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A genome-wide association study in autoimmune neurological syndromes with anti-GAD65 autoantibodies

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Autoimmune neurological syndromes (AINS) with autoantibodies against the 65 kDa isoform of the glutamic acid decarboxylase (GAD65) present with limbic encephalitis, including temporal lobe seizures or epilepsy, cerebellitis with ataxia, and stiff-person-syndrome or overlap forms. Anti-GAD65 autoantibodies are also detected in autoimmune diabetes mellitus, which has a strong genetic susceptibility conferred by human leukocyte antigen (HLA) and non-HLA genomic regions. We investigated the genetic predisposition in patients with anti-GAD65 AINS.

We performed a genome-wide association study (GWAS) and an association analysis of the HLA region in a large German cohort of 1214 individuals. These included 167 patients with anti-GAD65 AINS, recruited by the German Network for Research on Autoimmune Encephalitis (GENERATE), and 1047 individuals without neurological or endocrine disease as population-based controls. Predictions of protein expression changes based on GWAS findings were further explored and validated in the CSF proteome of a virtually independent cohort of 10 patients with GAD65-AINS and 10 controls.

Our GWAS identified 16 genome-wide significant ($P < 5 \times 10^{-8}$) loci for the susceptibility to anti-GAD65 AINS. The top variant, rs2535288 [$P = 4.42 \times 10^{-16}$, odds ratio (OR) = 0.26, 95% confidence interval (CI) = 0.187–0.358], localized to an intergenic segment in the middle of the HLA class I region. The great majority of variants in these loci (>90%) mapped to non-coding

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regions of the genome. Over 40% of the variants have known regulatory functions on the expression of 48 genes in disease relevant cells and tissues, mainly $CD4^+$ T cells and the cerebral cortex. The annotation of epigenomic marks suggested specificity for neural and immune cells. A network analysis of the implicated protein-coding genes highlighted the role of protein kinase C beta (*PRKCB*) and identified an enrichment of numerous biological pathways participating in immunity and neural function. Analysis of the classical HLA alleles and haplotypes showed no genome-wide significant associations. The strongest associations were found for the DQA1*03:01-DQB1*03:02-DRB1*04:01HLA haplotype ($P = 4.39 \times 10^{-4}$, OR = 2.5, 95%CI = 1.499–4.157) and DRB1*04:01 allele ($P = 8.3 \times 10^{-5}$, OR = 2.4, 95%CI = 1.548–3.682) identified in our cohort. As predicted, the CSF proteome showed differential levels of five proteins (HLA-A/B, C4A, ATG4D and NEO1) of expression quantitative trait loci genes from our GWAS in the CSF proteome of anti-GAD65 AINS.
These findings suggest a strong genetic predisposition with direct functional implications for immunity and neural function in anti-GAD65 AINS, mainly conferred by genomic regions outside the classical HLA alleles.

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Introduction

Autoimmune neurological syndromes (AINS) are immune-mediated disorders affecting the peripheral or central nervous system or both. In the peripheral blood (PB), CSF and brain parenchyma of these patients, B cell-derived autoantibodies but also T cells recognizing a variety of neural auto-antigens can be detected, illustrating the presence of specific, humoral and cellular, adaptive immune responses.[1] AINS with autoantibodies against the 65 kDa isoform of the glutamic acid decarboxylase (GAD65) present with three clinical syndromes that can partially overlap: limbic encephalitis (LE) with temporal lobe seizures (TLS) or epilepsy (TLE), cerebellitis (CB) with cerebellar ataxia (CA), and stiff-person-syndrome (SPS).[2] All forms of anti-GAD65 AINS exhibit a chronic, slowly progressive disease course with limited response to immunological and symptomatic treatments, leading to severe and life-long neurologic disability. Whilst the vast majority of anti-GAD65 AINS occur in a non-paraneoplastic context, some cases may be driven by a peripheral tumour.[2,3]

Anti-GAD65 autoantibodies also characterize autoimmune diabetes mellitus (type 1A according to the American Diabetes Association classification, T1DM). Consistently, many patients with anti-GAD65 AINS suffer from T1DM and other autoimmune endocrine disorders.[4] Patients with anti-GAD65 AINS typically exhibit more than 100-fold higher autoantibody serum titres when compared to T1DM patients. Moreover, intrathecal autoantibody synthesis is detected in 85–100% of anti-GAD65 AINS but not in patients with T1DM only.[2,3]

Familial cases of anti-GAD65 AINS have been associated with a rare human leukocyte antigen (HLA class II) haplotype (DRB1*15:01:01~DQA1*01:02:01~DQB1*05:02:01).[5] In sporadic cases, both an association with HLA class II haplotypes (HLA DQA1*05:01–DQB1*02:01–DRB1*03:01)[4] and non-HLA genes (i.e. cytotoxic T-lymphocyte-associated protein 4, CTLA4)[6] has been found.

Here, we performed a genome-wide association study (GWAS) and analysis of the HLA region in a large German cohort of 1214 individuals, including 167 patients with sporadic anti-GAD65 AINS, to investigate the genetic basis of these disorders.

Materials and methods

Ethics, consent and permissions

Initial ethics approval was given by the ethics committee of the University of Lübeck, Germany (reference number: 13–162) and consecutively by the ethics committees of all participating centers of the German Network for Research on Autoimmune Encephalitis (GENERATE; www.generate-net.de). All participants gave written informed consent to the study.

Study population

Clinical and paraclinical data, together with DNA samples of 205 German patients of Caucasian ethnicity with anti-GAD65 AINS, were collected by the German Network for Research on Autoimmune Encephalitis (for contributing scientists see Supplementary Table 1).

We prioritized a typical clinical phenotype as primary inclusion criterion. Patients were assessed by experienced neurologists in the referring centers and classified to a phenotype, based on the involvement of either the temporal lobe (including both LE and TLE), cerebellum (CB) and spinal cord (SPS), or overlap, if more than one localization was involved.[1–3] Clinical diagnosis was confirmed by detection of anti-GAD65 IgG autoantibodies in serum and/or CSF at least once during the disease course through a qualitative detection in a tissue-based assay (TBA) with typical staining pattern on rodent or non-human primate brain (and pancreas). To quantify antibody titres and to allow for assessment of intrathecal synthesis, a second method either a cell-based assay (CBA), enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) was added. Intrathecal synthesis of GAD65-specific IgG antibodies was assessed using the antibody-specificity index (ASI), according to Reiber with a method-specific cut-off of 4.0 (CBA) or 1.5 (ELISA, RIA).[7]

Whole blood or peripheral blood mononuclear cells from included patients were collected individually at all participating centers in EDTA tubes. DNA isolation from all patient samples was carried out using standard protocols in the Core Facility Genomics at the Medical Faculty of the University of Münster, Germany.

The control population used in this study consisted of a cohort of 1081 individuals, part of the PopGen cohort, a population-based biobank in Germany.[8] This subset of individuals corresponded to healthy subjects without neurological or endocrine disease, age-matched to our anti-GAD65 AINS patients, with available genotype data generated on the Infinium Global Screening Array (Illumina).

