




Genetic determinants of type 1 diabetes in individuals with weak evidence of islet autoimmunity at disease onset

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Received: 18 March 2022 / Accepted: 31 October 2022 / Published online: 24 January 2023
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Abstract

Aims/hypothesis Islet autoantibodies (AABs) are detected in >90% of individuals with clinically suspected type 1 diabetes at disease onset. A single AAB, sometimes at low titre, is often detected in some individuals, making their diagnosis uncertain. Type 1 diabetes genetic risk scores (GRS) are a useful tool for discriminating polygenic autoimmune type 1 diabetes from other types of diabetes, particularly the monogenic forms, but testing is not routinely performed in the clinic. Here, we used a type 1 diabetes GRS to screen for monogenic diabetes in individuals with weak evidence of autoimmunity, i.e. with a single AAB at disease onset.

Methods In a pilot study, we genetically screened 142 individuals with suspected type 1 diabetes, 42 of whom were AAB-negative, 27 of whom had a single AAB (single AAB-positive) and 73 of whom had multiple AABs (multiple AAB-positive) at disease onset. Next-generation sequencing (NGS) was performed in 41 AAB-negative participants, 26 single AAB-positive participants and 60 multiple AAB-positive participants using an analysis pipeline of more than 200 diabetes-associated genes.

Results The type 1 diabetes GRS was significantly lower in AAB-negative individuals than in those with a single and multiple AABs. Pathogenetic class 4/5 variants in MODY or monogenic diabetes genes were identified in 15/41 (36.6%) AAB-negative individuals, while class 3 variants of unknown significance were identified in 17/41 (41.5%). Residual C-peptide levels at diagnosis were higher in individuals with mutations compared to those without pathogenetic variants. Class 3 variants of unknown significance were found in 11/26 (42.3%) single AAB-positive individuals, and pathogenetic class 4/5 variants were present in 2/26 (7.7%) single AAB-positive individuals. No pathogenetic class 4/5 variants were identified in multiple AAB-positive individuals, but class 3 variants of unknown significance were identified in 19/60 (31.7%) patients. Several patients across the three groups had more than one class 3 variant.

Conclusions/interpretation These findings provide insights into the genetic makeup of patients who show weak evidence of autoimmunity at disease onset. Absence of islet AABs or the presence of a single AAB together with a low type 1 diabetes GRS may be indicative of a monogenic form of diabetes, and use of NGS may improve the accuracy of diagnosis.

Keywords Endotypes · Genetic risk score · HLA · Islet autoantibodies · Targeted exome sequencing · Type 1 diabetes · Whole exome sequencing

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Abbreviations

AAB	Autoantibody
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
GADA	Autoantibodies to GAD65
GRS	Genetic risk score
GWAS	Genome-wide association study

Research in context

What is already known about this subject?

- Some patients have weak evidence of autoimmunity at disease onset, i.e. a single islet autoantibody (AAb), making their diagnosis uncertain
- Use of a type 1 diabetes genetic risk score (GRS) can distinguish type 1 diabetes from MODY, permanent neonatal diabetes mellitus, monogenic autoimmunity and type 2 diabetes

What is the key question?

- Is it possible to improve the patient selection algorithm for screening for monogenic diabetes using next-generation sequencing (NGS)?

What are the new findings?

- Exome sequencing coupled with an analysis pipeline of more than 200 genes identified pathogenetic class 4/5 variants in AAb-negative and single AAb-positive individuals
- Rare pathogenetic variants of class ≥ 3 were identified in all groups, including multiple AAb-positive individuals with a disease onset at ≥ 13 years of age. Some patients had several class ≥ 3 variants, suggesting an oligogenic form of type 1 diabetes

How might this impact on clinical practice in the foreseeable future?

- Weak evidence of autoimmunity, combined with a low type 1 diabetes GRS, may be indicative of monogenic diabetes, and use of NGS can improve diagnosis. A workflow to help clinicians diagnose monogenic diabetes is provided

IAA	Autoantibodies to insulin
IA-2A	Autoantibodies to insulinoma antigen-2
ICI	Insulin-containing islet
MAID	Monogenic autoimmune disease
NGS	Next-generation sequencing
PNDM	Permanent neonatal diabetes mellitus
ROC	Receiver operating characteristic
STAT	Signal transducer and activator of transcription
T1DE	Type 1 diabetes endotypes
TSPAN7A	Autoantibodies to tetraspanin-7
ZnT8A	Autoantibodies to zinc transporter 8

Introduction

Type 1 diabetes is a disorder of glucose homeostasis, and patients require lifelong daily insulin injections. Type 1 diabetes is considered a multifactorial autoimmune disease in which several common genetic variants combined with environmental factors are thought to contribute to disease risk [1, 2]. Type 1 diabetes is caused by T cell-mediated killing of insulin-producing beta cells, and risk of type 1 diabetes is associated with the presence of measurable autoantibodies (AABs) in the patients' sera [3, 4]. Even though the pathogenesis of type 1

diabetes involves T cells, the immune markers are primarily serum AABs to pancreatic islet antigens. To date, five islet-specific AABs have been reported: against insulin (IAA), GAD65 (GADA), insulinoma antigen-2 (IA-2A), zinc transporter 8 (ZnT8A) and tetraspanin-7 (TSPAN7A) [5, 6]. Patients who are positive for at least one of these AABs are diagnosed with T1Da, which is the most frequent form of type 1 diabetes diagnosed in paediatric age groups [7, 8]. Conversely, AAb-negative patients are diagnosed as having T1Db, and account for up to 10% of newly diagnosed patients [9, 10]. Recent studies have shown that AAb-negative patients often have MODY, or, less frequently, permanent neonatal diabetes mellitus (PNDM) or syndromic forms, e.g. Wolfram syndrome [11–15]. The most common subtypes of MODY are caused by mutations in glucokinase (*GCK*), hepatocyte nuclear factor-1 α (*HNF1A*) and hepatocyte nuclear factor-4 α (*HNF4A*) genes. The former needs no treatment, while the latter two may be treated with low-dose sulfonylureas [16]. Hence, diagnosing MODY may lead to use of specific non-insulin targeted treatments and avoidance of insulin, with a positive impact on the quality of life of the affected individuals and their families, while reducing costs for the healthcare system.

Very-early-onset diabetes often occurs in patients with monogenic autoimmune disease (MAID) due to mutations in genes encoding forkhead box P3 (FOXP3), autoimmune

regulator (AIRE), IL-2 receptor alpha (IL-2R α), signal transducer and activator of transcription (STAT)-1, STAT-3 and STAT-5B, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and lipopolysaccharide-responsive and beige-like anchor protein (LRBA) [17–22]. Approximately 20–50% of these patients have no detectable islet-specific AAbs, although the disease is clearly immune-mediated. In several of these patients, diabetes and other autoimmune features are often the first clinical manifestations. Diagnosing MAID is crucial for patients' prognosis and clinical management. For example, it may enable personalised treatment using abatacept, a CTLA-4 mimetic, in patients with mutations in *CTLA4* and *LRBA* [23, 24].

