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ORIGINAL ARTICLE

Genetic variations in low-to-medium-affinity Fcγ receptors and autoimmune neutropenia in early childhood in a Danish cohort

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Abstract

Autoimmune neutropenia (AIN) in early childhood is caused by autoantibodies directed against antigens on the neutrophil membrane and is a frequent cause of neutropenia in children. Association of AIN with Fcγ receptor (FCGR) 3B variants is well described. In this study, we investigate genetic variations in the FCGR locus and copy number variation of *FCGR3B*. A total of 130 antibody-positive AIN patients, 64 with specific anti-HNA-1a antibodies and 66 with broad-reacting anti-FcγRIIIb antibodies, were genotyped with a multiplex ligation probe assay and compared with healthy controls. Positive findings were confirmed with real-time q-PCR. We determined copy numbers of the FCGR2 and FCGR3 genes and the following SNPs: *FCGR2A* Q62W (rs201218628), *FCGR2A* H166R (rs1801274), *FCGR2B* I232T (rs1050501), *FCGR3A* V176F (rs396991), haplotypes for *FCGR2B/C* promoters (rs3219018/rs780467580), *FCGR2C* STOP/ORF and HNA-1 genotypes in *FCGR3B* (rs447536, rs448740, rs52820103, rs428888 and rs2290834). Generally, associations were antibody specific, with all associations being representative of the anti-HNA-1a-positive group, while the only association found in the anti-FcγRIIIb group was with the HNA-1 genotype. An increased risk of AIN was observed for patients with one copy of

Abbreviations: AIN, autoimmune neutropenia; BCR, B cell receptor; CI, 95% confidence interval; CNR, copy number region; CNV, copy number variation; FcγR/FCGR, fc gamma receptor; GIFT, granulocyte immunofluorescence test; HNA, human neutrophil antigens; IgG, immunoglobulin G; IGIW, International Granulocyte Immunology Workshops; MLPA, multiplex ligation probe assay; OR, odds ratio; ORF, open reading frame; PCR, polymerase chain reaction; RA, rheumatoid arthritis; SLE, systematic lupus erythematosus; SNP, singe nucleotide polymorphism.

FCGR3B; the HNA genotypes HNA-1a, HNA-1aa or HNA-1aac; the *FCGR2A* 166H and *FCGR2B* 232I variations; and no copies of *FCGR2B* 2B.4. A decreased risk was observed for HNA genotype HNA-1bb; *FCGR2A* 166R; *FCGR2B* 232T; and one copy of *FCGR2B* promoter 2B.4. We conclude that in our Danish cohort, there was a strong association between variation in the FCGR locus and AIN. The findings of different genetic associations between autoantibody groups could indicate the presence of two different disease entities and disease heterogeneity.

KEYWORDS
AIN, autoimmunity, copy number variation, fc gamma receptors, neutropenia

1 | INTRODUCTION

Primary autoimmune neutropenia (AIN) in early childhood is caused by antibodies directed against neutrophil-specific antigens, mostly located on immunoglobulin G (IgG) Fc receptor type 3b (FcγRIIIb) (Flesch & Reil, 2018). The condition is often benign, and most patients are in remission after 2–3 years (Bux et al., 1998). Information regarding the cause of this disease is limited due to the scarce data on the triggering aetiology.

IgG is the most abundant Ig class, constituting over 75% of circulating Ig (Bournazos et al., 2009). Fc-gamma receptors (FcγRs) are the cellular receptors for IgG, and binding of IgG complexes triggers various cellular immune effector functions. Six classic FcγRs are known in humans and are divided into one high-affinity receptor (FcγRI) and five low- to medium-affinity receptors (FcγRIIa, -b and -c and FcγRIIIa and -b). Most FcγRs are activating receptors capable of inducing a cellular response, with the exception of FcγRIIb, which is an inhibitory receptor (Nagelkerke, Schmidt, et al., 2019). The five genes (*FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A* and *FCGR3B*) encoding the low- to medium-affinity Fcγ receptors are located in a single cluster on chromosome 1q23.3, the FCGR2/3 locus. Genetic variations, causing functional changes, have been found in all five genes. These variations are associated with several conditions, such as autoimmune (Breunis et al., 2008; Lee et al., 2009; Lee et al., 2008; Li et al., 2010; Yuan et al., 2009), autoinflammatory (Asano et al., 2009; Khor et al., 2011; Onouchi et al., 2012) and infectious diseases (Adu et al., 2012; Chai et al., 2012), and the efficacy of immunotherapy (Ahlgrimm et al., 2011; Cartron et al., 2002; Hurvitz et al., 2012; Tamura et al., 2011; Treon et al., 2011). Genetic analysis of these variants is complex, deriving from a high degree of homology and linkage disequilibrium (Nagelkerke et al., 2019).