Genome-wide association analysis

All patient samples were genotyped using the Infinium Global Screening Array-24 v3.0 (Illumina). Variant calling was performed with Illumina's GenomeStudio 2.0. Quality control (QC) criteria were applied separately to both patient and control cohorts and consisted of the exclusion of extra-chromosomes, of individuals with more than 1% missing genotypes, and of variants with more than 10% missingness, minor allele frequency (MAF) less than 1% and a Hardy–Weinberg equilibrium (HWE) P-value below 1×10^{-5}. Short insertion/deletion (indel) and multi-allelic markers were allowed. After individual QC, case and control datasets were merged, keeping only overlapping variants. The merged dataset consisted of 406 448 variants, and 1271 individuals (199 cases and 1072 controls), with a genotyping rate of 0.99899. This dataset was imputed using the Michigan Imputation Server[9] (MIF), with the 1000 Genomes Project phase 3 v5 as reference panel, and the Minimac4 pipeline. Strand and allele flips were managed as appropriate upon QC in the MIF. Additionally, the Genotype Imputation HLA Playground (1000G deep) tool of the MIF was used to impute the HLA region.[9] QC criteria were applied to the imputed dataset as before, with the addition of an Rsq filter to exclude variants of lesser quality (Rsq < 0.8). Outlier individuals were detected based on |Z| values

obtained for neighbour distances. Relatedness among individuals was assessed according to identity-by-descent, where a proportion threshold of 0.05 was used. For individuals showing relatedness, one individual from each pair was selected for removal according to their outlier status (|Z| threshold: 6).

The final GWAS dataset consisted of 7 012 247 variants [including single nucleotide polymorphisms (SNPs) and indels] and 1214 individuals, from which 167 were cases and 1047 were controls (see Table 1 for a description of effective study population). We tested for genetic associations with anti-GAD65 AINS using an additive logistic regression model, adjusting for age, sex and the first seven dimensions coming from a principal component analysis. All QC procedures of the genotypes datasets and association analyses were performed with Plink 1.9.[10] Genomic coordinates are given in the genome build GRCh37/hg19.

GWAS downstream analyses

Downstream processing of the GWAS summary statistics consisted in the definition of genomic loci that consider linkage disequilibrium (LD) information, and in performing variant annotation. Genomic loci were delineated using the SNP2GENE function of the Functional Mapping and Annotation of Genome-Wide Association Studies (FUMA GWAS) platform.[11] LD was defined by $r^2 \geq 0.6$ and a window of 500 kb. LD blocks were formed with variants under the genome-wide threshold of significance ($P < 5 \times 10^{-8}$) as lead variants, and all nominally significant ($P < 0.05$) variants in the dataset that were in LD with the lead variants. As genomic loci that are constituted by a single variant, showing no support of other variants in LD with at least nominal significance, might represent false positives, unsupported loci have been omitted from this report.

Variant annotations for all variants in genomic loci were obtained with FUMA GWAS and SNPnexus.[12] These included: (i) the mapped gene up to 1 kb from gene boundaries; (ii) the localization of the variant with respect to genes (functional consequence); (iii) expression quantitative trait loci [eQTLs; false discovery rate (FDR) < 0.05] effects from the BRAINEAC (Brain eQTL Almanac) and DICE (Database of Immune Cell Expression, eQTLs and Epigenomics) databases; as well as (iv) regulatory feature reports from the ENCODE (Encyclopedia of DNA Elements) database and the Roadmap Epigenomics Project.

To shed light onto the biological context of the identified loci, a protein-protein interaction (PPI) network was created with the ReactomeFIViz app for Cytoscape v.3.7.1[13,14] using the mapped and eQTL genes (excluding unsupported loci) as the input and adding linker genes. Clumping and module functions from this app were used to perform pathway enrichment analysis of network modules. Enriched terms were considered those with $P < 0.01$, as well as

Table 1 Basic description of the effective GWAS sample

	GWAS sample			Proteomics samples		
	Total	Males	Females	Total	Males	Females
Total	1214	653	561	20	5	15
Age (±SD)	53.1 (±14.4)	53.8 (±13.4)	52.3 (±15.4)	44.3 (±16.3)	46.2 (±20.4)	43.6 (±15.5)
Controls	1047	602	445	10	3	7
Age (±SD)	53.5 (±14.3)	54.2 (±13.3)	52.6 (±15.4)	49.9 (±18.2)	56.3 (±19.9)	47.1 (±18.1)
Cases	167	51	116	10	2	8
Age (±SD)	50.5 (±14.9)	49.1 (±13.7)	51.1 (±15.4)	38.6 (±12.8)	31 (±9.9)	40.5 (±13.3)
Limbic encephalitis	101	35	66	10	2	8
Stiff-person syndrome	48	9	39	1	0	1
Cerebellitis	33	10	23	0	0	0
Overlap	17	3	14	1	0	1
Type 1 diabetes mellitus	39	9	30	1	1	0
AID	46	13	33	0	0	0
Neoplasm	15	6	9	0	0	0
Anti-GAD65 IgG high	120	33	87	5	1	4
Anti-GAD65 IgG low	47	18	29	5	1	4
Intrathecal synthesis	123	33	90	3	1	2
Associated autoimmune diseases						
Autoimmune thyroid disease	34	–	–	–	–	–
Hashimoto's disease	31	–	–	–	–	–
Graves' disease	3	–	–	–	–	–
Vitiligo	8	–	–	–	–	–
Inflammatory bowel disease	4	–	–	–	–	–
Ulcerative colitis	3	–	–	–	–	–
Crohn's disease	1	–	–	–	–	–
Autoimmune gastritis	6	–	–	–	–	–
Sjögren syndrome	3	–	–	–	–	–
Addison's disease	2	–	–	–	–	–
Ankylising spondylitis	1	–	–	–	–	–
Primary biliary cholangitis	1	–	–	–	–	–
Polymyalgia rheumatica	1	–	–	–	–	–
Primary sclerosing cholangitis	1	–	–	–	–	–
Psoriasis	1	–	–	–	–	–
Rheumatoid arthritis	1	–	–	–	–	–

AID = autoimmune disorders other than type 1 diabetes mellitus; Overlap = any combination of LE, SPS and CB; SD = standard deviation.

number of overlapping genes (n) > 2 in modules 0–3 and $n = 2$ in modules 4–5. Moreover, pathway terms that define specific types of infections or processes in the context of a particular disease were excluded.

After defining genomic loci, the association of all variants within GWAS loci (858 variants) with a set of clinical phenotypes in anti-GAD65 AINS patients was tested, namely the presence of a disease subtype (LE with TLS or TLE, CB with CA, SPS or any combination of these), comorbid T1DM, other autoimmune disorders or neoplasms, as well as high compared to low serum concentration of anti-GAD65 IgG (high: CBA ≥ 100, ELISA ≥ 2000 U/ml, RIA ≥ 20 nmol/l)[2,5,15] and absence compared to presence of intrathecal anti-GAD65 IgG synthesis. This analysis was adjusted for covariates as before but considered a nominal P-value ($P < 0.05$) to be suggestive of an association.

SNP heritability and LD score regression

We calculated the proportion of variance in sporadic anti-GAD65 AINS explained by our GWAS using the genome-based restricted maximum likelihood (GREML)-LDMS (LD- and MAF-stratified GREML) method implemented in GCTA.[16,17] For all autosomal variants in the imputed dataset, we calculated the 200 kb segment-based LD scores, stratified variants according to LD scores of individual SNPs, computed one genetic relationship matrix for each quartile of the stratified variants, and performed a restricted maximum likelihood analysis using these four matrices. The variance explained was estimated in a case-control setting, adjusting for covariates as before, and assuming a disease prevalence of 1.9/100 000 individuals.[15] Additionally, to confirm that our findings were due to a polygenic architecture instead of batch effects, we calculated the LD score regression intercept using the LDSC software[18] with LD scores pre-computed in 1000 Genomes European data, as suggested by the authors.