From a clinical and epidemiological standpoint, patients who are AAb-positive comprise a heterogeneous group of patients. For example, studies on the natural history of diabetes performed by TrialNet and other consortia showed that some patients develop type 1 diabetes very rapidly when aged younger than 5 years and with low C-peptide levels that decline faster than in adults after diagnosis [25, 26]. Studies conducted post mortem on pancreases from individuals in the JDRF Network for Pancreatic Organ Donors revealed significant variability in the proportion of residual insulin-containing islets (ICIs) in individuals with recent-onset disease [27]. Histopathological evaluation of donors with recent-onset type 1 diabetes showed that insulinitis differs according to age, suggesting the existence of two type 1 diabetes endotypes (T1DE): T1DE1, with onset at age <13 years, characterised by CD20^{high} insulinitis and with islets rich in CD8⁺ T cells, with few residual ICIs, and evidence of abnormal insulin processing in the remaining beta cells, and T1DE2, with onset at age \geq 13 years, characterised by CD20^{low} insulinitis with fewer CD8⁺ T cells, more residual ICIs, and normal insulin processing [28–30]. Hence, there is a definite need to develop genetic testing that is able to effectively diagnose type 1 diabetes, differentiate it from other forms of diabetes, and perhaps identify disease endotypes.

Recently, several type 1 diabetes genetic risk scores (GRS) have been developed based on SNPs in HLA and non-HLA type 1 diabetes risk genes (approximately 30–40 SNPs). Such scores are able to discriminate type 1 diabetes from type 2 diabetes, monogenic diabetes from type 1 diabetes, and MAID from early-onset type 1 diabetes associated with poly-autoimmunity [31, 32]. The type 1 diabetes GRS is much lower in individuals with no islet AAbs at disease onset, especially in those with MODY [33]. Thus, the absence of islet AAb at disease onset and a low type 1 diabetes GRS are good indicators of MODY and monogenic forms of diabetes. However, some patients have a single and/or low-titre islet AAbs at disease onset, and there are no recommendations as to whether they should be genetically tested. The aim of the present pilot study was to identify the discriminatory power of a type 1 diabetes GRS in identifying monogenic forms of

diabetes in a cohort of 142 diabetic patients with either multiple AAbs, a single AAb or no evidence of autoimmune diabetes (islet AAbs). The patients' DNA was sequenced using a next-generation sequencing (NGS) panel, and analysed using a pipeline of more than 200 diabetes-associated genes, including those known to cause MODY, PNDM, MAID, primary immunodeficiency and diabetes-associated syndromes.

Methods

Study population Individuals with diabetes were recruited retrospectively from patients referred to San Raffaele Hospital between 2013 and 2021 (see electronic supplementary material [ESM] Fig. 1) who met the following criteria: a diagnosis of hyperglycaemia (i.e. fasting plasma glucose concentration \geq 7.0 mmol/l or random venous plasma glucose concentration \geq 11.1 mmol/l or a plasma glucose concentration \geq 11.1 mmol/l 2 h after an OGTT) at paediatric or adult age and no reported prior family history of monogenic diabetes. The selected patients included 73 patients with multiple islet AAbs, 27 individuals who were positive for a single islet AAb, and 42 without islet AAbs. General characteristics of study participants are reported in ESM Table 1. Of the 142 individuals included in our study, 65 were female (45.8%). DNA samples (saliva or peripheral blood) were collected, and clinical characteristics and symptoms at diagnosis were obtained from medical records. The DNA from 41 AAb-negative patients, 26 single AAb-positive patients and 60 multiple AAb-positive patients was sequenced by NGS. Genetic data from 330 non-diabetic individuals were included as control.

AAb analysis Analysis of IAA, GADA, IA-2A and ZnT8A was performed centrally at the Autoimmunity Laboratory of San Raffaele Hospital using either commercial kits (RSR, Cardiff, UK) or in-house luciferase immunoprecipitation systems (LIPS) and radiobinding assay (RBA) tests. The cut-off for positivity was set in each test as indicated by the manufacturer for commercial kits or to the 99th percentile of values measured in a cohort of blood donors.

C-peptide measurement Serum C-peptide levels in random non-fasting blood samples were measured at San Raffaele Hospital using standard methods.

Calculation of the type 1 diabetes GRS We calculated a type 1 diabetes GRS, as a measure of an individual's genetic susceptibility to type 1 diabetes, by typing 30 common genetic variants associated with type 1 diabetes [31]. DNA was extracted from saliva or peripheral blood samples using QIAamp DNA

Mini and Blood Mini kits (Qiagen, Germany) or the Maxwell RSC automatic extractor (Promega, USA) according to the manufacturer's instructions. The analysis of four SNPs associated with HLA risk haplotypes DR3 and DR4-DQ8 was performed by droplet digital PCR (Bio-Rad, USA). The remaining 26 SNPs located in other susceptibility genes were typed using the high-throughput OpenArray system (Life Technologies, USA) [34]. The score was generated by summing the effective allele dosage for each variant multiplied by the natural log (\log_e) of the odds ratio as previously described [31].

Next-generation sequencing Whole-exome sequencing was performed in 67 samples of genomic DNA (ESM Fig. 2) using a Nextera DNA Flex for Enrichment kit (Illumina, USA) and xGen Exome Hybridization panel version 1.0 probes (Integrated DNA Technologies, USA), according to the manufacturer's instructions. The libraries were sequenced on the NovaSeq 6000 platform (2×100 bp) (Illumina). Clinical exome-targeted enrichment sequencing of genomic DNA, including more than 4800 genes, was performed on 60 samples using TruSight One (Illumina) (ESM Fig. 2). Targeted resequencing including exon–intron 50-base boundaries was performed on the Illumina NextSeq500 platform, according to the manufacturer's protocol, with paired-end 300-cycle sequencing.

Reads obtained by whole-exome sequencing were aligned using the BWA-MEM aligner version 0.7.15 (<https://github.com/lh3/bwa>) on the hg19 reference genome [35]. Annotation was performed using databases such as dbSNP version 151 [36] and dbNSFP version 4.0a [37, 38]. Selection of genes associated with hereditary forms of diabetes, type 1 diabetes-associated polymorphisms and immune-mediated diseases was performed *in silico* and included the 212 genes listed in ESM Table 2. Functional effect prediction was performed using SnpEff software version 4.3t [39], and only variants with moderate or high impact were considered, together with those that had an effect involving a splice site. Read alignment and variant calling in clinical exome-sequenced samples were performed using BaseSpace Onsite (Illumina), and then variants were annotated using Variant Studio (Illumina). BAM files were visualised using Integrative Genome Viewer software (<http://broadinstitute.org/software/igv/>). Among the called variants, all the common variants with a MAF $>1\%$ in the GnomAD database (<https://gnomad.broadinstitute.org/>) [40] were filtered out. Classification of variants with respect to their clinical significance and according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology standards [41] was performed based on evidence from genomic and locus-specific databases, *in silico* predictions and experimental data in the literature. Variants of unknown significance (class 3), those that are probably

pathogenetic (class 4) or pathogenetic (class 5) were included in the study and confirmed by Sanger sequencing.

Ethics approval The study was approved by the San Raffaele Hospital Ethics Board (study titles: Gene T1D and TIGET004-DRI003). All participants and parents of participants who were less than 18 years old provided a signed informed consent for participation in the study.

Statistical analysis All analyses were performed using R software version 4.2.1 (<https://www.R-project.org>) [42]. One-way ANOVA was used to compare the means of continuous variables across multiple groups (>2), e.g. AAb groups (with or without the combination of family history and other autoimmune diseases). The post hoc Tukey honest significant differences test was used for comparisons across two groups, with correction of p values for the number of comparisons between groups. To compare the frequency of categorical variables across groups, Fisher's exact test was used.

