

Common variants in the *TCF7L2* gene help to differentiate autoimmune from non-autoimmune diabetes in young (15–34 years) but not in middle-aged (40–59 years) diabetic patients

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Received: 11 August 2008 / Accepted: 20 August 2008 / Published online: 7 October 2008
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Abstract

Aims/hypothesis Type 1 diabetes in children is characterised by autoimmune destruction of pancreatic beta cells and the presence of certain risk genotypes. In adults the same situation is often referred to as latent autoimmune diabetes in adults (LADA). We tested whether genetic markers associated with type 1 or type 2 diabetes could help to discriminate between autoimmune and non-autoimmune diabetes in young (15–34 years) and middle-aged (40–59 years) diabetic patients.

G. Sundkvist died in September 2006.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-008-1161-2) contains supplementary material, which is available to authorised users.

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Methods In 1,642 young and 1,619 middle-aged patients we determined: (1) *HLA-DQB1* genotypes; (2) *PTPN22* and *INS* variable-number tandem repeat (VNTR) polymorphisms; (3) two single nucleotide polymorphisms (rs7903146 and rs10885406) in the *TCF7L2* gene; (4) glutamic acid decarboxylase (GAD) and IA-2-protein tyrosine phosphatase-like protein (IA-2) antibodies; and (5) fasting plasma C-peptide.

Results Frequency of risk genotypes *HLA-DQB1* (60% vs 25%, $p=9.4\times 10^{-34}$; 45% vs 18%, $p=1.4\times 10^{-16}$), *PTPN22* CT/TT (34% vs 26%, $p=0.0023$; 31% vs 23%, $p=0.034$), *INS* VNTR class I/I (69% vs 53%, $p=1.3\times 10^{-8}$; 69% vs 51%, $p=8.5\times 10^{-5}$) and *INS* VNTR class IIIA/IIIA (75% vs 63%, $p=4.3\times 10^{-6}$; 73% vs 60%, $p=0.008$) was increased in young and middle-aged GAD antibodies (GADA)-

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positive compared with GADA-negative patients. The type 2 diabetes-associated genotypes of *TCF7L2* CT/TT of rs7903146 were significantly more common in young GADA-negative than in GADA-positive patients (53% vs 43%; $p=0.0004$). No such difference was seen in middle-aged patients, in whom the frequency of the CT/TT genotypes of *TCF7L2* was similarly increased in GADA-negative and GADA-positive groups (55% vs 56%).

Conclusions/interpretation Common variants in the *TCF7L2* gene help to differentiate young but not middle-aged GADA-positive and GADA-negative diabetic patients, suggesting that young GADA-negative patients have type 2 diabetes and that middle-aged GADA-positive patients are different from their young GADA-positive counterparts and share genetic features with type 2 diabetes.

Keywords C-peptide · *HLA-DQB1* · *INS* VNTR · Islet antibodies · *PTPN22* · *TCF7L2*

Abbreviations

DASP	Diabetes Autoantibody Standardization Program
DISS	Diabetes Incidence Study in Sweden
GADA	glutamic acid decarboxylase antibodies
IA-2A	IA-2-protein tyrosine phosphatase-like protein antibodies
ICA	islet cell antibodies
LADA	latent autoimmune diabetes in adults
RU	relative units
SNP	single nucleotide polymorphism
VNTR	variable-number tandem repeat

Introduction

Type 1 diabetes in children is mostly caused by an autoimmune process, characterised by T cell-mediated destruction of pancreatic beta cells. Common allelic variants at the HLA class II loci account for the major genetic risk in children and young adults [1–3]. Islet cell antibodies (ICA), glutamic acid decarboxylase antibodies (GADA), IA-2-protein tyrosine phosphatase-like protein antibodies (IA-2A) and insulin autoantibodies are established autoimmune markers of type 1 diabetes, but it is not clear whether they identify the same disease in young and adult diabetic patients [4–6]. It is generally thought that autoimmune diabetes in adults shows a slower progression towards insulin deficiency and no insulin requirement at diagnosis, which has led to the term latent autoimmune diabetes in adults (LADA) [7] or slowly progressing type 1 diabetes [8]. However, adult patients may also have rapid disease onset and ketoacidosis [9]. LADA is defined as GADA-positive diabetes in adults, but the definition of adult age has varied from 25 (UKPDS) to 40 years [10, 11].