Analysis of the HLA region

In total, 6351 variants were extracted from the HLA region at chromosomal coordinates chr6:29-34 Mb. HLA allele genotypes for

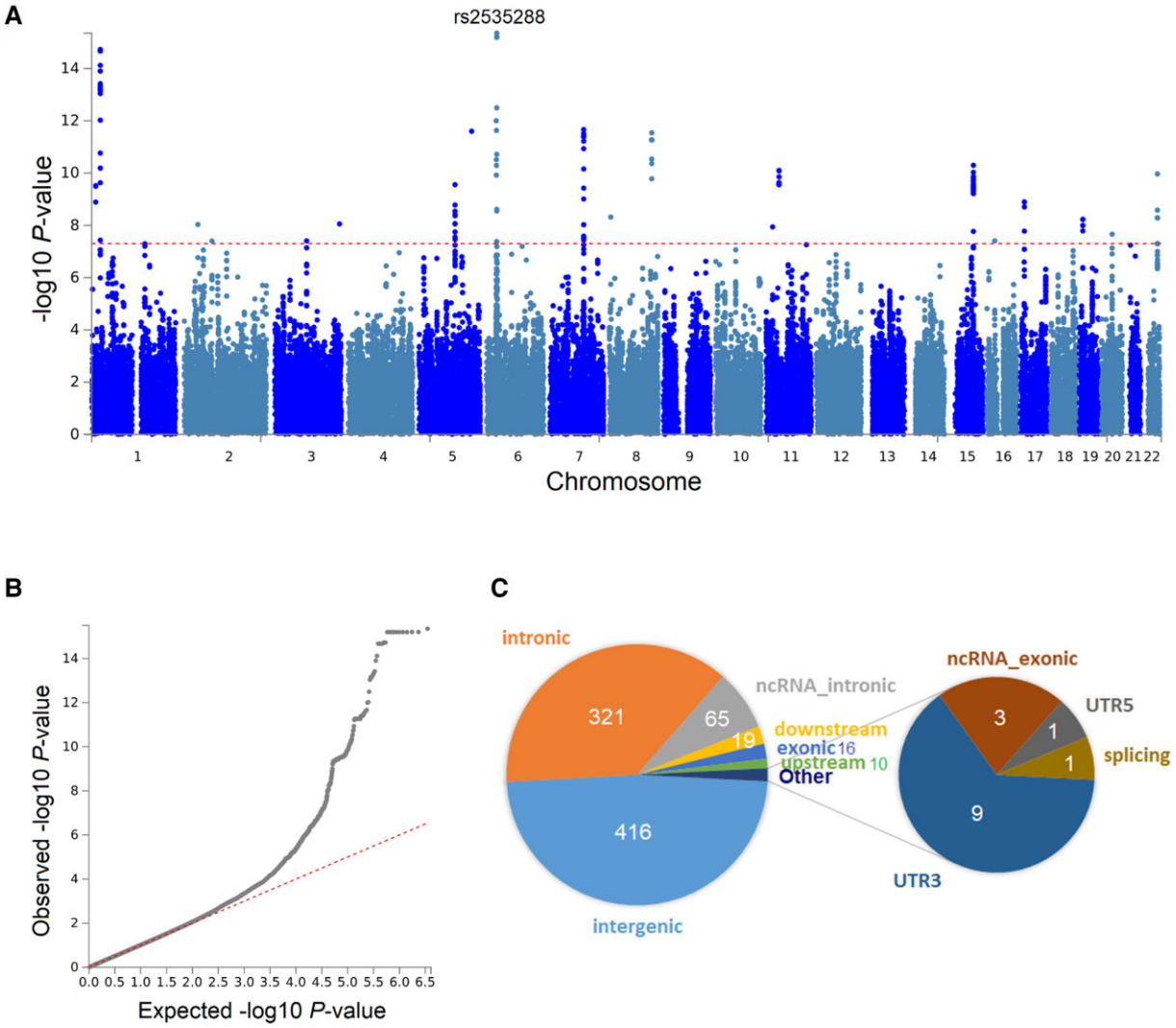

Figure 1 Overview of findings for the anti-GAD65 AINS GWAS. (**A**) Manhattan and (**B**) quantile-quantile plots of the anti-GAD65 AINS GWAS. The red dotted line in **A** depicts the threshold of genome-wide significance ($P < 5 \times 10^{-8}$). The total number of variants reaching this threshold was 191. The top variant was rs2535288 (chr6:31064007, $P = 4.42 \times 10^{-16}$, OR = 0.26, 95%CI = 0.187–0.358). (**C**) Functional consequences of all genome-wide and genomic loci variants in LD (r2 ≥ 0.6 and ±500 kb from lead, $P < 0.05$). The greatest proportion of variants locates to intergenic and intronic regions of the genome.

the classical HLA class I loci HLA-*A*, -*B*, -*C* and class II loci HLA-*DQA1*, -*DQB1*, -*DPB1* and -*DRB1* were imputed using the Broad HLARES models for the Illumina Infinium Global Screening Array v2.0 using the R-package HLA genotype imputation without attribute bagging (HIBAG) (Version 1.20.0) at 4-digit resolution.[19] We additionally imputed HLA nucleotides and amino acids, as well as performing HLA genotype phasing based on SHAPEIT2.[20,21] A significance threshold of $P < 0.001$ was considered suggestive of an association for the purposes of our study.

Analysis of CSF proteome

To further substantiate the predictions on changes of protein expression levels in disease-relevant cells and tissues obtained from our GWAS, we added a CSF proteome study. We retrospectively identified a cohort of 10 patients with anti-GAD65 AINS, eight of whom were not part of the initial GWAS analysis, from our data base. The control group consisted of 10 age- and sex-matched patients with somatoform disorders. All participants gave written informed consent prior to sample acquisition and the study was approved by the ethics committee of the University of Muenster (2016-053-f-S and 2013-350-f-S). Liquid-chromatography ultra-high definition mass spectrometry (LC-UDMSe) analysis was performed in 1 ml CSF. Complete methods can be found in the Supplementary material. The proteomics data was processed using Progenesis for Proteomics (Waters) and the Uniprot human database. Statistical analyses were additionally performed in R (http://www.R-project.org/). We compared groups with unpaired Mann–Whitney U-test. Volcano plots were created by plotting the log2 fold change (LFC) of protein intensity values between both depicted experimental groups against their –log10 *P*-value. Significantly regulated proteins were defined as having a *P*-value of < 0.05. The respective cut-offs are indicated as dotted lines.

Availability of data and materials

The datasets analysed in the current study are available from the authors on reasonable request. The results generated during this study are included in this published article and its supplementary information files.

Results

The initial study population included 205 anti-GAD65 AINS patients (62 males, 143 females) from the GENERATE network. After quality control and elimination of outliers and related individuals, 167 effective cases and 1047 controls remained. The cases consisted of 51 (31%) males versus 116 (69%) females, and the controls of 602 (57%) males and 445 (43%) females. In the anti-GAD65 AINS group, 101 (60%) patients had LE with TLS or TLE, 33 (20%) had CB with CA and 48 (29%) had SPS. An overlap syndrome was found in 17 (10%) patients, 39 (23%) patients had associated T1DM and 46 (28%) other autoimmune diseases, including autoimmune thyroid disease ($n = 34$; 20%), vitiligo ($n = 8$, 4.7%) and pernicious anaemia and/or autoimmune gastritis ($n = 6$, 3.5%) (for details, see Table 1).