Results

AAb-negative diabetic individuals with low type 1 diabetes GRS have pathogenetic class 4/5 variants Over a period of 8 years (2013–2021), 810 children newly diagnosed with diabetes have been referred to the Pediatric Diabetology Unit of the Pediatrics and Neonatology Department of San Raffaele Hospital. Individuals suspected of having type 1 diabetes underwent AAb testing for four major islet AAbs (IAA, IA-2A, GADA and ZnT8A) as a part of routine clinical care. The majority of patients with diabetes (83.3%) were diagnosed as having T1Da (one or more AAb), with the remainder being diagnosed with T1Db (no AAbs, 7.8%), type 2 diabetes (2.5%), MODY (2.2%), PNDM (0.6%) and other types (e.g. Wolfram syndrome, 0.6%) (ESM Fig. 1a). Within the paediatric cohort suspected of having type 1 diabetes, 89 had a single islet AAb at disease onset (ESM Fig. 1b).

In this pilot study, we randomly selected 69 individuals with weak evidence of autoimmunity, i.e. AAb-negative ($n=42$) or single AAb-positive (one AAb, $n=27$), irrespective of age at disease onset. Two additional cohorts of individuals diagnosed with type 1 diabetes at a paediatric age (<18 years) with multiple AAbs ($n=73$) or not diabetic ($n=330$) were used as controls. The characteristics of the diabetic patients are provided in ESM Table 1. All 142 diabetic patients were analysed using the type 1 diabetes GRS, and 127 (89.4%) were also analysed using NGS (either clinical or whole-exome sequencing) (ESM Fig. 2).

No significant differences in sex or age at disease onset were observed across the three groups (ESM Fig. 3a, b).

Children who were multiple AAb-positive showed a similar prevalence of all AAbs except for IAA (GADA 85%; IA-2A 75%; ZnT8A 81%; IAA 52%), while most individuals with a single AAb were positive for GADA (63%) followed by IA-2A, ZnT8A and IAA (7%, 15% and 11%, respectively) (ESM Fig. 3c, d). The type 1 diabetes GRS was calculated as previously described [31], and was significantly different across the three groups ($p=4.4 \times 10^{-8}$; ANOVA) (Fig. 1a). AAb-negative participants had a lower type 1 diabetes GRS compared with both single and multiple AAb-positive patients (adjusted p values =0.00008 and $<10^{-7}$, respectively; ANOVA post hoc tests) (Fig. 1a). AAb-negative participants showed also higher residual C-peptide levels at onset compared with multiple AAb-positive patients (adjusted p value =0.0003; ANOVA post hoc tests) (Fig. 1b), in line with previous reports [33, 43].

A genetic pipeline comprising genes causing MODY, PNDM, MAID, primary immunodeficiency, syndromic diabetes-associated diseases and type 1 diabetes risk genes identified in genome-wide association studies (GWAS), was

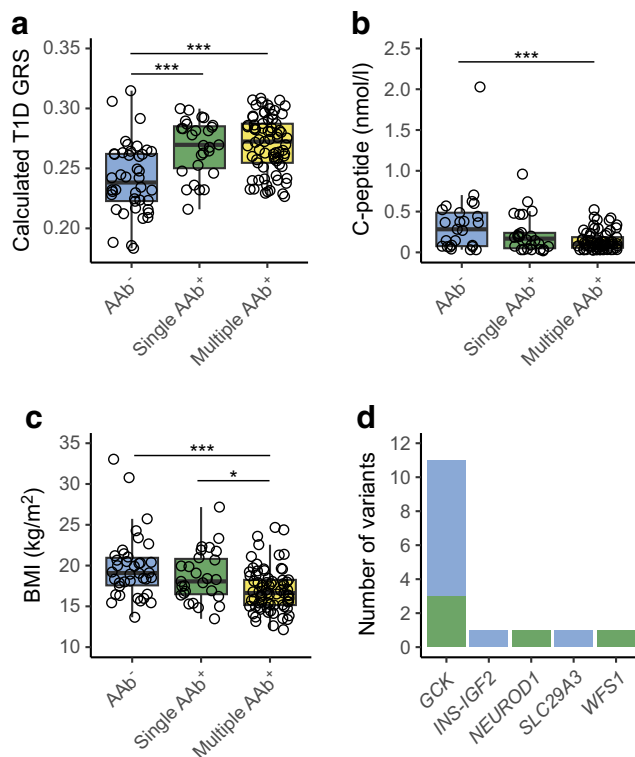


Fig. 1 AAb-negative individuals show a lower type 1 diabetes GRS, with higher BMI and residual C-peptide levels at onset, and in several cases show a clear genetic cause for their diabetes. Patients were classified as AAb-negative (AAb⁻, $n=42$), single AAb-positive (single AAb⁺, $n=27$) and multiple AAb-positive (multiple AAb⁺, $n=73$). (a) Calculated type 1 diabetes GRS; (b) residual C-peptide levels at onset; (c) BMI; (d) the number of class 5 variants (green bars) and class 4 variants (blue bars) found in each reported gene among AAb-negative patients. Circles indicate values for each patient; boxplots show the median and IQR for each group. * $p<0.05$, *** $p<0.001$ across two groups as shown. T1D, type 1 diabetes

used to genetically screen 127 samples (ESM Table 2). Class 4 and 5 pathogenetic variants in MODY genes and syndromic diabetes genes were identified in 15/41 AAb-negative individuals (36.6%; one adult and 14 children). The MODY subtypes identified were: MODY type 2 (*GCK*) in 11 patients (26.8%), MODY type 10 (*INS*) in one patient (2.4%) and MODY type 6 (*NEUROD1*) in one patient (2.4%) (Fig. 1d and Table 1). Pathogenetic variants in the *SLC29A3* and *WFS1* syndromic genes were identified in two individuals (Fig. 1c and Table 1), with the latter having two *WFS1* variants in compound heterozygosity. Among the AAb-negative patients, 17/41 (41.5%) had class 3 variants of unknown significance. In 13 of these patients (three adults, ten children), class 3 variants were present in the absence of class 4/5 variants. In seven patients, class 3 variants were present in more than one gene (Table 1). No significant differences in the type 1 diabetes GRS, age at onset and residual C-peptide levels were observed between AAb-negative patients with no variants and those with class 3 or class 4/5 variants (ESM Fig. 4).

AAb-negative and single and multiple AAb-positive individuals were further divided according to their family history of diabetes and the presence of an additional autoimmune disease (ESM Table 3). The type 1 diabetes GRS showed an almost linear median increase from AAb-negative individuals to those with multiple AAbs having a family history for type 1 diabetes and/or additional autoimmunity (ESM Fig. 5a). A lack of type 1 diabetes HLA risk alleles (absence of either DR3 or DR4-DQ8, X/X) was observed in AAb-negative individuals, particularly those without a family history for type 1 diabetes and/or an additional autoimmunity (ESM Fig. 5b). In summary, AAb-negative individuals without a family history of diabetes and/or additional autoimmunity had mutations that are rare in the general population, in genes that predominantly cause MODY.