Although type 1 diabetes is the predominant form of diabetes in young European diabetic patients, type 2 diabetes is increasing in young adults worldwide. *TCF7L2* is by far the most important type 2 diabetes gene to date. The mechanisms by which it increases the risk of type 2 diabetes seem to include impaired beta cell function, possibly through an impaired incretin effect [12]. No association has been reported between variants in the *TCF7L2* gene and type 1 diabetes [13].

Unfortunately, only few large studies have characterised antibody-positive diabetes in both young and middle-aged patients [11, 14, 15]. Also, to date, diagnosis of type 2 diabetes has been by exclusion, with patients not presenting with genetic and autoimmune markers of type 1 diabetes being considered to have type 2 diabetes [3, 11, 16, 17]. Apart from HLA, no other genetic markers have been used to distinguish type 1 and type 2 diabetes in young and middle-aged adults.

This study was designed to address the question of whether genetic markers (*HLA-DQB1*, *INS* variable-number tandem repeat (VNTR) alleles, *PTPN22* and common variants in the *TCF7L2* gene) can help distinguish between autoimmune and non-autoimmune diabetes in young (15–34 years) and middle-aged (40–59 years) patients.

Methods

Participants

In 1,642 of 1,824 diabetic patients (age at onset 15–34 years) from the Diabetes Incidence Study in Sweden (DISS) [3], islet antibodies (ICA, GADA, IA-2A) at diagnosis were measured. Fasting plasma C-peptide was measured at 3 to 6 months after diagnosis in 1,353 participants and *HLA-DQB1*, *INS* VNTR, *PTPN22* and the *TCF7L2* single nucleotide polymorphisms (SNPs), rs7903146 and rs10885406 were genotyped in 1,564 of these Swedish diabetic patients. In 1,619 of 1,636 diabetic patients (age at onset 40–59 years) from a local diabetes registry in southern Sweden (Scania Diabetes Registry), GADA were measured. Fasting plasma C-peptide was measured in 1,628 while *HLA-DQB1* was genotyped in 1,365 and *INS* VNTR and *PTPN22* in 1,312, and *TCF7L2* in 1,614 patients. There was a 43% (701/1,632) overlap between the current data set of middle-aged type 2 diabetic patients from the Scania Diabetes Registry and a previous paper by Cervin et al. [18]. Informed consent was obtained from all patients.

A total of 216 non-diabetic participants from the county of Skaraborg, Sweden served as controls for *HLA-DQB1* genotyping. In them, the *HLA-DQB1* locus was amplified by PCR, followed by dot-blotting on to nitrocellulose filters and hybridisation using the radioactively end-labelled

sequence-specific oligonucleotide probes, after which autoradiography was done [19].

In addition, non-diabetic control individuals (age at visit >40 years) with no family history of diabetes or antihypertensive treatment were selected from the Malmö Preventive Project [20]. A total of 1,000 controls were available for *INS* VNTR (rs689 and rs3842755) and *PTPN22* (rs2476601), and 11,923 controls for *TCF7L2* (rs7903146 and rs10885406) genotyping.

Islet cell antibodies

ICA were determined in the young adult participants by a prolonged two-colour immunofluorescence assay [21]. The detection limit for ICA was 4 Juvenile Diabetes Foundation (JDF) units for the first pancreas used in samples tested until April 1999 and 5 JDF units for the second pancreas used in samples tested from April 1999 and onwards. In the last ICA Proficiency Test (13th) our ICA assay performed with 100% sensitivity and 100% specificity (ICA is not included in the Diabetes Autoantibody Standardization Program [DASP]).