No distortions of the observed or imputed genotypes due to independent genotyping of cases and controls were observed. There was good correlation of our dataset with the reference panel and of cases with control individuals (Supplementary Fig. 1). Our GWAS of anti-GAD65 AINS identified 191 associations at the genome-wide threshold of significance (Fig. 1A and B) and explained about 14.4% of the variance (h^2) on the liability scale. The genomic inflation factor, λ=1.026, and the LD score regression intercept of 1.0037, corroborated that our findings were due to polygenicity. The top variant, rs2535288 [g.31064007C>A, $P = 4.42 \times 10^{-16}$, odds ratio (OR) = 0.26, MAF = 0.45, 95% confidence interval (CI) = 0.187–0.358], localizes to

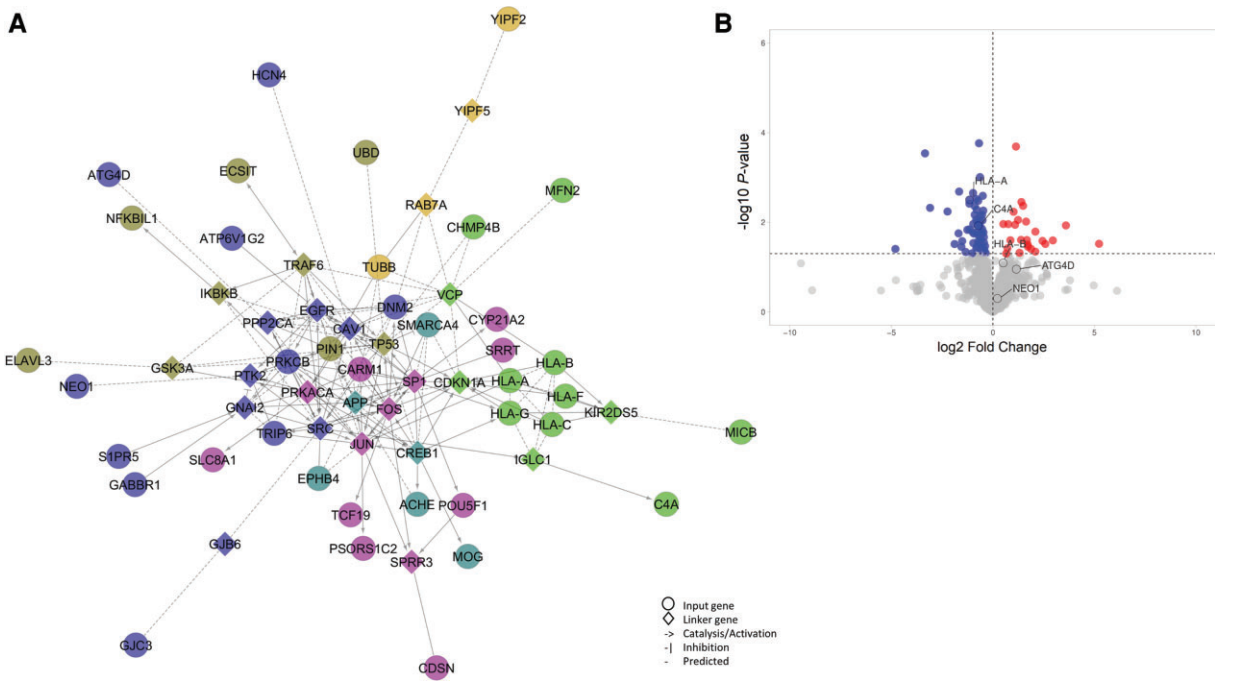

Figure 2 Investigation of GWAS findings at the protein level. (**A**) Protein-protein interaction network of GWAS coding genes. The network shows six functionally related gene clusters. Important connectors are *SP1*, *FOS*, *TP53*, *JUN* and *SRC*. (B) Volcano plot illustrating differential protein expression profiles in the CSF of anti-GAD65 AINS patients (red) compared to healthy controls (blue). HLA-A and C4A are differentially ($P < 0.05$) downregulated in anti-GAD65 AINS patients.

Table 2 Supported genomic loci for GAD65-AINS identified by our GWAS

Locus	Index variant	Index alleles	Index location (bp)	Index P-value	Index OR	Chromosomal location	Start (bp)	End (bp)	Size (bp)	# Variants	# Ind.Sig. Variants	# Lead variants	Effects of variants	Mapped gene(s)
1	rs28718032	T/C	12 046 880	3.03×10^{-10}	2.58	chr1p36.22	12 046 089	12 068 546	22 457	6	1	1	Risk	*MFN2*
2	rs3091240	T/G	25 676 935	1.88×10^{-15}	3.53	chr1p36.11	25 663 071	25 851 977	188 906	31	2	1	Risk + Protective	*C1orf63; RP11-335G20.7; TMEM50A; RHCE; TMEM57/MACO1; LDLRAP1*
3	rs11124749	C/G	40 821 576	9.29×10^{-9}	2.27	chr2p22.1	40 819 830	40 841 140	21 310	5	1	1	Risk	*SLC8A1*
4	rs2566748	T/C	82 292 908	3.99×10^{-8}	2.19	chr2p12	82 256 797	82 304 801	48 004	18	1	1	Risk + Protective	*AC079896.1*
5	rs1822385	C/A	94 758 570	3.94×10^{-8}	2.40	chr3q11.2	94 749 987	94 760 796	10 809	4	1	1	Risk	*LINC00879*
6	rs6887291	G/T	106 438 742	2.78×10^{-10}	0.35	chr5q21.3	106 404 866	106 557 132	152 266	103	1	1	Protective	*CTC-254B4.1; PSMC1P5*
7	rs2535288	C/A	31 064 007	4.42×10^{-16}	0.26	chr6p21.33, chr6p22.1	29 488 249	31 105 310	1 617 061	244	10	3	Risk + Protective	*LINC01015; OR2I1P; UBD; GABBR1; MICG; HLA-W; MICD; HCG9; HCG17; TRIM26BP; PPP1R18; TUBB; MUC22; HCG22; RNU6-1133P; C6orf15; PSORS1C1; CDSN*
8	rs314304	G/A	100 378 221	2.16×10^{-12}	0.34	chr7q22.1	100 368 671	100 523 241	154 570	113	3	1	Mostly protective*	*ZAN; EPHB4; SLC12A9; RP11-126L15.4; TRIP6; SRRT; UFSP1; ACHE; RN7SL549P; RP13-650G11.1*
9	rs12674939	T/A	126 479 907	2.87×10^{-12}	2.84	chr8q24.13	126 479 907	126 489 809	9902	16	1	1	Risk	*RP11-136O12.2*
10	rs12806812	A/G	38 401 216	7.90×10^{-11}	0.37	chr11p12	38 400 126	38 429 604	29 478	18	1	1	Mostly protective*	*RP11-63D14.1*

(continued)

Table 2 (continued)

