview of the protective-associated alleles, haplotypes, and SE's is presented in Table 4.

### 3.5 | Comparing HLA association and antibody specificity in AIN patients

Comparison of the two antibody specificity groups individually to the control group revealed that for class I, anti-HNA-1a antibody positivity had a higher risk for *C\*01:02:01G* ( $p < 0.0001$ , OR = 4.34 [2.04–8.52]), while anti-FcγRIIIb antibody positivity had a higher risk for *A\*02:17:01G* ( $p = 0.0001$ , OR = NA [not found in controls]). For HLA class II alleles, the risk-associated alleles for anti-HNA-1a antibody positivity were *DRB1\*01:01:01G* ( $p = 0.0006$ , OR = 2.52 [1.47–4.19]), *DRB1\*16:01:01G* ( $p = 0.0006$ , OR = 9.66 [2.54–31.56]), *DQB1\*05:01:01G* ( $p = 0.0008$ , OR = 2.37 [1.41–3.87]), *DQB1\*05:02:01G* ( $p = 0.0006$ , OR = 7.29 [2.28–20.10]), and *DPB1\*10:01:01G* ( $p = 0.001$ , OR = 6.13 [1.96–16.38]) (Table 1). For anti-FcγRIIIb antibody positivity, the following alleles were risk associated: *DRB1\*10:01:01* ( $p \leq 0.0001$ , OR = 10.85 [3.22–32.64]) and *DQB1\*05:02:01* ( $p = 0.0005$ , OR = 7.44 [2.33–20.51]) (Table 1). One HLA class II allele was found to be protective against AIN for anti-HNA-1a antibody

positivity, *DQB1\*06:04:01* ( $p = 0.002$ , OR = NA [not found in patients]) and none was observed for anti-FcγRIIIb antibody positivity. The following *DRB1-DQB1* two-locus haplotypes were associated with a higher risk for anti-HNA-1a antibody positivity: *DRB1\*01:01:01-DQB1\*05:01:01* ( $p = 0.0006$ , OR = 2.52 [1.54–4.13]) and *DRB1\*16:01:01-DQB1\*05:02:01* ( $p = 0.0006$ , OR = 9.66 [3.24–28.78]). For anti-FcγRIIIb antibody positivity, a higher risk was associated with *DRB1\*10:01:01-DQB1\*05:01:01*, ( $p = 0.0001$ , OR = 10.85 [3.93–29.93]) and *DRB1\*11:01:02-DQB1\*05:02:01* ( $p = 0.002$ , OR = NA [not found in controls]). No two-locus haplotypes were associated with a lower risk for any of the antibody specificity groups. Among the *DRB1* SE's, the S3P (Q- or R-RRAA) ( $p = 0.0022$ , OR = 2.07 [1.27–3.29]) SE was found to be associated with a higher risk for anti-HNA-1a antibody positivity, while S3D (DRRAA) ( $p = 0.014$ , OR = 2.03 [1.11–3.53]) was a risk factor for anti-FcγRIIIb antibody positivity (Table 1). S2 (Q- or D-KRAA) was protective for both antibody specificities ( $p = 0.027$ , OR = 0.38 [0.12–0.92]) and ( $p = 0.027$ , OR = 0.38 [0.38–0.94]) (Table 3). An overview of the associated alleles, two-locus haplotypes, and SE's for the two antibody-specific groups is presented in Table 4.

## 4 | DISCUSSION

In this study of primary AIN in early childhood among Danish patients, we analyzed the frequency of class I and II HLA alleles with G-group resolution in 107 patients and 1000 healthy controls and determined the associated two-locus haplotypes and SE's. As expected, the associated HLA class II alleles in *DRB1* and *DQB1* were similar to those that have been previously described for AIN,<sup>5,10–12</sup> but we are the first to report associations with class I alleles. This is also the first study to investigate HLA association

TABLE 2 Significant *DRB1-DQB1* haplotypes among all AIN patients, anti-HNA-1a antibody, and anti-FcγRIIIb antibody positive.