GAD 65 antibodies

In the young participants GADA were measured by a radioligand binding assay, based on human ³⁵S-labelled recombinant GAD 65 [22]. The results are presented as GADA index = 100 × (cpm of mean activity of all four measurements for sample – cpm of the negative control) / (cpm of the positive control – cpm of the negative control). A GADA index >4.6 was considered as positive (97.5 percentile of 165 non-diabetic controls aged 7–34 years). In the first DASP (2000) the GADA assay showed a sensitivity of 80% and a specificity of 96%, in the second (2002) a sensitivity of 88% and a specificity of 87%, and in the third DASP (2003) a sensitivity of 82% and a specificity of 93%.

In the middle-aged participants GADA were measured by a radioligand binding assay using ³⁵S-labelled recombinant human GAD 65 produced by in vitro transcription/translation [23]. The results are presented as relative units (RU): RU = 100 × (cpm of sample – mean cpm of three negative controls) / (cpm of a positive internal reference serum – mean cpm of three negative controls). The cut-off limit for positivity was 5 RU, which is comparable to 32 IU/ml according to the new WHO standard [24] and represents mean + 3 SD of 296 healthy control participants. In the first DASP (2000) the GADA assay showed a sensitivity of 76% and a specificity of 94%, in the second (2002) a sensitivity of 88% and a specificity of 96%, and in the third DASP (2003) a sensitivity of 88% and a specificity of 94% [25]. From the beginning of the year 2000 (in 41% of registry patients), the results were given as IU/ml [26].

IA-2-protein tyrosine phosphatase-like protein antibodies

IA-2A were measured in young adults by a similar assay based on human ³⁵S-labelled recombinant IA-2 [27]. IA-2A index >1.0 was considered as positive (97.5 percentile of 165 non-diabetic controls aged 7–34 years). In the first DASP (2000), the IA-2A assay showed a sensitivity of 58% and a specificity of 100%, in the second (2002) a sensitivity of 62% and a specificity of 100%, and in the third DASP (2003) a sensitivity of 64% and a specificity of 100%.

Plasma C-peptide

An RIA was used to determine fasting plasma C-peptide (Peninsula Laboratories, Belmont, CA, USA) in young (age 15–34 years) [3] and middle-aged (age 40–59 years) diabetic patients [26]. The reference range for healthy participants after 12 h fasting was 0.25 to 0.75 nmol/l.

HLA-DQB1 genotyping

Using a primer pair with biotinylated 3' primers, the 158 bp second exon of *HLA-DQB1* gene was amplified by PCR. The amplification product was bound to streptavidin-coated microtitration plates and denatured with NaOH. After washing, bound DNA was assessed using two different hybridisation mixtures with lanthanide (III) chelate-labelled DNA probes specific for the *HLA-DQB1* alleles. One mixture contained europium (Eu)-labelled and samarium (Sm)-labelled internal reporter probes for *DQB1*0602* and **0603* alleles (**0602–*0603*) and *DQB1*0603* and **0604* (**0603–*0604*) alleles respectively; a terbium (Tb)-labelled consensus sequence-specific probe (Tb-*DQB1* control) was used as control of PCR amplification. The other mixture contained Tb-, Sm- and Eu-labelled probes specific for *DQB1*0201*, *DQB1*0301* and *DQB1*0302* alleles, respectively. To measure probe hybridisation, microtitration plates were evaluated by time-resolved fluorescence (Delfia Research Fluorometer; Wallac OY, Turku, Finland). Different emission wavelengths and delay times were used to distinguish the signals of each lanthanide label [28]. From 1,564 genotyped DISS patients, genotyping success rate was 1,537 (98%) and from 1,365 diabetes registry patients 1,350 (99%). The risk *HLA-DQB1* genotypes include **02/*0302*, **0302/X*, **0302/*604* [3] and in the following we refer to risk *HLA-DQB1* without any further risk genotype specification.