Locus	Index variant	Index alleles	Index location (bp)	Index P-value	Index OR	Chromosomal location	Start (bp)	End (bp)	Size (bp)	# Variants	# Ind.Sig. Variants	# Lead variants	Effects of variants	Mapped gene(s)
11	15:73543854	C/CA	73 543 854	5.05×10^{-11}	2.66	chr15q24.1	73 331 183	73 626 970	295 787	199	3	1	Mostly risk*	*NEO1*; *HCN4*
12	16:24042219	C/CA	24 042 219	3.92×10^{-8}	0.43	chr16p12.2	24 042 219	24 053 704	11 485	5	1	1	Risk + Protective	*PRKCB*
13	rs8082548	G/A	12 418 790	1.28×10^{-9}	2.41	chr17p12	12 412 583	12 428 515	15 932	25	2	1	Risk	*LINC00670*
14	19:10882215	C/CT	10 882 215	5.93×10^{-9}	2.30	chr19p13.2	10 870 866	10 898 413	27 547	9	1	1	Risk	*DNM2*
15	rs2747537	G/A	32 405 217	2.19×10^{-8}	2.32	chr20q11.22	32 399 200	32 470 133	70 933	16	1	1	Risk + Protective	*CHMP4B*
16	rs62231498	A/G	44 914 879	1.08×10^{-10}	3.53	chr22q13.31	44 888 124	44 926 599	38 475	45	2	2	Risk + Protective	*LDOC1L*

bp = base pairs; Ind.Sig. = individually significant. *Most variants of the higher significance have these effects, although there are variants of lower significance with the opposite effects.

an intergenic genomic segment in the middle of the HLA class I region on chromosome 6. The variant exerts regulatory effects on multiple nearby genes (*HCG22*, *TCF19*, *HLA-B*) in brain tissue (frontal and temporal cortices) and immune cells (B cells and $CD4^+$ T cells). It also shows various epigenetic marks (H3K4me1, H3K27me3, ZNF207 transcription factor) in brain cells (astrocytes, bipolar neurons), as well as in immune organs (thymus, spleen) and cells (T cells, $CD14^+$ monocytes, common myeloid progenitors). Moreover, diverse genome-wide variants in LD with rs2535288 supported this association signal.

According to the applied criteria (see 'Methods' section), 16 genomic loci for anti-GAD65 AINS were defined by the combination of our GWAS summary statistics and LD information (Supplementary Figs 2–17). In total, these genomic loci contained 858 variants, of which 32 were independently significant, and 19 were lead variants, mapping to 48 genes in total (Table 2). All genome-wide and loci variants (including those genome-wide variants that were excluded from further analysis due to a lack of signal support) are listed in Supplementary Table 2. In their majority, these variants were intergenic (48.3%) or localized to introns of coding (37.3%) and non-coding (7.6%) genes (Fig. 1C). The annotation of variants in genomic loci showed that 358 variants have 1972 associations with the expression levels of 47 genes, including 39 non-mapped genes, in the brain (preferentially frontal cortex) and immune cell types (preferentially $CD4^+$ T cells) from the BRAINEAC and DICE eQTL datasets. This suggests that the regulation of gene expression in disease relevant cells and tissues is one of the molecular mechanisms by which the identified associations contribute to the pathobiology of anti-GAD65 AINS (for the genomic locus context of these eQTL annotations see Supplementary Table 3 and Supplementary Figs 2–17).

In addition, over 6000 annotations from the Roadmap and ENCODE epigenomics datasets that were obtained for 728 variants in the GWAS genomic loci gave insights into cell type and tissue specificity. The most frequent epigenomes encountered were neuronal cells (including neural stem progenitor cells) and immune cells (including $CD14^+$ monocytes, $CD34^+$ common myeloid progenitors and T cells). The most frequent epigenetic marks were the histone modifications H3K4me1, H3K36me3 and H3K27me3 (Table 3). Epigenetic annotations specific for each GWAS locus are presented in Supplementary Table 4.

The most significant and largest locus (#7), located in the HLA class I region, harboured 244 variants with both risk and protective effects, of which 10 were independent signals and three were lead variants. Eighteen genes mapped to this locus (Table 2). In addition, variants within this locus have been reported as eQTLs in brain and/or immune cells of 21 non-mapped genes. Of note, five eQTL genes (*HLA-F*, *HCG18*, *MICB*, *C4A*, *VARS2*) were found exclusively in the brain dataset, while *HCG22* showed the largest numbers of eQTLs in both the BRAINEAC (149 eQTLs in the average of all regions) and DICE (23 eQTLs in naïve B cells) datasets (Supplementary Table 3). Expectedly, epigenetic marks, particularly H3K4me1, were more common in epigenomes of diverse immune cells, most prominently of common myeloid progenitors.

The second (locus #2, chromosome 1, index variant: rs3091240, $P = 1.88 \times 10^{-15}$, OR = 3.53, 95%CI = 2.587–4.819) and third (locus #8, chromosome 7, index variant: rs314304, $P = 2.16 \times 10^{-12}$, OR = 0.34, 95%CI = 0.254–0.461) most significant loci mapped to more than two genes (Table 2). Mapped and eQTL genes in these loci implicated the Rh blood group cluster (*RHCE*, *RHD*, *TMEM50A*) and genes with important functions in neuronal development and function (*MACO1*, *ACHE*, *TRIP6*, *SRRT*, *EPHB4*) in the biology of anti-GAD65

Table 3 Summary of epigenetic marks annotated for variants in GWAS loci

Feature	Database	*n*	%
Epigenome: cell types			
Bipolar neuron	ENCODE	825	13.42
Neuron	Roadmap	279	4.54
Monocytes-CD14+	ENCODE	511	8.31
CD14 positive monocyte	ENCODE	413	6.72
Monocytes-CD14+ (peripheral blood)	Roadmap	305	4.96
Common myeloid progenitor CD34 positive (ENCSR337XXD 1)	Roadmap	434	7.06
Common myeloid progenitor CD34 positive	Roadmap	350	5.69
Common myeloid progenitor CD34 positive (ENCSR722JRY)	Roadmap	141	2.29
Neural stem progenitor cell	Roadmap	431	7.01
Neural progenitor cell	ENCODE	88	1.43
T cells (peripheral blood)	Roadmap	386	6.28
T cell	Roadmap	13	0.21
T helper 17 cell	Roadmap	104	1.69
Neutrophil	Roadmap	181	2.94
B cells (peripheral blood)	Roadmap	176	2.86
B cell (ENCSR682AXR)	ENCODE	126	2.05
B cell	Roadmap	16	0.26
B cell (ENCSR241CUA 1)	Roadmap	14	0.23
Natural Killer cells (peripheral blood)	Roadmap	171	2.78
CD4 positive alpha beta T cell (ENCSR948ZKZ)	Roadmap	145	2.36
CD4 positive alpha beta T cell	Roadmap	134	2.18
CD4 positive CD25 positive alpha beta regulatory T cell	Roadmap	104	1.69
Naive thymus derived CD4 positive alpha beta T cell	Roadmap	90	1.46
CD4 positive alpha beta memory T cell	Roadmap	55	0.89
Effector memory CD4 positive alpha beta T cell	Roadmap	17	0.28
Astrocyte	ENCODE	111	1.81
Astrocyte (ENCSR362MQF)	ENCODE	14	0.23
Astrocyte (ENCSR362MQF 1)	ENCODE	12	0.20
Cardiac muscle cell	ENCODE	84	1.37
Epigenome: organs			
Spleen	Roadmap	186	3.03
Heart	Roadmap	110	1.79
Brain	Roadmap	106	1.72
Brain (ENCSR189GMC-female embryo)	Roadmap	15	0.24
Marker			
H3K4me1 (histone)	Both	1780	28.96
H3K36me3 (histone)	Both	1479	24.06
H3K27me3 (histone)	Both	1376	22.38
H3K9me3 (histone)	Both	435	7.08
H3K27ac (histone)	Both	344	5.60
H3K4me3 (histone)	Both	262	4.26
DNase1 (open chromatin)	Both	206	3.35
CTCF (transcription factor)	Both	177	2.88
H4K20me1 (histone)	Both	54	0.88
H3K4me2 (histone)	Both	19	0.31
H3K9ac (histone)	Both	11	0.18
EZH2 (transcription factor)	Both	4	0.07