FcγRIIa (CD32a) induces many different cellular defence mechanisms and is found on monocytes, macrophages, dendritic cells, neutrophils and platelets. FcγRIIb (CD32b) is expressed on B cells, where it constitutes the only surface-expressed FcγR, and co-crosslinking of FcγRIIb with the B-cell receptor (BCR) inhibits activating signals induced by the BCR. FcγRIIb expression can also be detected on neutrophils but only in individuals with a *2B.4* promoter haplotype (Su et al., 2007). FcγRIIc (CD32c) is expressed on natural killer (NK) cells, neutrophils, monocytes and macrophages, but only in individuals who carry an open reading frame (ORF) of this receptor. However, in the majority of individuals, the gene is a pseudogene and is not expressed (Nagelkerke et al., 2019). The FcγRII class shares a characteristic structure that includes functional signalling motifs in their cytoplasmic domains and an immunoreceptor tyrosine-based activating motif for FcγRIIa and -c and an immunoreceptor tyrosine-based inhibition motif for FcγRIIb (Bournazos et al., 2009).

FcγRIIIa (CD16a) and FcγRIIIb (CD16b) share high levels of sequence homology, but they exhibit distinct structural differences. FcγRIIIa is a transmembrane protein, while FcγIIIb is a glycosylphosphatidylinositol-anchored protein. FcγRIIIa is expressed by several leukocyte cell types, including macrophages, NK cells and subsets of T cells and monocytes, while FcγRIIIb is highly expressed on neutrophils (Bournazos et al., 2009).

Epitopes of the FcγRIIIb receptor belong to the human neutrophil antigen (HNA) family, specifically HNA-1. The *FCGR3B* gene consists of five exons, and polymorphisms that define the described alleles of the HNA-1 system are all encoded in exon 3 and are distinguished by five single nucleotide polymorphisms (SNPs) (Flesch & Reil, 2018; Hargreaves et al., 2015; Qiu et al., 1990; Sachs et al., 2016). HNA-1 has at least four alleles, *FCGR3B*01* (HNA-1a), *FCGR3B*02* (HNA-1b, 1d) and *FCGR3B*03* (HNA-1c). As a result of gene duplication and recombination, copy number variation (CNV) is observed, and an individual can express between zero and four different HNA-1 alleles (Flesch & Reil, 2018). 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 (Nielsen et al., 2021).

The focus of this study is to investigate genetic variations in the low- to medium-affinity receptors and their association with AIN and the specificity of autoantibodies.

2 | METHODS

2.1 | Study cohort

The study cohort included 130 patients diagnosed with autoantibody-positive AIN between 2004 and 2022 at the National Centre for Diagnostic AIN Testing, Department of Clinical Immunology, Aalborg

University Hospital, Denmark. The inclusion criteria were the presence of neutropenia, an absolute neutrophil count below 1.5×10^9 cells/L in two repeated tests, age under 5 years at the time of diagnosis as suggested by Fioredda et al. (2022), and the presence of anti-neutrophil antibodies in the flow cytometric indirect granulocyte immunofluorescence test (Flow-GIFT) as previously described (Nielsen et al., 2021). Patients with initial negative antibody screening were retested as suggested by Bux et al. (1998). Patients with congenital neutropenia, neutropenia related to inborn syndromes, post-infection neutropenia or haematological malignancies were excluded. Genetic material was available for all patients for genotyping. As control groups we used anonymous healthy adult Danish blood donors from the Aalborg University Hospital blood bank, Aalborg, Denmark. Only exception was the control group to the *FCGR2B* and *FCGR2C* haplotypes were we used published data from Nagelkerke et al. (2019), constituting controls of European descent. Both patient and control groups consisted primarily of European ancestry individuals. 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-stabilised 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, USA).

2.3 | Multiplex ligation-dependent probe amplification

CNVs and SNPs in the low-affinity FCGR genes *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A* and *FCGR3B* were determined with an FCGR-specific multiplex ligation-dependent probe amplification (MLPA) assay using SALSA MLPA probe mixes P110-C1 FCGR mix 1 and P111-C1 FCGR mix 2, according to the manufacturer's protocol (MRC-Holland, The Netherlands). Amplified MLPA fragments were separated against a LIZ GS 5500 size standard using an ABI-3500 instrument for capillary electrophoresis. Data analysis was performed with Coffalyser.Net analysis software (MRC-Holland, The Netherlands). The FCGR MLPA includes gene-specific probes to determine the CNV of the FCGR2/3 genes. It also includes probes to detect the following SNPs: *FCGR2A* 184C>T and 185A>G, together forming *FCGR2A* Q62W (rs9427397 and rs9427398 (combined rs201218628)); *FCGR2A* 497A>G H166R (rs1801274); *FCGR2B* 695T>C I232T (rs1050501); *FCGR3A* 526G>T V176F (rs396991); haplotypes for *FCGR2B/C* promoters (−386G/C and −120T/A) (rs3219018/rs780467580); *FCGR2C* STOP/ORF; and HNA-1 genotypes in *FCGR3B* (rs527909462, rs448740, rs5030738 and rs2290834). These haplotypes were constructed according to the manufacturer's protocol and as previously described (Breunis et al., 2008; Nagelkerke, Tacke, et al., 2019).