VNTR polymorphisms in the insulin gene and in *PTPN22* and *TCF7L2* polymorphisms

Two SNPs associated with *INS* VNTR were genotyped, i.e. rs689 (–23*HphI* variant, a surrogate for the subdivision

of VNTR into class I [A] and III [T] alleles) and rs3842755, which also allows subdivision of class III into IIIA (C) and IIIB (A) alleles [29–31]. The SNP rs2476601 (also denoted 1858C/T) was genotyped in the *PTPN22* gene [32, 33]. Genotyping was performed by an allelic discrimination method (7900HT system; Applied Biosystems, Foster City, CA, USA). From young diabetic patients 1,564 DNA samples were available and the genotyping success rate was 98% for *PTPN22*, 98% for *INS* VNTR rs689 and 98% for *INS* VNTR rs3842755. In genotyped 1,312 middle-aged diabetic patients genotyping success rate was 99% for *PTPN22*, 98% for *INS* VNTR rs689 and 96% for *INS* VNTR rs3842755. Random samples (3.2%) for each SNP were re-genotyped, with reproducibility of 99.9%.

SNP rs7903146 in *TCF7L2* gene, previously shown to be associated with type 2 diabetes, and a more recently described microsatellite marker, captured by SNP rs10885406, were genotyped [34, 35].

Genotyping was performed by an allelic discrimination method (7900HT system; Applied Biosystems). In young diabetic patients 1,564 DNA samples were available and the genotyping success rate was 98% for *TCF7L2* rs7903146 and 98% for *TCF7L* rs10885406. In middle-aged diabetic patients 1,614 DNA samples were available and the genotyping success rate was 96% for *TCF7L2* rs7903146 and 99% for *TCF7L* rs10885406. Random samples (3.2%) for each SNP were re-genotyped for quality control. The T allele of *TCF7L2* rs7903146 was

in strong linkage disequilibrium with the G allele of *TCF7L2* rs10885406 ($D'=0.99$; $r^2=1.0$). (Electronic supplementary material [ESM] Table 1).

Statistical analysis

Comparison of genotype frequencies between GADA-positive and GADA-negative diabetic patients and non-diabetic controls was tested by the two-tailed Fisher's exact test or χ^2 test with Bonferroni adjustment for multiple comparisons (the normal p value of 0.05 multiplied by the number of tests being performed). Odds ratios and 95% CIs were calculated as previously described [3]. Continuous data are presented as median and 1st and 3rd quartile. The significance of differences in continuous variables between groups was assessed by the non-parametric Mann–Whitney U test. All statistical tests were performed by SPSS version 13.0 (SPSS, Chicago, IL, USA) or JMP version 5 for MAC OS X (SAS Institute, Cary, NC, USA).

Results

Comparison between antibody-positive and antibody-negative young (15–34 years old) diabetic patients

Out of 1,642 young diabetic patients, 1,013 (62%) had GADA, 829 (50%) had ICA and 716 (44%) had IA-2

Table 1 Clinical characteristics of young (15–34 years at onset) and middle-aged (40–59 years at onset) diabetic patients with and without GADA

Clinical characteristic	Young (15–34 years old)			Middle-aged (40–59 years old)		
	GADA-positive	GADA-negative	p value ^a	GADA-positive	GADA-negative	p value ^b
Men/women, n/n (% men)	588/425 (58)	309/195 (61)	0.24	104/101 (51)	916/498 (65)	0.0001
Age at onset (years)	25 (20; 30)	28 (24; 32)	<0.0001	50 (45; 55)	51 (47; 56)	0.006
BMI (kg/m ²)	22 (20; 24)	29 (23; 35)	<0.0001	25 (22; 29)	29 (26; 33)	<0.0001
Family history of diabetes, n (%)	175/777 (23)	152/355 (43)	2.8×10^{-12}	44/70 (63)	244/349 (70)	0.26
Diabetes symptoms ≤ 3 months, n (%)	372/837 (44)	161/409 (39)	0.1	NA	NA	NA
Diabetes duration ≤ 1 year, n (%)	NA	NA	NA	147/205 (72)	1,078/1,413 (76)	0.16
Insulin treatment, n (%)	934/1,004 (93)	294/500 (59)	1.1×10^{-57}	113/187 (60)	301/1,303 (23)	9.4×10^{-81}
Fasting C-peptide (nmol/l)	0.23 (0.14; 0.38)	0.70 (0.38; 1.1)	<0.0001	0.27 (0; 0.65)	0.90 (0.60; 1.3)	<0.0001
HbA _{1c} (%)	NA	NA	NA	7.7 (6.6; 8.9)	7.1 (5.9; 8.4)	<0.0001
Total cholesterol (mmol/l)	NA	NA	NA	5.0 (4.5; 5.9)	5.3 (4.6; 6.1)	0.003
HDL-cholesterol (mmol/l)	NA	NA	NA	1.3 (1.0; 1.6)	1.0 (0.8; 1.3)	<0.0001
LDL-cholesterol (mmol/l)	NA	NA	NA	3.0 (2.2; 3.6)	3.0 (1.6; 3.7)	0.55
Triacylglycerol (mmol/l)	NA	NA	NA	1.0 (0.8; 1.5)	1.8 (1.2; 2.6)	<0.0001