Single AAb-positive diabetic individuals test positive for monogenic causes of diabetes

When AAb-positive individuals were divided into those with family history for type 1 diabetes and/or the presence of additional autoimmunity, no differences were observed in the distribution of islet AAbs (ESM Fig. 6). Most single AAb-positive individuals had islet AAb levels that were much lower than those observed in multiple AAb-positive children with type 1 diabetes (ESM Fig. 7). When single AAb-positive individuals were genetically tested by NGS, pathogenetic variants (class 5), probably pathogenetic variants (class 4) and variants of unknown significance (class 3) were identified in 12/26 individuals (46.2%) (Fig. 2a and Table 2). MODY class 5 pathogenetic variants were identified in 2/26 patients (7.7%), comprising one case of MODY type 3 (*HNF1A*) and one case of *HNF1B* MODY (a gene that is also associated with type 2 diabetes). Interestingly, the individual with *HNF1B* MODY had a large

Table 1 Pathogenetic (class 4/5) and candidate (class 3) variants in AAb-negative patients

Patient	Gene	DNA sequence change	Amino acid change	Effect	Class
1	<i>DROSHA</i>	NM 013235.4:c.1883C>T	NP 037367.3:p.Thr628Met	Missense variant	3
	<i>GCK</i>	NM 033507.1:c.1115G>T	NP 277042.1:p.Cys372Phe	Missense variant	4
2	<i>AFF3</i>	NM 001025108.1:c.1261G>A	NP 001020279.1:p.Ala421Thr	Missense variant, splice region variant	3
	<i>ATG7</i>	NM 006395.2:c.1588G>A	NP 006386.1:p.Ala530Thr	Missense variant	3
	<i>GLIS3</i>	NM 001042413.1:c.232C>T	NP 001035878.1:p.Arg78Cys	Missense variant	3
	<i>LYST</i>	NM 000081.3:c.3286A>G	NP 000072.2:p.Ser1096Gly	Missense variant	3
	<i>MYC</i>	NM 002467.4:c.64T>C	NP 002458.2:p.Phe22Leu	Missense variant	3
	<i>PRF1</i>	NM 005041.4:c.368G>A	NP 005032.2:p.Arg123His	Missense variant	3
	<i>STK39</i>	NM 013233.2:c.430G>A	NP 037365.2:p.Gly144Ser	Missense variant, splice region variant	3
	<i>TMPRSS6</i>	NM 153609.2:c.1033C>A	NP 705837.1:p.Leu345Ile	Missense variant	3
3	<i>INS-IGF2</i>	NM 001042376.2:c.153delA	NP 001035835.1: p.Lys53ArgfsTer39	Frameshift variant	4
4	<i>CACNA1C</i>	NM 199460.2:c.911T>C	NP 955630.2:p.Ile304Thr	Missense variant	3
	<i>THADA</i>	NM 022065.4:c.5221G>A	NP 071348.3:p.Glu1741Lys	Missense variant	3
	<i>UNC13D</i>	NM 199242.2:c.610A>G	NP 954712.1:p.Met204Val	Missense variant	3
5	<i>GCK</i>	NM 033507.1:c.109C>T	NP 277042.1:p.Arg37Trp	Missense variant	4
6	<i>GCK</i>	NM 033507.1:c.686C>T	NP 277042.1:p.Thr229Met	Missense variant	5
7	<i>GCK</i>	NM 033507.1:c.686C>T	NP 277042.1:p.Thr229Met	Missense variant	5
8	<i>ABCC8</i>	NM 000352.3:c.2483A>G	NP 000343.2:p.Asn828Ser	Missense variant	3
	<i>GCK</i>	NM 033507.1:c.784G>A	NP 277042.1:p.Gly262Arg	Missense variant	4
9	<i>GCK</i>	NM 033507.1:c.1023-1G>A		Splice acceptor variant	4
10	<i>WFS1</i>	NM 006005.3:c.316-1G>A		Splice acceptor variant	5
	<i>WFS1</i>	NM 006005.3:c.757A>T	NP 005996.2:p.Lys253Ter	Stop gained	5
11	<i>BLK</i>	NM 001715.2:c.391C>T	NP 001706.2:p.Arg131Trp	Missense variant	3
	<i>GCK</i>	NM 033507.1:c.565G>A	NP 277042.1:p.Ala189Thr	Missense variant	4
12	<i>BLK</i>	NM 001715.2:c.974A>C	NP 001706.2:p.Lys325Thr	Missense variant	3
	<i>PRKDC</i>	NM 006904.6:c.3112A>G	NP 008835.5:p.Lys1038Glu	Missense variant	3
	<i>WFS1</i>	NM 006005.3:c.2390-2392dupACG	NP 005996.2:p.Asp797dup	In-frame insertion	3
13	<i>IFIH1</i>	NM 022168.3:c.2807+1G>A		Splice donor variant	3
	<i>KLF11</i>	NM 003597.4:c.546C>A	NP 003588.1:p.Ser182Arg	Missense variant	3
	<i>RFX6</i>	NM 173560.3:c.2039C>A	NP 775831.2:p.Thr680Lys	Missense variant	3
14	<i>GCK</i>	NM 033507.1:c.110G>C	NP 277042.1:p.Arg37Pro	Missense variant	4
15	<i>WFS1</i>	NM 006005.3:c.461G>A	NP 005996.2:p.Gly154Asp	Missense variant, splice region variant	3
	<i>WFS1</i>	NM 006005.3:c.961A>C	NP 005996.2:p.Thr321Pro	Missense variant	3
16	<i>GCK</i>	NM 033507.1:c.1090G>A	NP 277042.1:p.Asp364Asn	Missense variant	3
	<i>SLC29A3</i>	NM 018344.5:c.1309G>A	NP 060814.4:p.Gly437Arg	Missense variant	4
17	<i>WFS1</i>	NM 006005.3:c.1294C>G	NP 005996.2:p.Leu432Val	Missense variant	3
18	<i>GLIS3</i>	NM 001042413.1:c.893C>A	NP 001035878.1:p.Ser298Tyr	Missense variant	3
	<i>KCNJ11</i>	NM 000525.3:c.1084G>A	NP 000516.3:p.Ala362Thr	Missense variant	3
	<i>WFS1</i>	NM 006005.3:c.1277G>A	NP 005996.2:p.Cys426Tyr	Missense variant	3
19	<i>HNF1A</i>	NM 000545.5:c.490A>C	NP 000536.5:p.Thr164Pro	Missense variant	3
20	<i>GLIS3</i>	NM 001042413.1:c.893C>A	NP 001035878.1:p.Ser298Tyr	Missense variant	3
	<i>SIRT1</i>	NM 012238.4:c.300A>C	NP 036370.2:p.Glu100Asp	Missense variant	3
21	<i>ABCC8</i>	NM 000352.3:c.2264G>C	NP 000343.2:p.Arg755Pro	Missense variant	3
22	<i>GCK</i>	NM 033507.1:c.995-1022+6del AGACGCGCTTCGTGTCGCA GGTGGAGAGGTGTGC		Splice donor variant, coding sequence variant, intron variant	5
23	<i>GCK</i>	NM 033507.1:c.1115G>T	NP 277042.1: p.Cys372Phe	Missense variant	4
24	<i>GCK</i>	NM 033507.1:c.504G>A	NP 277042.1:p.Trp168Ter	Stop gained	4
25	<i>WFS1</i>	NM 006005.3:c.2563T>C	NP 005996.2:p.Ser855Pro	Missense variant	3

Table 1 (continued)

Patient	Gene	DNA sequence change	Amino acid change	Effect	Class
26	<i>NEUROD1</i>	NM_002500.4:c.616dupC	NP_002491.2:p.His206ProfsTer38	Frameshift variant	5
27	<i>AIRE</i>	NM_000383.3:c.1322C>T	NP_000374.1:p.Thr441Met	Missense variant	3
	<i>INS</i>	NM_001042376.2:c.56C>T	NP_001035835.1:p.Pro19Leu	Missense variant	3
28	<i>PDX1</i>	NM_000209.3:c.509A>G	NP_000200.1:p.Tyr170Cys	Missense variant	3