	HLA- <i>DRB1-DQB1</i> haplotypes		Controls		OR (95% CI)	<i>p</i> -value <sup>a</sup>
			<i>n</i> = 1000 (%)	Patients (%)		
All AIN patients <i>n</i> = 107 (%)	<i>DRB1*10:01:01G</i>	<i>DQB1*05:01:01G</i>	11 (0.5)	7 (3.2)	6.11 (2.35–15.94)	<b>0.0008</b>
	<i>DRB1*16:01:01G</i>	<i>DQB1*05:02:01G</i>	10 (0.5)	8 (3.7)	7.73 (3.02–19.80)	<b>0.0001</b>
Anti-HNA-1a positive <i>n</i> = 54 (%)	<i>DRB1*01:01:01G</i>	<i>DQB1*05:01:01G</i>	184 (9.2)	22 (20.3)	2.52 (1.54–4.13)	<b>0.0006</b>
	<i>DRB1*16:01:01G</i>	<i>DQB1*05:02:01G</i>	10 (0.5)	5 (4.6)	9.66 (3.24–28.78)	<b>0.0006</b>
Anti-FcγRIIIb positive <i>n</i> = 53 (%)	<i>DRB1*10:01:01G</i>	<i>DQB1*05:01:01G</i>	11 (0.5)	6 (5.6)	10.85 (3.93–29.93)	<b>0.0001</b>
	<i>DRB1*11:01:02G</i>	<i>DQB1*05:02:01G</i>	0 (0)	2 (1.8)	NA	<b>0.002</b>

Note: NA = not applicable (only observed in one group).

Abbreviations: AIN, autoimmune neutropenia; CI, confidence interval; HNA, human neutrophil antigens; OR, odds ratio.

<sup>a</sup>*p*-value using Fisher's exact test. Significance level after Bonferroni correction to  $\alpha = 0.05$ : All AIN patients  $p = \alpha/13 = 0.004$ , anti-HNA-1a positive

$p = \alpha/10 = 0.005$ , anti-FcγRIIIb positive  $p = \alpha/17 = 0.003$ . Significant *p* values are highlighted with bold, but only for haplotypes with case frequency >1%.

with AIN at the high resolution (3-field, 6-digit), as well as SE's.

Interestingly, we found associations with alleles for *HLA-A* and *-C*. The *A\*02:17:01G* allele is rare, and while it was not observed in the control group, we did observe it in the patient group. Thus far, there is no previous disease association with this allele in the literature, and investigation of the patients carrying it did not reveal any ethnic explanation. However, the strongest *HLA-A* binders with virus peptides have been found to belong to the *A\*02* lineage.<sup>26</sup> For *HLA-C*, both a risk allele and a protective allele were identified. *C\*01:02:01G* was more frequent in AIN patients than in healthy controls. The proposed function of *HLA-C* protein in autoimmune and inflammatory diseases is to present antigens to T cells and to drive innate immunity through binding activating or inhibitory receptors on natural killer (NK) cells.<sup>27</sup> The proposed interaction among viral infection, *C\*01*, is through the killer cell immunoglobulin-like receptors of NK cells.<sup>28</sup> In this study, protection against AIN was observed for the allele *C\*03:04:01G*, and the *C\*03:04* allele has previously been linked to sarcoidosis in Korean patients.<sup>29</sup>

For *HLA* class II, we replicated all our previously published low-resolution findings of associated *DRB1* alleles in this cohort of Danish AIN patients<sup>5,12</sup> and, at a G-group resolution, found the risk alleles to be *DRB1\*10:01:01G*, *DRB1\*14:01:01G*, *DRB1\*16:01:01G* and the protective alleles to be *DRB1\*04:01:01G* and *DRB1\*13:02:01G*. For *DQB1*, we similarly replicated our previous findings for *DQB1\*03* and *DQB1\*05*, and we found an association with *DQB1\*03:02:01G*, *DQB1\*05:02:01G*, and *DQB1\*05:03:01G*. In addition, we also observed a protective effect of *DQB1\*06:04:01G*.