Patients with only IA-2A or ICA or both were excluded from analysis

Unless otherwise stated, data are shown as median (1st quartile; 3rd quartile)

^a χ^2 test p value comparing young GADA-positive with GADA-negative diabetic patients

^b χ^2 test p value comparing middle-aged GADA-positive with GADA-negative diabetic patients

NA, not available

Table 2 Prevalence of *HLA-DQB1*, *PTPN22*, *INS* VNTR and *TCF7L2* in young (15–34 years at onset) and middle-aged (40–59 years at onset) GADA-positive vs GADA-negative diabetic patients

Genotype	n (total)	Young (15–34 years)				Middle-aged (40–59 years)			
		GADA- positive	GADA- negative	<i>p</i> value ^a	<i>p</i> value ^b	GADA- positive	GADA- negative	<i>p</i> value ^c	<i>p</i> value ^d
<i>HLA-DQB1</i>	2,760								
Risk (*02/*0302,*0302/X, *0302/*604)		572 (60)	115 (25)	9.4×10^{-34}	5.7×10^{-33}	78 (45)	208 (18)	1.4×10^{-16}	8.3×10^{-16}
Non-risk		387 (40)	341 (75)			94 (55)	965 (82)		
<i>PTPN22</i> (rs2476601)	2,719								
CC		631 (66)	344 (74)	0.0023	0.014	86 (69)	908 (77)	0.034	0.2
CT + TT ^e		324 (34)	120 (26)			39 (31)	267 (23)		
<i>INS</i> VNTR (rs689)	2,700								
AA (class I/I) ^e		634 (69)	248 (53)	1.3×10^{-8}	8×10^{-8}	87 (69)	588 (51)	8.5×10^{-5}	0.0005
AT + TT (class I/III + III/III)		295 (31)	216 (47)			39 (31)	573 (49)		
<i>INS</i> VNTR (rs3842755)	2,669								
CC (class IIIA/IIIA) ^e		707 (75)	290 (63)	4.3×10^{-6}	2.6×10^{-5}	71 (73)	691 (60)	0.008	0.048
AC + AA (class IIIA/IIIB + IIIB/IIIB)		242 (25)	173 (37)			26 (27)	469 (40)		
<i>TCF7L2</i> (rs7903146)	2,948								
CC		538 (57)	217 (47)	0.0004	0.0024	88 (44)	610 (45)	0.88	NS
CT + TT ^e		407 (43)	246 (53)			109 (56)	733 (55)		
<i>TCF7L2</i> (rs10885406)	3,002								
GG ^e		183 (19)	103 (22)	0.18	NS	54 (27)	321 (23)	0.21	NS
AA + AG		773 (81)	360 (78)			144 (73)	1,064 (77)		

Patients with only IA–2A or ICA or both were excluded from analysis

Data are shown as absolute numbers (%)

^a χ^2 test *p* value comparing young GADA-positive with GADA-negative diabetic patients; ^b adjusted *p* value for multiple testing comparing young GADA-positive with GADA-negative diabetic patients; ^c χ^2 test *p* value comparing middle-aged GADA-positive with GADA-negative diabetic patients; ^d adjusted *p* value for multiple testing comparing middle-aged GADA-positive with GADA-negative diabetic patients; ^e diabetes risk genotypes

antibodies. Of the young diabetic patients, 33% had all three antibodies (GADA, ICA, IA-2A), whereas 7.6% had either ICA or IA-2A or both, but not GADA and 31% were antibody-negative.