Variants are grouped by patient and the corresponding change in the cDNA and putative protein reference sequence is indicated (Human Genome Variation Society nomenclature; varnomen.hgvs.org). The effect of each variant and the American College of Medical Genetics and Genomics/Association for Molecular Pathology classification are shown (class 3, unknown clinical significance; class 4, probably pathogenetic; class 5, pathogenetic)

deletion (exons 1–8) in *HNF1B* and two additional variants in the PNDM genes *ABCC8* (class 4) and *GATA6* (class 3). This patient had an age at onset of 39 years and positivity for ZnT8A. Diabetes in the patient with the *HNF1A* mutation appeared at 13 years of age in the presence of GADA only. Class 3 variants in genes such as *IFIH1*, *GCKR*, *UCP2* and *SH2B3*, four genes that are associated with diabetes risk, were identified in 11/26 (42.3%) single AAb-positive patients. Two patients had variants in *AIRE*, one in association with variants in the *IL6* gene; one patient had variants in the *GLIS3* and *SLC5A1* genes; one patient had a variant in *RFX6*, a beta cell gene; one patient had a variant in *KLF11*, a MODY gene; one patient had a variant in *SLC29A3*, a gene previously described in an individual with syndromic diabetes [44] (Table 2). Of

note, the class 3 variants identified in single AAb-positive diabetic individuals were either absent in the 330 non-diabetic individuals or had a low allele frequency (allele frequency range 0.45–0.9%) (Table 2).

The type 1 diabetes GRS in single AAb-positive patients with variants of class ≥ 3 was generally high, and comparable to that observed in single AAb-positive individuals without variants and in multiple AAb-positive patients (Fig. 2b). Interestingly, the two individuals with a single AAb and pathogenetic MODY mutations showed the lowest type 1 diabetes GRS (< 0.27). No significant differences were found in the age at disease onset or the residual C-peptide levels between the single AAb-positive group (with or without class ≥ 3 variants) and multiple AAb-positive individuals (Fig. 2c, d).

Table 2 Pathogenetic (class 4/5) and candidate (class 3) variants in single AAb-positive patients

Patient	Gene	DNA sequence change	Amino acid change	Effect	Class	Frequency n (%)
1	<i>SH2B3</i>	NM_005475.2:c.772C>T	NP_005466.1:p.Gln258Ter	Stop gained	3	0
2	<i>HNF1A</i>	NM_000545.5:c.1028_1029delCA	NP_000536.5:p.Thr343SerfsTer75	Frameshift variant	5	0
3	<i>GLIS3</i>	NM_001042413.1:c.1994G>A	NP_001035878.1:p.Ser665Asn	Missense variant	3	0
	<i>SLC5A1</i>	NM_000343.3:c.1693A>G	NP_000334.1:p.Asn565Asp	Missense variant	3	0
4	<i>RFX6</i>	NM_173560.3:c.1733G>C	NP_775831.2:p.Arg578Pro	Missense variant	3	6 (0.9)
5	<i>KLF11</i>	NM_003597.4:c.673A>C	NP_003588.1:p.Ser225Arg	Missense variant	3	0
6	<i>AIRE</i>	NM_000383.3:c.827A>G	NP_000374.1:p.Gln276Arg	Missense variant	3	0
7	<i>GCKR</i>	NM_001486.3:c.1618C>T	NP_001477.2:p.Arg540Ter	Stop gained	3	3 (0.45)
	<i>UCP2</i>	NM_003355.2:c.427C>T	NP_003346.2:p.Arg143Ter	Stop gained	3	0
8	<i>AIRE</i>	NM_000383.3:c.718G>A	NP_000374.1:p.Gly240Ser	Missense variant	3	0
	<i>IL6</i>	NM_000600.3:c.17C>T	NP_000591.1:p.Thr6Ile	Missense variant, splice region variant	3	0
9	<i>GATA4</i>	NM_002052.3:c.73G>A	NP_002043.2:p.Ala25Thr	Missense variant	3	0
10	<i>IFIH1</i>	NM_022168.3:c.2807+1G>A		Splice donor variant	3	3 (0.45)
11	<i>SLC29A3</i>	NM_018344.5:c.419C>T	NP_060814.4:p.Thr140Met	Missense variant	3	0
12	<i>ABCC8</i>	NM_000352.3:c.4055G>A	NP_000343.2:p.Arg1352His	Missense variant	4	0
	<i>GATA6</i>	NM_005257.4:c.1774C>G	NP_005248.2:p.Leu592Val	Missense variant	3	0
	<i>HNF1B</i>		Deletion exons 1–8		5	0

Variants are grouped by patient and the corresponding change in the cDNA and putative protein reference sequence is indicated (Human Genome Variation Society nomenclature; varnomen.hgvs.org). The effect of each variant and the American College of Medical Genetics and Genomics/Association for Molecular Pathology classification are shown (class 3, unknown clinical significance; class 4, probably pathogenetic; class 5, pathogenetic). Frequency indicates the number (percentage) of times that allele is found in 330 control non-diabetic individuals (660 control alleles)

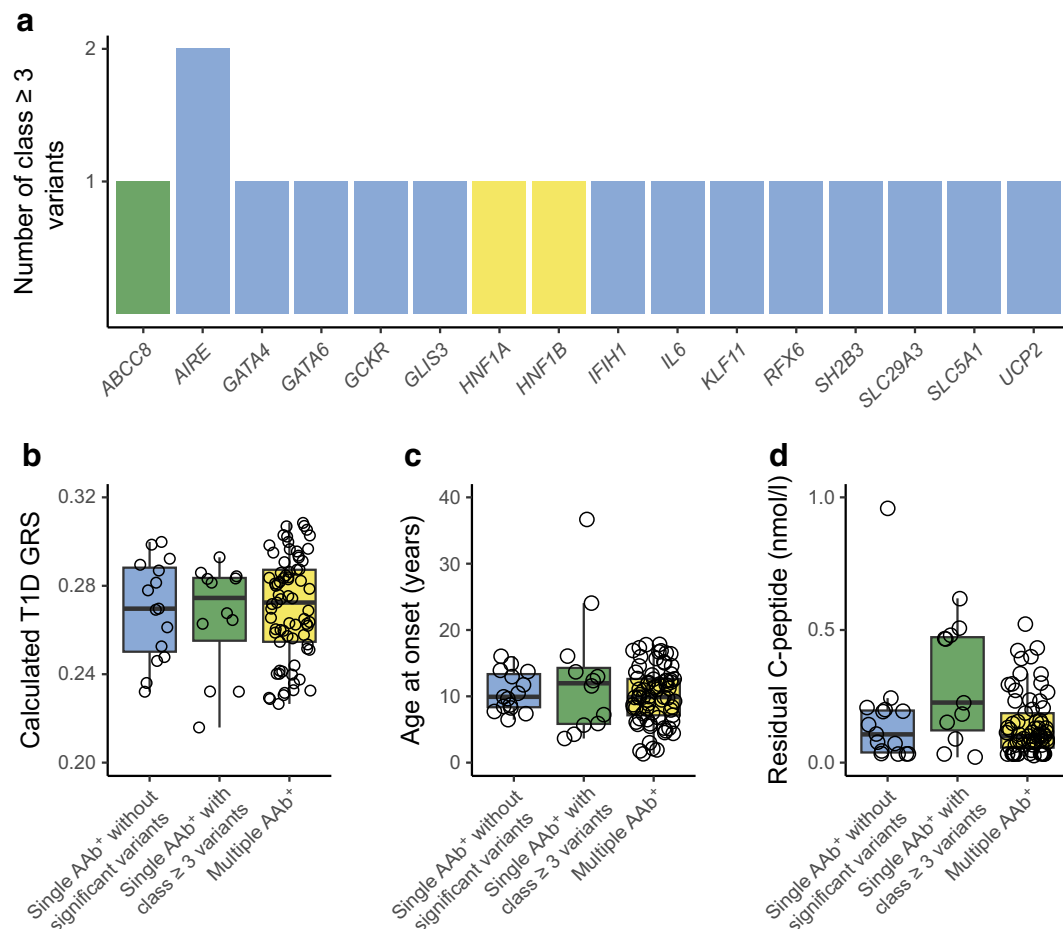


Fig. 2 Single AAb-positive patients harbour pathogenetic variants in MODY genes. Seventeen probably pathogenetic variants (class ≥ 3) were found in 12/26 single AAb-positive patients by clinical exome sequencing. Patients were stratified according to the presence of variants: single AAb-positive without a significant variant ($n=14$), single AAb-positive with at least one variant of class ≥ 3 ($n=12$) and multiple AAb-positive