Interpretation of MLPA data does not only include a statistical estimation of the significance of the result, but also look at the probe ratio obtained and compare this with the arbitrary borders in Coffalyser.Net and with the thresholds given in the product description. This excludes situations in which a probe is seemingly statistically significantly different by coincidence. Similarly, the reaction quality and the variability of the reference probes and samples is evaluated with Coffalyser.Net. This protects against interpreting experimentally induced variability as true results. The SNP genotypes are concluded based on two or more mutation-specific probes from two individual tests confirming the result (Coffalyser.Net™ Reference Manual, MRC-Holland, The Netherlands).

2.4 | Genotyping

Real-time polymerase chain reaction (PCR) using TaqMan probes was used for genotyping of Danish control groups for *FCGR2A* H166R (rs1801274, C__9077561_20), *FCGR3A* V158F (rs396991, C__25815666_10) and HNA-1 (*FCGR3B*) (rs527909462, rs448740, rs5030738, rs147574249 and rs2290834, as previously described[9]) and for validation of genotypes found with MLPA for *FCGR2A* H166R and HNA-1 in patients. The HNA-1 analysis is performed routinely on AIN patients in Denmark and is ISO accredited (DANAK, Denmark) and validated continuously in the International Granulocyte Immunology Workshops (IGIW) and INSTAND eV.

The copy number of *FCGR3B* was determined for the controls using a real-time q-PCR TaqMan Copy Number Assay, *FCGR3B* (Hs04211858_cn), and TaqMan Copy Number Reference Assay, RNase P, according to the manufacturers' protocol (Applied Biosystems, USA). Copy numbers of *FCGR3B* found by MLPA in patients were also validated with this method, while controls found to have a copy number >2 with real-time q-PCR had their HNA-1 genotypes determined with MLPA. Data analysis was performed with CopyCaller® Software (Applied Biosystems).

2.5 | Statistics

Statistical analysis was conducted using the statistical program Stata (Version 17.0; StataCorp, College Station, Texas). Gene frequencies were estimated by direct counting and they were compared using the Fisher exact test (Yao et al., 2019). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Only SNPs with a frequency ≥1% are reported in this study. SNPs were tested for Hardy–Weinberg equilibrium with p values calculated based on the χ^2 distribution with 1 degree of freedom (Table S2). Genotype frequencies were compared with Cochran-Armitage trend test with p values calculated based on the χ^2 distribution with 1 degree of freedom. Bonferroni correction was used to adjust p values in case of multiple statistical testing.

3 | RESULTS

3.1 | Baseline characteristics

We included 130 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 64 patients which were anti-HNA-1a-positive, and the remaining 66 patients which will be referred to as anti-FcγRIIIb-positive.

3.2 | SNPs in FCGR genes

The genotype frequency was significantly different between Danish AIN patients and healthy Danish controls for *FCGR2A* H166R ($p = .0015$). The frequency of the *FCGR2A* 166H allele was significantly higher in patients (OR = 1.59 (1.21–2.08)) (see Table 1). The genotypes 166HH (OR = 1.94 (1.30–2.91)) and 166RR (OR = 0.52 (0.32–0.85)) both differed significantly between patients and controls, while there was no difference in the distribution of 166HR. Individual comparison of the anti-HNA-1a-positive and the remaining anti-FcγRIIIb-positive patients to the control group revealed, that only the anti-HNA-1a-positive patients differed significantly ($p = .0001$); 166H (OR = 2.17 (1.47–3.20)), 166HH (OR = 2.40 (1.42–4.05)) and 166RR (OR = 0.31 (0.14–0.70))).