There was no sex-related difference between young antibody-positive and antibody-negative diabetic patients (ESM Table 2). Young antibody-positive patients had lower BMI (22 vs 29 kg/m²; $p < 0.0001$), were less likely to have a family history of diabetes (22% vs 43%; $p = 2.9 \times 10^{-13}$), were more frequently treated with insulin (93% vs 59%; $p = 6.0 \times 10^{-62}$) and had lower fasting plasma C-peptide concentrations (0.24 vs 0.70 nmol/l; $p < 0.0001$) than their antibody-negative counterparts. At diagnosis, 83% of the patients were on insulin therapy; this rose to 91% one month later when information on antibody status was available.

To allow comparison with GADA-positive middle-aged patients, we restricted the analysis of the young patients to the 1,013 GADA-positive participants (89% of all 1,138

antibody-positive patients) and excluded from the analysis those who were positive for only IA-2A or ICA or both. A full analysis including even these antibodies is given as ESM Tables 3 and 4.

There was no sex-related difference between young GADA-positive and GADA-negative diabetic patients (Table 1). Young GADA-positive patients had younger age at onset (25 vs 28 years; $p < 0.0001$), lower median BMI (22 vs 29 kg/m²; $p < 0.0001$), were less likely to have a family history of diabetes (23% vs 43%; $p = 2.8 \times 10^{-12}$), were more frequently treated with insulin (93% vs 59%; $p = 1.1 \times 10^{-57}$) and had lower median fasting plasma C-peptide concentrations (0.23 vs 0.70 nmol/l; $p < 0.0001$) than their GADA-negative counterparts (Table 1).

HLA-DQB1, *PTPN22* and *INS* VNTR As expected, young GADA-positive diabetic patients had higher frequency of risk *HLA-DQB1* (60% vs 25%; $p = 9.4 \times 10^{-34}$), *PTPN22* CT/TT (34% vs 26%; $p = 0.0023$), *INS* VNTR (class I/I) AA (69% vs 53%; $p = 1.3 \times 10^{-8}$) and *INS* VNTR

Table 3 Prevalence of *HLA-DQB1*, *PTPN22*, *INS* VNTR and *TCF7L2* in young (15–34 years at onset) and middle-aged (40–59 years at onset) GADA-positive and GADA-negative diabetic patients

Genotype	n (total)	GADA-positive				GADA-negative			
		Young	Middle-aged	p value ^a	p value ^b	Young	Middle-aged	p value ^c	p value ^d
<i>HLA-DQB1</i>	2,760								
Risk (*02/*0302, *0302/X, *0302/*604)		572 (60)	78 (45)	0.0006	0.0036	115 (25)	208 (18)	0.0007	0.004
Non-risk		387 (40)	94 (55)			341 (75)	965 (82)		
<i>PTPN22</i> (rs2476601)	2,719								
CC		631 (66)	86 (69)	0.6	NS	344 (74)	908 (77)	0.2	NS
CT + TT ^e		324 (34)	39 (31)			120 (26)	267 (23)		
<i>Ins</i> VNTR (rs.689)	2,700								
AA (class I/I) ^e		654 (69)	87 (69)	1.0	NS	248 (53)	588 (51)	0.3	NS
AT + TT (class I/III + III/III)		295 (31)	39 (31)			216 (47)	573 (49)		
<i>INS</i> VNTR (rs3842755)	2,669								
CC (class IIIA/IIIA) ^e		707 (75)	71 (73)	0.8	NS	290 (63)	691 (60)	0.3	NS
AC + AA (class IIIA/IIIB + IIIB/IIIB)		242 (25)	26 (27)			173 (37)	469 (40)		
<i>TCF7L2</i> (rs7903146)	2,948								
CC		538 (57)	88 (45)	0.002	0.012	217 (47)	610 (45)	0.6	NS
CT + TT ^e		407 (43)	109 (55)			246 (53)	733 (55)		
<i>TCF7L2</i> (rs10885406)	3002								
GG ^e		183 (19)	54 (27)	0.01	0.06	103 (22)	321 (23)	0.7	NS
AA + AG		773 (81)	144 (73)			360 (78)	1,064 (77)		