AINS. The majority of the identified loci mapped to only one protein-coding or non-coding gene (10/16) and contained variants with both, risk and protective effects (9/16). However, three of the 'mixed effects' loci might be assumed to have either mostly protective (loci #8 and 10) or mostly risk (#11) effects on the anti-GAD65 AINS phenotype, driven by the variants of highest significance within the locus. Six loci contained only risk variants, while one contained only variants with protective effects (locus #6). The biological implications of some of these loci are not clear from our analyses, not only because they mapped to non-coding genes, but also because no eQTL effects for these variants were found in the relevant datasets.

For the protein-coding genes that physically mapped to our anti-GAD65 AINS GWAS loci and/or are regulated by these variants (i.e. eQTL genes) in the relevant datasets, we created a PPI network to provide some biological context for our findings beyond the immune component driven by the HLA. The resulting network comprised 62 genes, of which 38 were GWAS input and 24 as linker genes (Fig. 2A). Unsurprisingly, the largest number of functional interactions in the network was observed for linker genes. These network hubs included *TP53*, *FOS* and *SP1*, with 18 interactions each, as well as *JUN* and *SRC*, with 17 interactions each. From the input genes, the most largely connected node was protein kinase C beta (*PRKCB*) from the GWAS locus #12, with 13 interactions. The clustering of the network defined six modules, with 17 genes in the largest and four genes in the smallest module (Table 4). In total, 115 module pathway enrichments passed our criteria (Supplementary Table 5). These included 85 unique pathway terms, with overlaps observed mainly between modules #0 (lilac) and #2 (magenta), including various cell signalling pathways related to immunity and neural function. As expected, all network modules were highly enriched in signalling pathways that relate to immunity, including antigen presentation and autoimmunity, which concentrated in the module #1 (green), as well as innate immune pathways, such as the Toll-like and RIG-I-like receptor cascades. Enrichment was found in signalling pathways of other molecules and activation of receptors, whose functions might not primarily be immune, but have known roles in immune regulation. These included lysophosphatidic acid (LPA) receptors, sphingolipids, oestrogen, oxytocin, gonadotropin-releasing hormone (GnRH) and relaxin (Supplementary Table 5).

In addition, a total of 411 associations with all nine clinical variables were found at the nominal level for 290 anti-GAD65 AINS loci variants. These highlighted the importance of locus #7 for almost all of the phenotypes tested (with the exception of the overlapping of LE/SPS/CB), but particularly for LE with TLS/TLE, T1DM and intrathecal synthesis, for which the lowest *P*-values were found in this GWAS locus (Table 5). Our results suggest that both loci #7 and #8 overlap between different types of anti-GAD65 AINS, as well as other autoimmune diseases and the presence of a neoplasm. Differences between these might be influenced by variants in other loci, particularly in loci #5, #6, #10 and #11. Interestingly, loci #5 and #11, which together showed associations with SPS and the presence of a neoplasm, have risk effects, while loci #6 and #10, which appear to influence CB, showed protective effects in our GWAS. This illustrates how variations in multiple regions of the genome and their interactions contribute to distinctive phenotypes.

Our analysis of the HLA classical alleles and haplotypes showed no associations at the genome-wide level and no overlaps with the GWAS genome-wide signals in the region (Fig. 3A). Nonetheless, because only a segment of the genome is tested in this analysis and considering the relatively small sample size of our study, we chose to lower the threshold for suggestive associations to $P < 0.001$. At this threshold, one significant haplotype (DQA1*03:01–DQB1*03:02-DRB1*04:01, $P = 4.29 \times 10^{-4}$, OR = 2.5, frequency = 0.064, 95%CI = 1.499–4.157) and one significant allele (DRB1*04:01, $P = 8.3 \times 10^{-5}$, OR = 2.39, frequency = 0.094, 95%CI = 1.548–3.682) conferring risk for anti-GAD65 AINS were found, together with two significant

Table 4 Summary of network modules

Module	Color	Nodes	Genes	Pathways	Top three pathways	Summary pathway class(es)
0	Lilac	17	**ATG4D**, **ATP6V1G2**, *CAV1*, **DNM2**, *EGFR*, **GABBR1**, *GJB6*, **GJC3**, *GNAI2*, **HCN4**, **NEO1**, *PPP2CA*, **PRKCB**, *PTK2*, **S1PR5**, *SRC*, **TRIP6**	49	Signalling events mediated by VEGFR1 and VEGFR2, LPA receptor mediated events, Thromboxane A2 receptor signalling	Cell signalling mediating immune and neuronal processes
1	Green	13	**C4A**, *CDKN1A*, **CHMP4B**, **HLA-A**, **HLA-B**, **HLA-C**, **HLA-F**, **HLA-G**, *IGLC1*, *KIR2DS5*, **MFN2**, **MICB**, *VCP*	16	Antigen processing and presentation, Allograft rejection, Immunoregulatory interactions between a lymphoid and a non-lymphoid cell	Antigen processing and presentation, Autoimmunity, interferon signalling
2	Magenta	13	**CARM1**, **CDSN**, **CYP21A2**, *FOS*, *JUN*, **POU5F1**, *PRKACA*, **PSORS1C2**, **SLC8A1**, *SP1*, *SPRR3*, **SRRT**, **TCF19**	30	ESR-mediated signalling, Calcium signalling in the CD4+ TCR pathway, ErbB2/ErbB3 signalling events	Cell signalling mediating immune and neuronal processes
3	Olive	9	**ECSIT**, **ELAVL3**, *GSK3A*, *IKBKB*, **NFKBIL1**, **PIN1**, *TP53*, *TRAF6*, **UBD**	11	Toll-like receptor pathway, Signal transduction through IL1R, p75(NTR)-mediated signalling	Inflammation, neurotrophin signalling
4	Aqua	6	**ACHE**, *APP*, *CREB1*, **EPHB4**, **MOG**, **SMARCA4**	7	ATF-2 transcription factor network, Glucocorticoid receptor regulatory network, Cholinergic synapse	Macrophage activity
5	Mustard	4	*RAB7A*, **TUBB**, **YIPF2**, *YIPF5*	2	Phagosome, Neutrophil degranulation	Innate immunity

Input genes for the network are highlighted in bold.

amino acids and three significant nucleotides conferring protection (Table 6 and Fig. 3B). The most common suggestive protective and risk HLA haplotypes in our cohort were DQA1*05:05-DQB1*03:01 (OR = 0.45, frequency = 0.1223, 95%CI = 0.264–0.759) and DQA1*05:01-DQB1*02:01-DRB1*03:01 (OR = 2.02, frequency = 0.121, 95%CI = 1.223–3.329), respectively.