For *FCGR2B* I232T, we observed a significantly difference in genotype frequency ($p = .015$) and higher frequency of the 232I allele (OR = 1.83 (1.14–2.94)) among Danish AIN patients compared with the control group of European descent published by Nagelkerke et al. (2019) (see Table 1). A significant difference was observed for the 232II (OR = 2.12 (1.24–3.60)) and 232IT (OR = 0.43 (0.24–0.77)) genotypes. Of the two antibody specificity groups, only the anti-HNA-1a-positive patients was significantly different from the European control group. This group had a significant difference in genotype frequency ($p = .013$) and a higher frequency of the 232I allele (OR = 2.63 (1.21–5.71)) and the 232II genotype (OR = 3.08 (1.31–7.23)), as well as a lower frequency of the 232IT genotype (OR = 0.30 (0.12–0.76)).

We observed a significantly lower frequency of the *FCGR2B* promoter variant 2B.4 among AIN patients compared with the control group published by Nagelkerke et al. (2019), but only in the anti-HNA-1a-positive ($p = .012$), zero *2B.4* (OR = 3.43 (1.23–9.56)) and one 2B.4 promoter variant (OR = 0.32 (0.12–0.90)).

No significant difference was observed for *FCGR2A* Q62W, *FCGR3A* V176F, *FCGR2C* promoter or FCGR2C STOP/ORF (Table S1).

3.3 | HNA-1 alleles and FCGR3B copy numbers

Copy numbers from one to four of *FCGR3B* were observed in both Danish AIN patients and healthy Danish controls (see Table 2). There was a significant difference in the frequency of patients with only one copy in the anti-HNA-1a-positive group compared with the control group (OR = 3.15 (1.23–8.06)). We did not observe any associations between copy number and AIN for the combined group or for the anti-FcγRIIIb-positive patients.

Using MLPA, we were able to define exact copy numbers and HNA-1 genotypes in the 130 AIN patients and a Danish control group, and three HNA genotypes showed a statistically significant higher risk of AIN: HNA-1a (OR = 6.49 (1.92–21.93)), HNA-1aa (OR = 9.89 (5.98–16.37)) and HNA-1aac (OR = 12.67 (1.40–114.42)) (see Table 3). For the HNA-1a genotype, the significance was limited to the combined and anti-HNA-1a-positive groups of patients (OR = 12.16 (3.45–42.8)), while the HNA-1aa genotype was significant in the combined group, as well as the two groups based on antibody specificity (anti-HNA-1a (OR = 16.31 (8.80–30.20)); anti-FcγRIIIb (OR = 5.95 (3.17–11.17)). The rare HNA-1aac genotype was only significant in the combined group.

One HNA genotype showed a statistically significant lower risk of AIN when comparing patients and controls: HNA-1bb (OR = 0.17 (0.09–0.30)). The HNA-1bb genotype was significantly different from the controls for both patient groups (anti-HNA-1a (OR = 0.01 (0.00–0.18)); anti-FcγRIIIb (OR = 0.38 (0.20–0.70)).

4 | DISCUSSION

In this study, we investigated the most relevant and functional SNPs and CNVs of FcγRII and FcγRIII polymorphisms in a Danish AIN cohort. Previous studies have focused on single SNPs, especially the HNA-1 genotypes in the *FCGR3B* gene, but these studies have not investigated the copy number of the *FCGR3B* gene. Low copy numbers of *FCGR3B* have previously been associated with several autoimmune diseases, including systemic lupus erythematosus (SLE) (McKinney & Merriman, 2012), Sjögren syndrome (Nossent et al., 2012), systemic sclerosis (McKinney et al., 2012) and rheumatoid arthritis (RA) (Graf et al., 2012; McKinney & Merriman, 2012; Rahbari et al., 2017), causing a functional change in the expression levels and uptake of immune complexes.

The HNA-1 genotypes, a result of polymorphisms in *FCGR3B*, are known to be associated with AIN. Bruin et al. (2005) suggested an association between the HNA-1a homozygous state and the risk of AIN. Audrain et al. (2011) reported similar findings in a French AIN cohort, and our group confirmed these findings and discovered that the presence of the HNA-1a genotype appeared to be responsible for all anti-HNA-1a-positive cases (Nielsen et al., 2021).

In the present study, we combined *FCGR3B* copy number and HNA-1 genotype data. With the use of MLPA, we were able to identify exact numbers and genotypes in our cohort of Danish AIN patients and compare them with a healthy control group. We observed an association with a lower number of copies of *FCGR3B* for the group of patients who were anti-HNA-1a positive (OR = 3.15 (1.23–8.06)), indicating similarities to other autoimmune diseases also associated with lower copy numbers. Consistent with our previously published data (Nielsen et al., 2021), the HNA-1a genotype appears to be present in all anti-HNA-1a-positive cases, and we can now extend this knowledge by

TABLE 1 Frequencies of functionally associated genetic variants at the FCGR2/3 locus, comparing 130 Danish AIN patients with healthy controls of European descent.