Patients with only IA–2A or ICA or both were excluded from analysis

Data are absolute numbers (%)

^a χ^2 test p value comparing young GADA-positive with middle-aged GADA-positive diabetic patients; ^b adjusted p value for multiple testing comparing young GADA-positive with middle-aged GADA-positive diabetic patients; ^c χ^2 test p value comparing young GADA-negative with middle-aged GADA-negative diabetic patients; ^d adjusted p value for multiple testing comparing young GADA-negative with middle-aged GADA-negative diabetic patients; ^e diabetes risk genotypes

(class IIIA/IIIA) CC (75% vs 63%; $p=4.3 \times 10^{-6}$) genotypes than young GADA-negative patients [36–39] (Table 2).

TCF7L2 The frequency of type 2 diabetes risk genotypes CT/TT of *TCF7L2* (rs7903146) was significantly increased in young GADA-negative (53% vs 43%; $p=0.0004$) compared with GADA-positive diabetic patients. Consequently, the frequencies of the wild-type CC genotypes were increased in young GADA-positive compared with GADA-negative patients (57% vs 47%) (Table 2).

To study whether the GADA level would influence the results, we re-analysed the data after dividing the GADA-positive patients into two subgroups with GADA levels below (low GADA) or above (high GADA) the median of GADA. Both groups showed the same increase in *HLA DQB1* (59% and 60% vs 25%; $p<0.0001$ for both) and the *PTPN22* (34% and 33% vs 26%; $p=0.0044$ and $p=0.013$), *INS* VNTR (class I/I) AA (68% and 70% vs 53%; $p<0.0001$ for both) and *INS* VNTR (class IIIA/IIIA) CC (73% and 76% vs 62%; $p=0.0008$ and $p<0.0001$) polymorphisms, as well as in CC genotypes of *TCF7L2* (56% and 58% vs 47%;

$p=0.0048$ and $p=0.0011$), as seen in the whole group compared with GADA-negative patients (ESM Table 5).

Comparison between middle-aged (40–59 years old) GADA-positive and GADA-negative diabetic patients

Out of 1,619 middle-aged diabetic patients, 205 (13%) were positive for GADA. There were more women among the GADA-positive than among GADA-negative middle-aged diabetic patients (49% vs 35%; $p=0.0001$). GADA-positive patients also had lower median BMI (25 vs 29 kg/m²; $p<0.0001$), were more frequently treated with insulin (60% vs 23%; $p=9.4 \times 10^{-81}$) and had lower median fasting plasma C-peptide concentrations (0.27 vs 0.90 nmol/l; $p<0.0001$) than their GADA-negative counterparts (Table 1). GADA-positive diabetic patients in this group had lower total cholesterol (median: 5.0 vs 5.3 mmol/l; $p=0.003$), higher HDL-cholesterol (1.3 vs 1.0 mmol/l; $p<0.0001$) and lower triacylglycerol (1.0 vs 1.8 mmol/l; $p<0.0001$), but higher HbA_{1c} (7.7% vs 7.1%; $p<0.0001$) concentrations than GADA-negative patients.

HLA-DQB1, *PTPN22* and *INS VNTR* Middle-aged GADA-positive patients also had a higher frequency of risk *HLA-DQB1* (45% vs 18%; $p=1.4\times 10^{-16}$), *PTPN22* CT/TT (31% vs 23%; $p=0.034$), *INS VNTR* (class I/I) AA (69% vs 51%; $p=8.5\times 10^{-5}$) and *INS VNTR* (class IIIA/IIIA) CC (73% vs 60%; $p=0.008$) genotypes than middle-aged GADA-negative patients (Table 2).