Finally, we identified five proteins (HLA-A/B, C4A, ATG4D and NEO1) of eQTL genes from our GWAS in the CSF proteome of anti-GAD65 AINS patients (Fig 2B, clinical data in Table 1). Moreover, compared to controls, these changes were consistent with the effect direction in immune cells for the corresponding gene annotated from DICE in our GWAS, including elevation of HLA-B (B cells) and NEO1 (B and T cells) as well as decrease in HLA-A (T cells). HLA-A ($P = 0.003$, LFC = −1.14) and C4A ($P = 0.012$, LFC = −0.72) showed differential levels in patients.

Discussion

We conducted a GWAS of sporadic anti-GAD65 AINS in a cohort of 167 patients and over 1000 population-based controls. The demographic characteristics of our study population are in line with previous studies regarding sex and age, as well as the distribution of clinical phenotypes.[2,3,6] The presence of overlapping clinical phenotypes in 10% of our patients underlines the view of anti-GAD65 AINS as a disease spectrum rather than separate disease entities, similar to what has been previously described.[2] We focused our inclusion on a typical clinical phenotype, confirmed by a typical staining pattern in a TBA on rodent or non-human primate brain (and pancreas), which is indicative of high autoantibody titres.[3] We included patients who fulfilled these criteria, even if they had 'low' titres in the tertiary quantitative analyses, to study the full spectrum of anti-GAD65 AINS.

Our GWAS identified 16 genomic loci for anti-GAD65 AINS at the genome-wide threshold of significance. These included mainly intergenic or intronic variants outside the classic HLA genomic region. The annotation of these variants with eQTL and epigenetic datasets, as well as the network analysis of the implicated protein-coding genes, consistently underscored variations in both CNS tissues and immune cells. T-cell infiltrates have been detected in close spatial proximity to neurons of the hippocampus in anti-GAD65 TLE,[22] genetic variations resulting in altered interaction between the immune cells and the neurons could facilitate this.

Our PPI network analysis further illustrated the complex interplay of immune and neural signalling pathways relevant for anti-GAD65 AINS pathophysiology. According to the information contained in the GWAS Catalog,[23] several of the non-classical HLA genes implicated in anti-GAD65 AINS by our study have known associations with the susceptibility to different autoimmune disorders, such as T1DM, multiple sclerosis, psoriasis, arthritis and Crohn's disease. Among these genes are *EPHB4*, *PRKCB* and *SMARCA4*. The most common associated autoimmune diseases in our sample besides T1DM, were autoimmune thyroid diseases and vitiligo, for which some genetic susceptibility traits overlapping with genetic variations in anti-GAD65 AINS have been reported.[6,24–27]

Previous genetic studies of anti-GAD65 AINS have focused on the HLA class II region, suggesting associations in both familial and sporadic cases.[4–6] Our study did not find associations in this genomic locus at the genome-wide level of significance, neither in our GWAS, nor the HLA association analysis. Therefore, we lowered the significance threshold to consider an HLA-association 'suggestive' and searched for the most common haplotypes, based on their frequencies in our cohort. Thereby we were able to detect one haplotype and one allele in the HLA class II genomic locus that might contribute to the risk for anti-GAD65 AINS in our cohort. The suggested haplotype, DQA1*03:01-DQB1*03:02-DRB1*04:01, has been identified as one of the susceptibility haplotypes for T1DM,[28] with DQB1*03:02 reported as one of the high-risk alleles,[29] while

Table 5 Summary of associations of variants in GWAS loci with clinical phenotypes

Clinical phenotype	Total cases	Effective cases	Nominal associations	GWAS loci[a]
Limbic encephalitis	125	101	58	**7**,8,11
Stiff-person syndrome	57	48	63	5,7,8,**11**
Cerebellitis	36	33	28	6,7,8,**10**
Any combination of the above	20	17	25	9,11,**12**,13
Type 1 diabetes mellitus (T1DM)	41	39	40	2,4,**7**,8,9,10
Autoimmune disorders other than T1DM	53	46	66	**7**,8,10
Presence of neoplasm	19	15	14	5,7,8,11,**15**
Anti-GAD65 IgG concentration (CBA: 1/Titre, ELISA: U/ml, RIA: nmol/l) in serum. High (CBA ≥ 100, ELISA ≥ 2000 U/ml, RIA ≥ 20 nmol/l)	149	120	98	**4**,7,14,16
Intrathecal synthesis (with method-based cut off CBA/TBA 4, ELISA/RIA 1.5)	140	123	19	**7**,11,15

[a]The locus showing the lowest *P*-values is highlighted in bold.

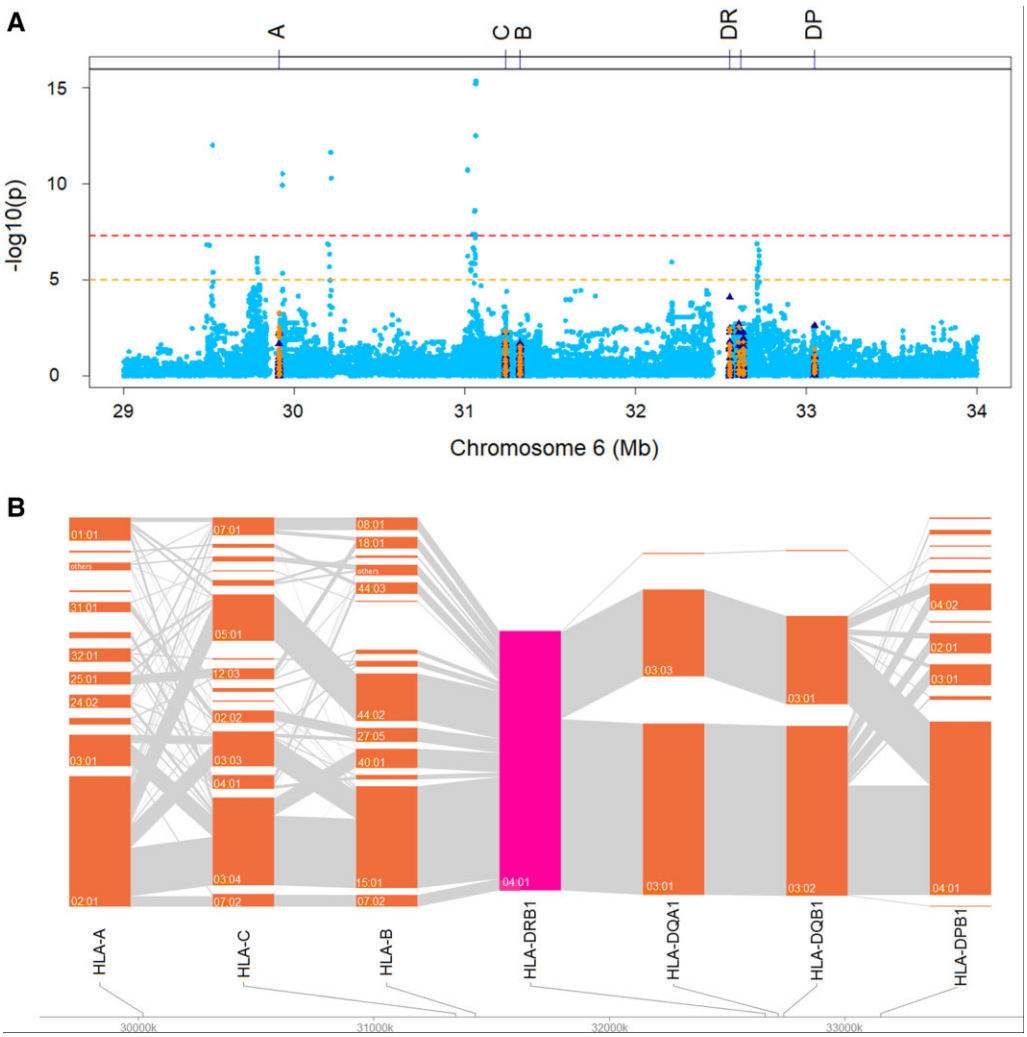

Figure 3 Analysis of the classical HLA alleles and haplotypes. (**A**) Manhattan and (**B**) disentangler plots of the analysis of the classical HLA alleles and haplotypes. In **A**, the GWAS variants are shown in light blue, the classical alleles in dark blue and the amino acid variants in orange. The yellow and red dotted lines indicate the commonly used suggestive ($P < 1 \times 10^{-5}$) and genome-wide ($P < 5 \times 10^{-8}$) significance thresholds, respectively. In **B**, the relationships of the allele of highest significance in our study, HLA-DRB1*04:01, with other classical alleles in the whole GWAS dataset are shown as disentangler plot.[36]