FCGR2A H166R (131†) rs1801274	Controls *n* (%)*	All AIN *n* (%)	Fisher *p*	OR (95%CI)	CA** *p*	Anti-HNA-1a *n* (%)	Fisher *p*	OR (95%CI)	CA** *p*	Anti-FcγRIIIb *n* (%)	Fisher *p*	OR (95%CI)	CA** *p*
Genotype frequency													
166HH (AA)	164 (26.9)	50 (38.5)	**.0104**	1.70 (1.14–2.53)	**.0015**	30 (46.9)	**0.0013**	2.40 (1.42–4.05)	**.0001**	20 (30.3)	.562	1.18 (0.68–2.06)	.363
166HR (AG)	275 (45.1)	58 (44.6)	1.000	0.98 (0.67–1.44)		27 (42.2)	.694	0.89 (0.53–1.50)		31 (47.0)	.796	1.08 (0.65–1.80)	
166RR (GG)	171 (28.0)	22 (16.9)	**.0082**	0.52 (0.32–0.85)		7 (10.9)	**.0026**	0.32 (0.14–0.70)		15 (22.7)	.388	0.76 (0.41–1.38)	
Allele frequency													
166H (A)	603 (49.4)	158 (60.8)	**.001**	1.59 (1.21–2.08)		87 (68.0)	**.0001**	2.17 (1.47–3.20)		71 (53.8)	.360	0.34 (0.83–1.71)	
166R (G)	617 (50.6)	102 (39.2)	**.001**	0.63 (0.48–0.83)		41 (32.0)	**.0001**	0.46 (0.31–0.68)		61 (46.2)	.360	0.84 (0.59–1.20)	
***FCGR2B* I232T (188†) rs1050501**													
Genotype frequency													
232II (TT)	697 (75.8)	113 (86.9)	**.005**	2.12 (1.24–3.60)	**.0146**	58 (90.6)	**.0054**	3.08 (1.31–7.23)	**.0132**	55 (83.3)	.180	1.59 (0.82–3.10)	.279
232IT (TC)	201 (21.9)	14 (10.8)	**.0025**	0.43 (0.24–0.77)		5 (7.8)	**.0063**	0.30 (0.12–0.76)		9 (13.6)	.122	0.56 (0.27–1.16)	
232TT (CC)	21 (2.3)	3 (2.3)	1.000	1.01 (0.30–3.43)		1 (1.6)	1.000	0.68 (0.09–5.13)		2 (3.0)	.663	1.34 (0.31–5.83)	
Allele frequency													
232I (T)	1595 (86.8)	240 (92.3)	**.012**	1.83 (1.14–2.94)		121 (94.5)	**.0086**	2.63 (1.21–5.71)		119 (90.2)	.347	1.39 (0.77–2.51)	
232T (C)	243 (13.2)	20 (7.7)	**.012**	0.55 (0.34–0.88)		7 (5.5)	**.0086**	0.34 (0.18–0.82)		13 (9.8)	.347	0.72 (0.40–1.29)	
FCGR2B* promoter* **rs3219018/ rs780467580**													
Variant frequency													
0 2B.4	748 (81.4)	116 (89.2)	.027	1.89 (1.06–3.38)	0.032	60 (93.8)	**.010**	3.43 (1.23–9.56)	**.0123**	56 (84.8)	.621	1.28 (0.64–2.56)	.533
1 2B.4	157 (17.1)	13 (10.0)	.042	0.54 (0.30–0.98)		4 (6.3)	**.022**	0.32 (0.12–0.90)		9 (13.6)	.609	0.77 (0.37–1.58)	
2 2B.4	14 (1.5)	1 (0.8)	1.000	0.50 (0.07–3.84)		0 (0.0)	1.000	0.48 (0.03–8.21)		1 (1.5)	1.000	0.99 (0.13–7.68)	
Allele frequency													
2B.4	187 (10.2)	15 (5.8)	**.024**	0.54 (0.31–0.93)		4 (3.1)	**.0053**	0.28 (0.10–0.78)		11 (8.3)	.652	0.80 (0.43–1.52)	

*Danish control group consisting of healthy Danish blood donors ($n = 610$).

**CA = Cochran-Armitage Trend test. *p* Value calculated based on the χ^2 distribution with 1 degree of freedom.

***European control group ($n = 919$) published by Nagelkerke et al.[11]

****Significance level after Bonferroni correction for haplotypes to $\alpha = 0.05$: $\alpha/2 = <0.025$.

†According to Human Genome Structural Variation Consortium (HGSV) guidelines, we use amino acid numbering from the full protein (including signal peptides) and have done this throughout the manuscript. The position in the mature protein (without signal peptides) is shown between brackets for some of the SNPs that are commonly known by that position.