($n=73$). **(a)** Number of class 5 (yellow bars), class 4 (green bar) and class 3 (blue bars) variants found in each of the indicated genes; **(b)** calculated type 1 diabetes GRS; **(c)** age at onset; **(d)** residual C-peptide levels at diabetes diagnosis. Circles indicate values for each patient; boxplots show the median and IQR for each group. T1D, type 1 diabetes

Rare class 3 variants of unknown significance are identified in multiple AAb-positive type 1 diabetes We applied our NGS pipeline in 60 multiple AAb-positive individuals diagnosed with type 1 diabetes at a paediatric age (<18 years) to assess the contribution of low-frequency and rare genetic variants to disease susceptibility. No class 4 or 5 pathogenetic variants were found, but low-frequency class 3 variants of unknown significance were identified in 19/60 individuals (31.7%), nine of whom presented with several variants in two or more genes (Fig. 3a and Table 3). Class 3 variants identified in multiple AAb-positive individuals were absent in the 330 non-diabetic individuals or had a very low allele frequency (7/34 variants, allele frequency range 0.15–1.5%) (Table 3).

Low-frequency coding variants in *CASP8* and *CASP10* genes that are associated with autoimmune lymphoproliferative syndrome [45, 46] were identified in three patients, with compound heterozygosity in one of them (Fig. 3a and Table 3). Class 3 variants of unknown significance in *LYST*, *RAG2*, *IL23R*,

UNC13D and *STIM1*, genes that are associated with primary immunodeficiency, were found in five patients (Fig. 3a and Table 3), with one patient having a variant in both *IL23R* and *UNC13D*. Class 3 variants in beta cell-associated genes, i.e. *GLIS3*, *EIF2AK3*, *SLC19A2*, *MYT1* and *SLC2A1*, were found in four patients, with one patient having a variant both in *GLIS3* and *EIF2AK3* (Fig. 3a and Table 3) [47, 48]. Class 3 variants in the type 1 diabetes genes *FUT2* and *RNLS*, identified by GWAS, and in genes that play critical roles in insulin resistance and glucose metabolism, *AKT2* and *SREBF1*, were identified in two patients for each category (Fig. 3a and Table 3) [49–51]. Some class 3 variants of unknown significance in genes that are suspected of involvement in diabetes were found, such as *SPTA1* in four patients and *DICER1* in two patients (Fig. 3a and Table 3). Except for one gene, *GLIS3*, there was no genetic overlap between the rare class ≥ 3 genetic variants identified in single and multiple AAb-positive individuals (Fig. 3b and ESM Fig. 8).

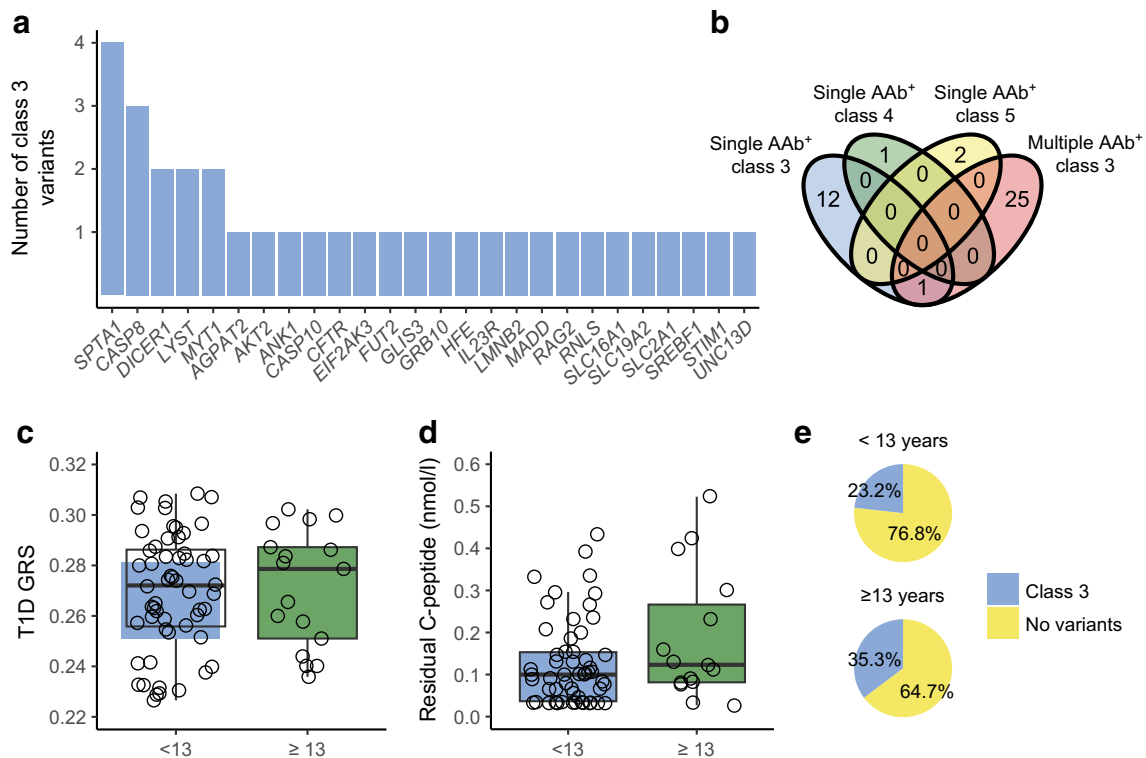


Fig. 3 Class 3 variants of unknown significance in multiple AAb-positive type 1 diabetes patients. **(a)** Number of class ≥ 3 variants found in each indicated gene in multiple AAb-positive patients analysed by NGS ($n=60$) (only class 3 variants found in this group). **(b)** Venn diagram of the class 3, class 4 and class 5 variants shared across single and multiple AAb-positive individuals. **(c, d)** Type 1 diabetes GRS **(c)** and residual C-

peptide levels **(d)** at onset of diabetes in multiple AAb-positive patients diagnosed before (blue) or after 13 years of age (green) (age <13 and age ≥ 13 being proxies of T1DE1 and T1DE2, respectively). **(e)** Pie charts showing the frequency of class 3 variants in multiple AAb-positive patients stratified by age at onset < or ≥ 13 years old. T1D, type 1 diabetes

T1DE1, an immune-driven type 1 diabetes endpoint, seems to manifest in individuals with disease onset at <13 years, while T1DE2, which is characterised by a less immune-driven and more beta cell-driven signature, manifests at an older age (≥ 13 years). To identify whether the type 1 diabetes GRS can distinguish T1DE1 from T1DE2, multiple AAb-positive individuals were separated according to their age of disease onset. No statistically significant differences were found between the type 1 diabetes GRS and residual C-peptide levels in multiple AAb-positive individuals with type 1 diabetes onset before or after 13 years of age (Fig. 3c, d). Similarly, the prevalence of class 3 variants was not significantly different between multiple AAb-positive individuals with disease onset before or after 13 years of age (23.2% vs 35.3%, respectively) (Fig. 3e).