One *HLA* class II allele, *DQB1\*05*, has been consistently found to be associated with AIN in all previous studies, unaffected by ethnicity.<sup>5,10-12</sup> The study from Taiwan is the only study that has reported more than 1 digit, and they reported an association with *DQB1\*05:03*.<sup>11</sup> Our results support that *DQB1\*05:03:01G* was indeed associated with a higher risk of AIN, but also *DQB1\*05:01:01G* and *DQB1\*05:02:01G* were found to be associated.

Other of the associated alleles have previously been associated to autoimmune diseases. *DRB1\*14* has previously been found to be strongly associated with a higher risk of the rare autoimmune disease pemphigus vulgaris in White Europeans.<sup>30,31</sup> In pemphigus vulgaris, IgG autoantibodies bind to the protein desmoglein 3 (*dsg3*), which is found in desmosomes in keratinocytes near the bottom of the epidermis.<sup>30,31</sup> Pemphigus vulgaris has also been associated with *DRB1\*04*, *DQB1\*03:02*, *DQB1\*05:01*, and *DQB1\*05:03*.<sup>30,31</sup>

The *DRB1\*10:01* allele has been found to be a strong binder of virus peptides.<sup>26</sup> It has also been associated with a relatively new disorder caused by neurodegenerative and autoimmune mechanisms named anti-IgLON5 disease. The main symptoms of the disease are sleeping disturbances, bulbar symptoms, and gait abnormalities. Similar to AIN, anti-IgLON5 disease is associated with a highly specific antibody and particular *HLA* alleles. The frequency of the *DRB1\*10:01* allele is very low and, when present, strongly segregates with *DQB1\*05:01*, which has also been found to be associated with IgLON5.<sup>32</sup> The two-locus haplotype of *DRB1\*16:01:01-DQB1\*05:02:01* has been associated with drug-induced liver injury.<sup>33</sup>

This differentiation between *HLA* associations and HNA-1a antibody specificities in AIN patients has been observed previously for both *DRB1* and *DQB1*<sup>5</sup> but also in regards to genetic variation in low-to-medium Fcγ receptors.<sup>6</sup> In this study, we observed differences regarding *HLA* class I and II alleles. Both antibody specificity groups share an association with *DQB1\*05:02:01G*. The association with the alleles *C\*01:02:01G*, *DRB1\*01:01:01G*, *DRB1\*16:01:01G*, *DQB1\*05:01:01G*, *DQB1\*06:04:01G*, and *DPB1\*10:01:01G* was unique to anti-HNA-1a-positive patients. The two-locus haplotypes *DRB1\*01:01:01G-DQB1\*05:01:01G* and *DRB1\*16:01:01G-DQB1\*05:02:01G* were also found to be specific to this group. Additionally, the group of patients with broadly reactive antibodies against the FcγRIIIb receptor had unique *HLA* associations different from those of anti-HNA-1a-positive patients, including the class I allele *A\*02:17:01G*, the class II allele *DRB1\*10:01:01G*, and the two-locus haplotypes *DRB1\*10:01:01G-DQB1\*05:01:01G* and *DRB1\*11:01:02G-DQB1\*05:02:01G*.

*DRB1\*01*, which is unique to anti-HNA-1a positivity, has been reported to be protective for sarcoidosis<sup>34-37</sup> and a predisposing factor in diseases such as recurrent lymphocytic meningitis and juvenile idiopathic arthritis in Hungarian patients,<sup>38,39</sup> exhibiting contrary effects depending on the disease. *HLA-DRB1\*01:01* has been shown to be a strong binder for viruses and is overrepresented in individuals who develop infectious mononucleosis compared with individuals with asymptomatic Epstein Barr virus (EBV) infection.<sup>26,40</sup> A more significant reduction in EBV copy number has been observed in individuals with *HLA-DRB1\*01:01*, suggesting that immune control of viral replication is more effective in these individuals.<sup>41</sup>

*DPB1* has not been studied in AIN to date, and we found that *DPB1\*10:01:01* was a risk factor for patients with anti-HNA-1a antibodies. *DPB1\*10* has been found to be associated with a higher risk of severe aplastic anemia.<sup>42</sup> The etiology of acquired severe aplastic anemia is



TABLE 3 Frequencies of DRB1-shared epitopes in AIN patients and controls.