TABLE 2 Frequencies of CNV of *FCGR3B* (CNR1) comparing 130 AIN patients and Danish controls.

CNV CNR1/ *FCGR3B*	Controls *n* (%)*	All AIN *n* (%)	*p* value	OR (95%CI)	Anti-HNA-1a *n* (%)	*p* Value	OR (95%CI)	Anti-FcγRIIIb *n* (%)	*p* Value	OR (95%CI)
1	15 (3.8)	10 (7.7)	0.092	2.14 (0.94–4.89)	7 (10.9)	**.0214**	3.15 (1.23–8.06)	3 (4.5)	0.730	1.22 (0.34–4.34)
2	352 (88.0)	113 (86.9)	.759	0.91 (0.50–1.64)	53 (82.8)	.232	0.66 (0.32–1.34)	60 (90.9)	.678	1.35 (0.56–3.33)
3	32 (8.0)	6 (4.6)	.242	0.56 (0.23–1.36)	3 (4.7)	.452	0.57 (0.17–1.90)	3 (4.5)	.452	0.55 (0.16–1.84)
4	1 (0.2)	1 (0.8)	.431	3.09 (0.19–49.8)	1 (1.6)	.257	6.33 (0.39–102.5)	0 (0.0)	1.000	2.00 (0.08–49.68)

*Danish control group consisting of healthy Danish blood donors ($n = 610$).

also comparing the number of copies of HNA-1a genotypes. Having only one copy of *FCGR3B*, which is an HNA-1a genotype, does indeed induce the risk of getting AIN (OR = 6.49 (1.92–21.93)), but the association is clearly driven by the group of patients who are anti-HNA-1a positive (OR = 12.16 (3.45–42.8)), while there appears to be no association for the anti-FcγRIIIb-positive patients. The results were different in patients with two copies of the *FCGR3B* gene, where two HNA-1a genotypes were a risk factor for both the anti-HNA-1a-positive (OR = 16.31 (8.80–30.20)) and anti-FcγRIIIb-positive (OR = 5.95 (3.17–11.17)) groups. Here, we observe that two copies of the HNA-1b genotype are an advantage against disease for both patient groups. Interestingly, in the anti-HNA-1a-positive group, the HNA-1b genotype also appears to protect individuals who have both an HNA-1a and HNA-1b genotype, so HNA-1b seems to offer protection even in the presence of an HNA-1a genotype. In the group of individuals with more than three copies, the HNA-1aab and HNA-1abb genotypes were the most frequent types in the healthy blood donor control group, but interestingly, we did not observe any of these HNA genotypes in our patient cohort. Instead, we observed several cases with HNA-1aac (OR = 12.7 (1.4–114.4)), which seems to be a rare genotype in the Danish population, also based on a low frequency of HNA-1c (*FCGR3B*03*) in the population (Nielsen et al., 2012). A correlation between the HNA-1c genotype and increased copy number in Europeans has previously been published (Nagelkerke, Tacke, et al., 2019). We observed no individuals homozygous for HNA-1c, but we did observe both HNA-1ac and HNA-1bc genotypes in the patients with two copies, which was confirmed by both real-time PCR and MLPA. However, we cannot exclude the possibility that this can be a result of ethnicity and all HNA-1c genotypes found in our control group are indeed linked to a higher copy number of *FCGR3B*.

Based on our results, harbouring the HNA-1a genotype (*FCGR3B*01*), whether one or more copies, seems to be a potential risk factor for AIN, while having an HNA-1b (*FCGR3B*02*) genotype or a heterogeneous combination of HNA-1a (*FCGR3B*01*) and HNA-1b (*FCGR3B*02*) genotypes protects against this disease. The frequency of the HNA-1c (*FCGR3B*03*) genotype in the Danish population is too low to make any conclusions about its possible involvement in the disease.

The interaction between FcγRs on phagocytic cells and antibodies plays a critical role in the innate immune response. When multiple IgG molecules are fixed close to each other, this results in cross-linking and activation of several FcγRs in the cell membrane. FcγRs play an important role in immunity by linking the adaptive and innate immune systems together. Immune cells, such as neutrophils, monocytes and dendritic cells, express multiple Fc receptors for IgG with overlapping ligand specificity. These receptors compete for the same target ligand and transduce both positive and negative signals to the cell. Human neutrophils express two types of FcγRs. In addition to FcγRIIIb, they also express FcγRIIa (Selvaraj et al., 2004).