Combined GRS/AAb algorithm to determine benefit of NGS in type 1 diabetes precision diagnosis The type 1 diabetes GRS applied in our cohort of patients discriminated polygenic type 1 diabetes from MODY, albeit with imperfect performance, as demonstrated by a receiver operating characteristic (ROC)–AUC analysis (Fig. 4a, b). Patient stratification according to GRS quartiles, as described by Patel et al [33], and AAb status

(ESM Table 4) suggested an improved algorithm for selection of patients who may benefit from an NGS analysis by including individuals with weak evidence of autoimmunity and low GRS in addition to those already recommended by current guidelines (Fig. 4c) [11].

Discussion

Type 1 diabetes is known to have a significant genetic component, as confirmed by family- and twin-based studies [52, 53]. The aetiology of type 1 diabetes in paediatric-onset multiple AAb-positive individuals is hypothesised to be driven mainly by the HLA and the compound effect of non-HLA SNPs identified by GWAS. These genetic associations represent common and mid-frequency genetic variants with small effect sizes, and can explain only a small proportion of heritability of type 1 diabetes. Our original hypothesis was that monogenic diabetes genes or other rare pathogenetic variants may also underlie some cases of type 1 diabetes. We therefore examined the genetic background of individuals with diabetes, with a particular focus on young individuals who presented with a

Table 3 Candidate (class 3) variants in multiple AAb-positive patients

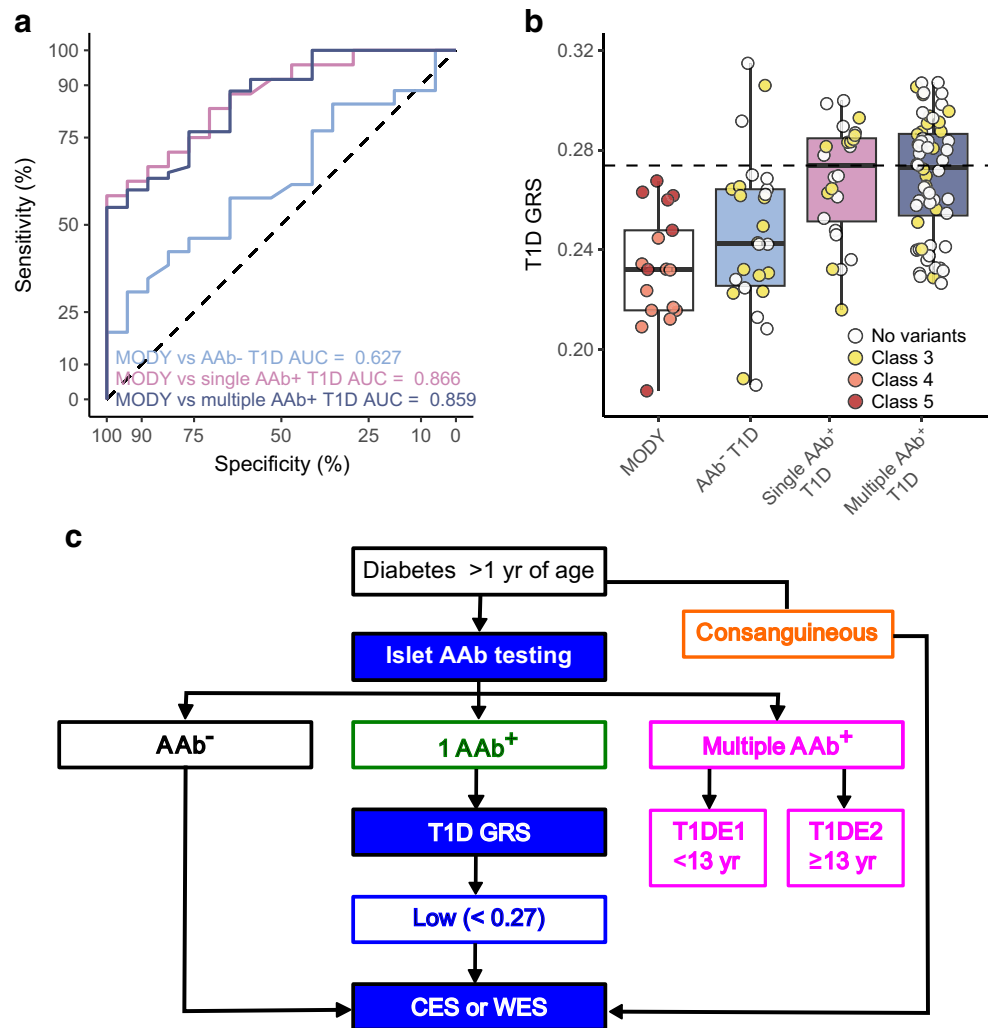
Patient	Gene	DNA sequence change	Amino acid change	Effect	Class	Frequency n (%)
1	<i>CASP8</i>	c.298G>A	p.Ala100Thr	Missense variant	3	0
	<i>CASP8</i>	c.1069A>G	p.Ile357Val	Missense variant	3	1 (0.15)
	<i>RAG2</i>	c.677A>G	p.Asn226Ser	Missense variant	3	0
2	<i>LYST</i>	c.9017A>G	p.Lys3006Arg	Missense variant	3	0
	<i>SPTA1</i>	c.4697T>C	p.Ile1566Thr	Missense variant	3	0
3	<i>GRB10</i>	c.314C>T	p.Pro105Leu	Missense variant	3	0
4	<i>FUT2</i>	c.412C>T	p.Arg138Cys	Missense variant	3	10 (1.5)
	<i>SLC16A1</i>	c.548G>T	p.Gly183Val	Missense variant	3	0
5	<i>AKT2</i>	c.641C>A	p.Ala214Glu	Missense variant, splice region variant	3	0
6	<i>DICER1</i>	c.2435A>C	p.Gln812Pro	Missense variant, splice region variant	3	0
	<i>MYT1</i>	c.2173C>T	p.Pro725Ser	Missense variant	3	0
7	<i>SPTA1</i>	c.4580C>G	p.Ser1527Cys	Missense variant	3	0
8	<i>CASP8</i>	c.620A>G	p.Lys207Arg	Missense variant	3	0
9	<i>SLC19A2</i>	c.42_47delGGCGGC	p.Ala15_Ala16del	Disruptive in-frame deletion	3	1 (0.15)
10	<i>CASP10</i>	c.259C>T	p.Arg87Trp	Missense variant	3	0
	<i>DICER1</i>	c.5276A>G	p.Lys1759Arg	Missense variant	3	0
	<i>SPTA1</i>	c.1667T>C	p.Ile556Thr	Missense variant	3	0
	<i>SREBF1</i>	c.680C>T	p.Pro227Leu	Missense variant	3	7 (1)
11	<i>CFTR</i>	c.2991G>C	p.Leu997Phe	Missense variant, splice region variant	3	2 (0.3)
12	<i>HFE</i>	c.18G>C	p.Arg6Ser	Missense variant	3	1 (0.15)
	<i>MADD</i>	c.422C>T	p.Ser141Phe	Missense variant	3	0
	<i>SPTA1</i>	c.2762T>C	p.Ile921Thr	Missense variant	3	0
13	<i>MYT1</i>	c.1670A>G	p.Tyr557Cys	Missense variant	3	0
14	<i>ANK1</i>	c.4606C>T	p.Arg1536Cys	Missense variant	3	0
15	<i>LYST</i>	c.11122G>A	p.Val3708Met	Missense variant	3	0
16	<i>AGPAT2</i>	c.70G>T	p.Ala24Ser	Missense variant	3	0
	<i>IL23R</i>	c.770A>G	p.Tyr257Cys	Missense variant	3	0
	<i>LMNB2</i>	c.619G>A	p.Asp207Asn	Missense variant	3	0
	<i>UNC13D</i>	c.610A>G	p.Met204Val	Missense variant	3	1 (0.15)
17	<i>RNLS</i>	c.223C>T	p.Arg75Cys	Missense variant, splice region variant	3	0
	<i>STIM1</i>	c.1510A>G	p.Thr504Ala	Missense variant	3	0
18	<i>SLC2A1</i>	c.652C>T	p.Arg218Cys	Missense variant	3	0
19	<i>EIF2AK3</i>	NM_004836.5:c.719G>A	NP_004827.4:p.Arg240His	Missense variant	3	0
	<i>GLIS3</i>	NM_001042413.1:c.669G>A	NP_001035878.1:p.Met223Ile	Missense variant	3	0