DRB1-SE	Controls		All AIN patients			Anti-HNA-1a antibodies			Anti-FcγRIIIB antibodies		
	<i>n</i> = 1000 (%)	<i>n</i> = 107 (%)	OR (95% CI)	<i>p</i> -value	<i>n</i> = 54 (%)	OR (95% CI)	<i>p</i> -value	<i>n</i> = 53 (%)	OR (95% CI)	<i>p</i> -value	
S1 (A- or E-RAA)	664 (33.2)	64 (29.9)	0.85 (0.62–1.18)	0.359	34 (31.5)	0.92 (0.59–1.42)	0.753	30 (28.3)	0.79 (0.50–1.24)	0.340	
S2 (Q- or D-KRAA)	229 (11.5)	10 (4.7)	0.38 (0.18–0.73)	<b>0.0016</b>	5 (4.6)	0.38 (0.12–0.92)	<b>0.027</b>	5 (4.7)	0.38 (0.12–0.94)	<b>0.027</b>	
S3D (DRRAA)	172 (8.6)	26 (12.1)	1.47 (0.91–2.30)	0.100	9 (8.3)	0.97 (0.42–1.95)	1.000	17 (16.0)	2.03 (1.11–3.53)	<b>0.014</b>	
S3P (Q- or R-RAA)	289 (14.5)	42 (19.6)	1.45 (0.98–2.09)	0.055	28 (25.9)	2.07 (1.27–3.29)	<b>0.0022</b>	14 (13.2)	0.90 (0.47–1.63)	0.880	
X	646 (32.3)	72 (33.6)	1.06 (0.78–1.44)	0.701	32 (29.6)	0.88 (0.56–1.37)	0.598	40 (37.7)	1.27 (0.83–1.93)	0.244	

Note: Significant *p* values are highlighted with bold.

Abbreviations: AIN, autoimmune neutropenia; CI, confidence interval; HNA, human neutrophil antigens; OR, odds ratio, SE, shared epitope.

TABLE 4 Overview of HLA association in combined, anti-HNA-1a-positive patients and anti-FcγRIIIb-positive patients.

Risk	Locus	All AIN patients (n = 107)	Anti-HNA-1a antibodies (n = 54)	Anti-FcγRIIIb antibodies (n = 53)
Class I	A	A*02:17:01G (OR = NA)	None	A*02:17:01G (OR = NA)
	C	C*01:02:01G (OR = 3.19)	C*01:02:01G (OR = 4.43)	None
Class II	DRB1	DRB1*10:01:01G (OR = 6.11)	DRB1*01:01:01G (OR = 2.52)	DRB1*10:01:01G (OR = 10.85)
		DRB1*14:01:01G (OR = NA)	DRB1*16:01:01G (OR = 9.66)	
		DRB1*16:01:01G (OR = 7.73)		
	DQB1	DQB1*05:02:01G (OR = 7.37)	DQB1*05:01:01G (OR = 2.37)	DQB1*05:02:01G (OR = 7.44)
		DQB1*05:03:01G (OR = 3.77)	DQB1*05:02:01G (OR = 7.29)	
	DPB1	None	DPB1*10:01:01G (OR = 6.13)	None
Haplotypes	DRB1*16:01:01G-DQB1*05:02:01G (OR = 7.73)	DRB1*01:01:01G-DQB1*05:01:01G (OR = 2.52)	DRB1*10:01:01G-DQB1*05:01:01G (OR = 10.85)	
	DRB1*10:01:01G-DQB1*05:01:01G (OR = 6.11)	DRB1*16:01:01G-DQB1*05:02:01G (OR = 9.66)	DRB1*11:01:02G-DQB1*05:02:01G (OR = NA)	
Shared epitopes	None	S3P (OR = 2.07)	S3D (OR = 2.03)	
Protective	Locus	All AIN patients (n = 107)	Anti-HNA-1a antibodies (n = 54)	Anti-FcγRIIIb antibodies (n = 53)
Class I	A	None	None	None
	C	C*03:04:01G (OR = 0.35)	None	None
Class II	DRB1	DRB1*04:01:01G (OR = 0.32)	None	None
		DRB1*13:02:01G (OR = 0.13)		
	DQB1	DQB1*03:02:01G (OR = 0.37)	DQB1*06:04:01G (OR = NA)	None
		DQB1*06:04:01G (OR = 0.07)		
	DPB1	None	None	None
	Haplotypes	None	None	None
Shared epitopes	S2 (OR = 0.38)	S2 (OR = 0.38)	S2 (OR = 0.38)	