our study defined this as DRB1*04:01 for anti-GAD65 AINS. In our cohort, 39 patients had an associated T1DM, out of which only 10 carried the DQA1*03:01-DQB1*03:02-DRB1*04:01 haplotype. This haplotype was also present in 17 cases that did not have T1DM, and vice versa, 26 cases had an associated T1DM but carried other HLA haplotypes. This haplotype has been reported as a genetic

Table 6 HLA region findings at the threshold $P < 0.001$

Type	Feature	Position	Effect	Non effect	n	P	OR	95% CI	Frequency
Suggestive threshold ($P < 0.001$)									
Haplotype	DQA1*03:01-DQB1*03:02-DRB1*04:01	–	P	A	1091	4.39×10^{-4}	2.496	1.499–4.157	0.0637
Allele	imputed_HLA_DRB1*04:01	32552086	P	A	1214	8.30×10^{-5}	2.387	1.548–3.682	0.09432
Amino acid	imputed_prot_A_166	166	D	E	1214	5.41×10^{-4}	0.4872	0.324–0.732	0.2801
Amino acid	imputed_prot_A_167	167	G	W	1214	5.41×10^{-4}	0.4872	0.324–0.732	0.2801
Nucleotide	imputed_nuc_A_29910761	29910761	A	G	1214	3.02×10^{-5}	0.498	0.359–0.691	0.3904
Nucleotide	imputed_nuc_A_29911271	29911271	C	G	1214	5.41×10^{-4}	0.4872	0.324–0.732	0.2801
Nucleotide	imputed_nuc_A_29911272	29911272	G	T	1214	5.41×10^{-4}	0.4872	0.324–0.732	0.2801
Other haplotypes of interest									
Haplotype	DQA1*05:05-DQB1*03:01	–	P	A	1108	2.84×10^{-3}	0.4477	0.264–0.759	0.1223
Haplotype	DPB1*04:01-DQA1*01:03-DQB1*06:03-A*02:01-C*05:01-DRB1*13:01-B*44:02	–	P	A	941	2.94×10^{-3}	17.38	2.647–114.1	0.002657
Haplotype	DQA1*05:01-DQB1*02:01	–	P	A	1108	5.14×10^{-3}	2.042	1.238–3.368	0.1196
Haplotype	DQA1*05:01-DQB1*02:01-DRB1*03:01	–	P	A	1091	6.00×10^{-3}	2.018	1.223–3.329	0.121
Haplotype	A*31:01-C*04:01-B*35:01	–	P	A	1168	8.36×10^{-3}	4.71×10^{-4}	1.59×10^{-6}–0.1399	0.0008562
Haplotype	DPB1*04:01-DQA1*02:01-DQB1*02:02-A*01:01-C*07:01-DRB1*07:01-B*08:01	–	P	A	941	8.70×10^{-3}	45.24	2.623–780.3	0.001063

risk factor in polyglandular autoimmune syndrome type 2.[30] The DRB1*04:01 allele as such has been reported in association with T1DM and rheumatoid arthritis.[31,32] Furthermore, it plays a role in the interaction of multiple sclerosis and other autoimmune diseases,[33] which supports the hypothesis that different autoimmune diseases share a genetic risk.[34]

Additionally, the most common HLA class II risk haplotype identified in our study (DQA1*05:01-DQB1*02:01-DRB1*03:01), was reported as the most frequent haplotype in a previous association study of the HLA in 32 anti-GAD65 patients.[4]

We assessed whether our GWAS signals were specific for particular anti-GAD65 AINS phenotype and, although we did not find any genome-wide significant associations, we observed about 400 associations at the nominal level of significance. Associations with the most prominent loci (locus #7 and #8), which mapped to both immune-related genes, including the HLA class I region, and to neural-related genes, including inhibitory neuronal signalling and neuronal structure and development, were present across all phenotypes (but not their combinations). There were subtle associations in loci #5, #6, #10 and #11, which could point towards a difference in genetic vulnerability for a certain phenotype. It is conceivable, that many subtle genetic signals with particular anti-GAD65 AINS phenotypes were lost in our study due to the small sample size when dividing into subgroups. Particular genetic loci might interact with each other, exerting a cumulative effect. Downstream effects (i.e. post-transcriptional and post-translational modifications) could explain differences among phenotypes. Additionally, our study did not take into consideration the effects of external factors, such as the living situation, exposure to different environments or preceding infections, which could have influenced the development of anti-GAD65 AINS, similar to what has been suggested for T1DM and Hashimoto's disease.[35] As this was not the main concern of the present work, it should be the focus of further studies. Another task for further studies will be translating the results to therapeutic strategies, since traditional immunosuppression has limited effects.[3] Our protein-protein interaction network highlighted the importance of PRKCB, a factor in cell death and cell survival, and SMARCA4 which is reported to be involved in the regulation of neural stem cell renewal and proliferation (GWAS Catalogue),[23] which may be potential targets for neuroprotective strategies.

There are other limitations to our study: Due to the rarity of the disease, we worked with a German, strictly Caucasian sample, which could have influenced the results. In the future, an international sample could provide further insights into the genetic architecture of anti-GAD65 AINS. Moreover, we acknowledge that focusing on common variants might result in the effect of important yet rare genetic variants being missed while the effects of the most common variants might be overestimated. We tried to overcome some of these limitations by applying stringent QC and performing an array of downstream analyses, including investigation in proteomics data from the CSF. Although our proteomics sample is still small, we believe the observed overlaps with our GWAS findings are encouraging.

In conclusion, we demonstrated that common variations at numerous genes involved in innate and adaptive immunity, as well as contributing to neural structure and function, modulate the susceptibility to anti-GAD65 AINS. The implicated protein-coding genes are part of a network of biological pathways with various cross-links between the CNS development and function and immunity. Although the most significant locus was located in the HLA class I region, classical HLA analysis could only point to

potential haplotype associations located to the HLA class II region. Our findings highlighted the involvement of different immune cell types and the brain itself in the pathogenesis of anti-GAD65 AINS. In individuals with a predisposition to aberrant immune responses and altered neuronal structure and function, a bidirectional interaction could explain the poor treatment response, which warrants a deeper analysis of the cause-and-effect relationship in anti-GAD65 AINS and other neurological disorders.

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Competing interests

The authors declare no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

Appendix 1

Members of the German Network for Research on Autoimmune Encephalitis (GENERATE)

For institutions and contact information, please refer to the Supplementary material.

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