We observed a statistically significant association between a SNP in FcγRIIa, *FCGR2A* H166R and AIN. In our group of patients, homozygous *FCGR2A* 166H individuals had a higher risk of disease (OR = 1.94 (1.30–2.91)), while homozygous *FCGR2A* 166R individuals had a lower risk (OR = 0.52 (0.32–0.85)). Interestingly, when dividing the patients based on antibody specificity, the *FCGR2A* variants were only risk factors for patients with anti-HNA-1a antibodies (166HH OR = 2.40 (1.42–4.05)), 166RR OR = 0.31 (0.14–0.70)).

Neutrophils from homozygous *FCGR2A* 166H individuals have been shown to have increased phagocytosis and degranulation in response to bacteria and a higher binding affinity for IgG1 and IgG2 compared with *FCGR2A* 166R (Bruhns et al., 2009; Park et al., 1993; Trinchieri & Valiante, 1993). In resting neutrophils, FcγRIIa is in a low-affinity state, while binding is engaged by FcγRIIIb, but once neutrophils have been activated, FcγRIIa is converted to a high-affinity receptor that leads to FcγRIIa-dependent ligand binding and signalling (Nagarajan et al., 2000). The *FCGR2A* 166H variant has been found to be associated with autoimmune diseases such as Kawasaki disease (Duan et al., 2014) and childhood immune thrombocytopenia (Wang et al., 2014), the latter having many similarities to AIN.

FcγRIIb is the only surface expressed FCGR on B cells, where it inhibits activation signals by crosslinking with BCRs. Other types of cells express *FCGR2B*, but at much lower levels. Expression of FcγRIIb can be detected on neutrophils but only in individuals with a 2B.4 promoter haplotype (Su et al., 2007). The less frequent 2B.4 promoter haplotype results in FcγRIIb expression on monocytes, neutrophils and myeloid dendritic cells, similar to expression on B lymphocytes, indicating that FcγRIIb expression on both myeloid and lymphoid cells is regulated by the naturally occurring regulatory SNPs in the *FCGR2B* promoter (Su et al., 2007). We observed that lacking a 2B.4 promoter haplotype was associated with AIN with almost twice as high a risk compared with healthy controls, and that having one 2B.4 promoter haplotype was associated with a lower risk (OR = 0.54 (0.30–0.98)). This is, however, only applicable for the group of patients with anti-HNA-1a antibodies, where over 94% of the patients lack a

TABLE 3 HNA-1 genotypes in 130 Danish AIN patients and Danish controls.

HNA-1	Controls *n* (%)*	All AIN *n* (%)	*p* Value**	OR (95%CI)	Anti-HNA-1a *n* (%)	*p* Value**	OR (95%CI)	Anti-FcγRIIIb *n* (%)	*p* Value**	OR (95%CI)
CNV 1										
a	4 (1.0)	8 (6.2)	**.0022**	6.49 (1.92–21.93)	7 (10.9)	**.0001**	12.16 (3.45–42.8)	1 (1.5)	.540	1.52 (0.17–13.84)
b	11 (2.8)	2 (1.5)	0.744	0.55 (0.12–2.53)	0 (0.0)	.375	0.26 (0.02–4.51)	2 (3.0)	.701	1.12 (0.24–5.18)
c	0 (0.0)	0 (0.0)	–	–	0 (0.0)	–	–	0 (0.0)	–	–
CNV 2										
aa	31 (7.8)	59 (45.4)	<**.00001**	9.89 (5.98–16.37)	37 (57.8)	<**.00001**	16.31 (8.80–30.20)	22 (33.3)	<**.00001**	5.95 (3.17–11.17)
ab	154 (38.5)	38 (29.2)	.059	0.66 (0.43–1.01)	15 (23.4)	.025	0.49 (0.27–0.90)	23 (34.8)	.682	0.85 (0.50–1.47)
ac	0 (0.0)	1 (0.8)	.245	9.28 (0.38–229.23)	1 (1.6)	.138	18.92 (0.75–469.60)	0 (0.0)	–	–
bb	167 (41.8)	14 (10.8)	<**.00001**	0.17 (0.09–0.30)	0 (0.0)	<**.00001**	0.01 (0.00–0.18)	14 (21.2)	**.0016**	0.38 (0.20–0.70)
bc	0 (0.0)	1 (0.8)	.245	9.28 (0.38–229.23)	0 (0.0)	–	–	1 (1.5)	.142	18.34 (0.74–455.13)
CNV 3										
aab	12 (3.0)	0 (0.0)	.045	0.12 (0.01–2.03)	0 (0.0)	.386	0.24 (0.01–4.12)	0 (0.0)	.231	0.23 (0.01–3.99)
abb	12 (3.0)	0 (0.0)	.045	0.12 (0.01–2.03)	0 (0.0)	.386	0.24 (0.01–4.12)	0 (0.0)	0.231	0.23 (0.01–3.99)
aac	1 (0.3)	4 (3.1)	**.014**	12.67 (1.40–114.42)	2 (3.1)	.051	12.87 (1.15–144.13)	2 (3.0)	.054	12.47 (1.11–139.52)
abc	4 (1.0)	2 (1.5)	.638	1.55 (0.28–8.54)	1 (1.6)	.526	1.57 (0.17–14.29)	1 (1.5)	0.536	1.52 (0.17–13.84)
bbb	3 (0.8)	0 (0.0)	1.000	0.44 (0.02–8.48)	0 (0.0)	1.000	0.88 (0.04–17.23)	0 (0.0)	1.000	0.85 (0.04–16.73)
CNV 4										
abac	1 (0.3)	0 (0.0)	1.000	1.02 (0.04–25.21)	0 (0.0)	1.000	2.06 (0.08–51.23)	0 (0.0)	1.000	2.00 (0.08–49.68)
aaac	0 (0.0)	1 (0.8)	.245	9.28 (0.38–229.22)	1 (1.6)	.138	6.35 (0.39–102.8)	0 (0.0)	1.000	2.01 (0.08–49.81)