Note: NA = not applicable (only observed in one group).

Abbreviations: AIN, autoimmune neutropenia; HNA, human neutrophil antigens; OR, odds ratio.

not understood but is likely related to abnormal immune responses and environmental exposures.

We investigated SE's for both HLA-B and -DRB1 and did not find an association with HLA-B SEs. AIN has been associated with several *DRB1* alleles, and we found associations with several *DRB1* SE groups. Both the combined group of AIN patients and the two antibody subgroups had protective associations with the S2 (Q- or D-KRAA) allele group. This corresponds with S2 containing *DRB1\*04* and *DRB1\*13*, which are both found to be protective. Anti-HNA-1a positivity had a risk association with S3P (Q- or R-RRAA), corresponding with the unique signal to *DRB1\*01* for this patient group. For anti-FcγRIIIb-positive patients, the S3D (DRRAA) group was associated with higher disease risk, which corresponds

with the *DRB1\*16* association. In the case of SE's, we see an indication that all patients share the same protective epitope but that the risk epitope is connected to antibody specificity.

In this study, we found several associations for different HLA alleles, although we applied a strict correction factor. As stated in the beginning of the discussion, we did not observe any difference from our previous findings of associated *DRB1* and *DQB1* alleles in this cohort, which is why we feel confident that the cohort is representative of Danish AIN patients. Nevertheless, our study has limitations, and due to the small number of patients, some of the observed associations, especially in comparisons with antibody specificity, could arise by chance and therefore should not be overinterpreted before

independent replication in another cohort. Our patients were selected according to strict criteria, and our findings can only be expected to represent this restricted portion of patients suffering from neutropenia. We did not obtain HWE in the control group for *HLA-A*, *-B* and *-DPB1*, which is why the findings in these loci should be interpreted with caution. The controls and patients are genotyped with two different methods and due to the high level of ambiguities in next generation sequencing data, the comparison was only possible at G-group resolution.

This study presents a high number of alleles associated with AIN. An explanation for this could be the wide range of infections that is observed among this group of patients. Previous studies have reported recurrent infectious episodes of skin, upper respiratory tract, and ear infections.<sup>43–47</sup> Additionally, among Danish AIN patients, a wide range of different infections is observed, including upper respiratory tract infections, otitis media, dermal infections/abscesses, gastroenteritis, oral infection, conjunctivitis, and pneumonia (unpublished data). Our results suggest that specific HLA alleles and haplotypes might play a role in susceptibility to and protection against AIN. We also observed different associations regarding FcγRIIIb antibody specificities, which could indicate disease heterogeneity and may be related to different infections.

#### AUTHOR CONTRIBUTIONS

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

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#### CONFLICT OF INTEREST STATEMENT

All authors declare that there are no conflicts of interest.

#### DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

#### ETHICS STATEMENT

All procedures performed in the study were in accordance with the ethical standard of the institution and/or national research committee, and with the 1964 Helsinki

Declaration, and its later amendments or comparable ethical standards. It was approved by the North Denmark Region Committee on Health Research Ethics (Approval number: N-20170026). Oral and written consents from the participants or their guardians were obtained as according to the Danish Healthcare Act.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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