Variants are grouped by patient and the corresponding change in the cDNA and putative protein reference sequence is indicated (Human Genome Variation Society nomenclature; varnomen.hgvs.org). The effect of each variant and the American College of Medical Genetics and Genomics/Association for Molecular Pathology classification are shown (class 3, unknown clinical significance). Frequency indicates the number (percentage) of times that allele is found in 330 control non-diabetic individuals (660 control alleles)

single or no islet AAbs at the time of disease diagnosis in comparison with patients with the classical multiple AAb-positive phenotype. Our pilot study corroborates previous findings showing that a significant proportion of AAb-negative individuals have pathogenetic class 4/5 variants in MODY genes and also in genes associated with PNDM and syndromic diseases such as Wolfram syndrome [14, 54]. Several of these AAb-negative patients showed no signs of additional autoimmunity, presented with elevated C-peptide

levels, and later were confirmed to have MODY despite the lack of a family history of monogenic diabetes. Moreover, our study shows that genetic testing of single AAb-positive individuals can identify MODY or other monogenic causes of diabetes in approximately 8% of such patients. Of note, the single AAb-positive individuals with MODY pathogenetic variants had a rather low type 1 diabetes GRS. On the other hand, no class 4/5 pathogenetic variants were identified in multiple AAb-positive individuals with paediatric onset of

Fig. 4 Development of a combined GRS/AAb algorithm to recommend NGS for precision diagnosis of type 1 diabetes. Patients were stratified according to the presence of class 4/5 mutations in MODY genes and the number of AAbs. **(a)** ROC curve analysis of GRS discriminates autoimmune, polygenic type 1 diabetes from MODY with acceptable performance. **(b)** Distribution of GRS and mutations/variants in new-onset patients with type 1 diabetes. **(c)** Proposed algorithm for selection of patients with potential monogenic diabetes for NGS analysis. Genetic testing is recommended in all individuals with diabetes onset at <1 year of age and/or consanguineous parents, those without islet AAbs or markers of type 2 diabetes at disease onset, and patients with one type 1 diabetes AAb and a GRS below the median for single AAb-positive individuals (i.e. <0.274). CES, clinical exome sequencing; T1D, type 1 diabetes; WES, whole exome sequencing; yr, year



diabetes. The inclusion of class 3 variants of unknown significance in the analysis provided additional information on the possible underlying genetic predisposition to type 1 diabetes. Across all patients and irrespective of AAb status, rare class 3 variants were identified in immune and beta cell genes that may predispose to diabetes. Hence, our genomic approach provides support for the concept that an oligogenic cause of type 1 diabetes may be present in a proportion of single and multiple AAb-positive individuals.

The originality and strength of our study stem from the fact that patients were sequenced for more than 4800 genes, with a high coverage (median 103 ×), and the gene discovery pipeline included 212 genes previously associated with various forms of diabetes, including MODY, PNDM, MAID, primary immunodeficiency and syndromic diseases, as well as genes predisposing to type 1 diabetes that were previously identified in GWAS. One of the limitations of our retrospective study was the fact that only a proportion of the patients who presented with type 1 diabetes at our institute were included, either because of a lack of archived biological samples, which are

necessary for NGS, or gaps in the clinical and AAb diagnostic records. Moreover, TSPAN7A, a recently discovered type 1 diabetes AAb [6], was not tested as part of routine diagnostics, suggesting that some patients may have been misclassified as AAb-negative. Nevertheless, our results identified syndromic conditions in individuals who were negative for two or more islet AAbs at disease onset, reinforcing previous hypotheses and suggesting new hypotheses. Importantly, some of the patients diagnosed with monogenic diabetes were switched to the recommended clinical management according to their mutation type. Based on data obtained from this and previous studies, we consider that strong islet seropositivity to more than one AAb at onset is a reasonable exclusion criterion for progressing to genetic testing in a person with diabetes. While we obviously advocate for testing for all four major AAbs in all individuals at the time of disease onset, positivity for a single AAb should constitute one of the criteria for requesting calculation of a type 1 diabetes GRS, to be potentially followed by an NGS analysis. The cost of genotyping individual SNPs has fallen dramatically, and calculation of a type 1

diabetes GRS may soon become a relatively accessible and inexpensive tool to aid disease diagnosis. Our present study adds to previous studies of genetic screening based on a GRS, and underlies the need to include individuals with single AAB positivity in the pipeline for identification of patients affected by monogenic forms of diabetes.

Similarly to type 2 diabetes, rare genetic variants may also contribute to disease risk and pathogenesis in autoimmune diabetes, and are probably distributed across the coding and non-coding regions of numerous genes [55]. In our small cohort of multiple AAB-positive patients, disease susceptibility was probably mostly driven by the individuals' HLA and known GWAS-based genetic risk loci. However, the exome signals we identified may include additional small contributors to autoimmune diabetes risk and/or pathogenesis. Despite its relatively small size, our study is a first step towards the discovery of rare genetic underpinnings of a complex disease and their potential association with pathophysiological mechanisms underlying disease endotypes. We believe that some of our observations are interesting and hopefully will stimulate further research in this direction. Although this will probably require study of extremely large cohorts of samples, it may lead to identification of unique causative factors and novel therapeutic targets.

Taken together, our study contributes to the roadmap for identifying diabetic patients who are suitable for genetic testing to define diabetes type. This will lead to more appropriate disease management and treatment, with an impact on quality of life. Furthermore, the healthcare system will benefit from reductions in the cost of insulin treatment and patient monitoring.

Supplementary Information The online version of this article (<https://doi.org/10.1007/s00125-022-05865-5>) contains peer-reviewed but unedited supplementary material.

Data availability The datasets generated and/or analysed during the current study are available from the corresponding authors on reasonable request.

Funding This study was supported by JDRF Innovative Award 1-INO-2019-789-S to GFo.

Authors' relationships and activities The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement PC, IM, MF, TJ, LM, SC, SBondesan, EZ, NC and SBonfiglio contributed to the acquisition, analysis and interpretation of genetic data. VDG, CB, VF, AL, AC, GFr, AR, CM, MS, FS, VC, GB, AS, AP, MPC and FU contributed to the acquisition, analysis and interpretation of clinical data. IM, VL and GFo drafted the article. PC, EB, RB, MB, AA, MS, LP, VL and GFo contributed to the conception and design of the study and revised it critically. All authors contributed to revising the article and approved the final version of the manuscript. GFo takes full responsibility for the work as a whole, including the study design, access to data and the decision to submit and publish the manuscript.

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