*Danish control group consisting of healthy Danish blood donors ($n = 400$).

**Significance level after Bonferroni correction for multiple testing $\alpha = 0.05$: $\alpha/2 = <0.025$.

2B.4 promoter haplotype (OR = 3.43 (1.23–9.65)) compared with 81% in the control group. This could indicate that it is beneficial to have FcγRIIb expression on neutrophils in regard to AIN triggered by specific anti-HNA-1a antibodies. Homozygosity of the 2B.4 promoter has, in previous studies, been shown to be associated with SLE in Europeans (Blank et al., 2005; Su et al., 2004). A variant in *FCGR2B*, *FCGR2B* T232, has also been associated with SLE (Floto et al., 2005). Individuals with *FCGR2B* T232 are unable to inhibit activation receptors because the receptor is excluded from sphingolipid rafts, resulting in the unopposed proinflammatory signalling thought to promote SLE (Floto et al., 2005). The mechanism by which this single amino acid change in the transmembrane domain prevents access to lipid rafts is unclear. We observed comparable findings in our AIN cohort, where homozygosity for *FCGR2B* T232 was significantly associated with a higher risk of disease (OR = 2.12 (1.24–3.60)), especially for the anti-HNA-1a patients (OR = 3.08 (1.31–7.23)), but not for the anti-FcγRIIIb patients.

The *FCGR2/3* locus contains a high degree of homology, and linkage disequilibrium needs to be taken into consideration for the identified associations. It is also important to highlight that CNV of *FCGR3B* includes deletion or duplication of a whole region, which also includes *FCGR2C*, and it is merely because of the HNA-1 association with AIN that we focus on CNV in *FCGR3B*. However, we cannot exclude the possibility that the association found for CNV in *FCGR3B* could also be related to other elements in the copy number region.

Homology between the three FcγRII receptor genes makes genotyping in this region difficult using standard techniques, which is why we chose to use the control group already published by Nagelkerke et al. (2019) to compare with the findings in our study where a validated commercial real-time PCR assay was not available. This control group consisted of healthy individuals from Austria, Australia, the Netherlands and the United Kingdom who had all self-reported to be of European descent. However, it is a limitation to the findings of associations for *FCGR2B* I232T and *FCGR2B* promoter 2B.4 that we did not compare them to a Danish control group or tried to replicate our findings with another method, and the association to AIN must therefore be taken with precaution, until these results have undergone further replication.

In general, SNPs of Fcγ receptors are accompanied by the loss of inhibitory Fcγ expression and altered functions may result in unbalanced immunity and subsequently cause autoinflammation, which might be associated with susceptibility to AIN.

A novel finding in this study is the highly significant relationship between autoantibody specificity and genetic risk factors, a phenomenon previously described for other autoimmune diseases, such as RA and SLE, where several discrete subgroups can be defined based on autoantibody specificity (Reed et al., 2020). These subgroups also differ significantly in relation to genetic risk factors (Diaz-Gallo et al., 2022; Padyukov, 2022). We propose a similar heterogeneity in AIN. The presence of two distinct subgroups of patients with childhood AIN based on serological findings at the time of diagnosis and different genetic risk factors warrants further study.

Overall, we can conclude that there are several associations for AIN in the FCGR locus. However, with the exception of HNA-1a, our findings seem to only concern half of the patients diagnosed with AIN, those that have specific antibodies against HNA-1a. It is very interesting that the other half of the group seemed to be distinguished in regard to their genetic background. This indicates that there might be two different diseases underlying AIN diagnosis, and future investigation is relevant to clarify what this means for the clinical outcome.

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The authors have nothing to report.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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