TCF7L2 There was no difference in type 2 diabetes-associated CT/TT genotypes of the *TCF7L2* gene between middle-aged GADA-positive and GADA-negative patients (Table 2), both of whom showed a significantly higher frequency than controls (55% vs 43%; $p=1.5\times 10^{-9}$ and 24% vs 20%; $p=0.0007$) (ESM Table 6).

We also subdivided the middle-aged GADA-positive group into two subgroups with GADA levels below (low GADA) or above (high GADA) the median. Both groups showed the same increase in *HLA-DQB1* (55% and 36% vs 18%; $p<0.0001$ and $p<0.0001$) compared with GADA-negative patients. However, the frequency of *INS VNTR* (class I/I) AA (76% vs 51%; $p<0.0001$) and *INS VNTR* (class IIIA/IIIA) CC (80% vs 60%; $p=0.003$) was increased only in the high GADA compared with GADA-negative group. Notably, there was no difference in the frequency of the *TCF7L2* CC genotypes between high and low GADA and GADA-negative groups, suggesting that the increase in the type 2 diabetes-associated CC genotypes was not restricted to those with low GADA levels (ESM Table 7).

Consequently, the frequency of the *TCF7L2* risk genotypes was higher in middle-aged than in young GADA-positive diabetic patients (CT/TT: 55% vs 43%; $p=0.002$; GG: 27% vs 19%; $p=0.01$) suggesting that middle-aged GADA-positive patients have partially different genetic backgrounds (Table 3). The prevalence of common variants in the *TCF7L2* gene did not differ between young and middle-aged GADA-negative diabetic patients (Table 3).

Discussion

We provide a comprehensive genetic and clinical characterisation of antibody-positive and -negative diabetes across the age groups 15 to 59 years, and one which is not inflated by subjective attempts to classify into type 1 and type 2 diabetes. The key finding of our study was that *TCF7L2* CT/TT genotypes helped distinguish between young GADA-positive and GADA-negative patients (age 15–34 years) but not between middle-aged (40–59 years) GADA-positive and GADA-negative diabetic patients. These findings may have important implications for diagnosis of diabetic subgroups. The clinical diagnosis of young adults is not always easy and even young patients classified as having type 2 diabetes can have islet antibodies, a condition sometimes referred to as latent

autoimmune diabetes in youth (LADY) [40]. Genetically, young and middle-aged antibody-positive patients were similar with respect to increased prevalence of risk genotypes *HLA-DQB1*, *PTPN22* CT + TT, *INS VNTR* class I/I and *INS VNTR* class IIIA/IIIA. These findings are consistent with previous reports [3, 41, 42]. Although HLA genotyping together with measurements of islet antibodies has been used to identify individuals at risk of type 1 diabetes, until now no genetic markers have been available for type 2 diabetes. This has changed with the identification of the strong association between common variants in the *TCF7L2* gene and type 2 diabetes, and may change further with the identification of novel genetic variants associated with type 2 diabetes in whole-genome association studies [34, 43–46]. Therefore, addition of *TCF7L2* to HLA genotyping in young adults could clearly help to improve the prediction of diabetic subtype.

Our results in young GADA-positive patients on the frequency of SNPs in the *TCF7L2* gene differ partially from recent findings in a large cohort of patients with type 1 diabetes [13]. While both studies showed no increase in the T allele of rs7903146 in apparent type 1 diabetic patients, we actually observed an increase of the C allele in GADA-positive young diabetic patients.

A possible explanation for this discrepancy could be age at onset of diabetes, which in the quoted study was below 17 years, but in our study ranged from 15 to 34 years. Under the age of 17 years type 2 diabetes is quite rare. In fact, when we subdivided the young adult patients into two age groups (15–25 and 25–34 years), we observed a stronger discriminatory effect of variants in the *TCF7L2* gene in the older than in the younger group (ESM Table 8).

It is likely that our young GADA-negative patients do have true type 2 diabetes, as they shared the increased frequency of the type 2 diabetes-associated T allele in rs7903146 with middle-aged GADA-negative type 2 diabetic patients. In support of this, they also showed increased frequency of *INS VNTR* class III genotypes, which have previously been shown to be associated with type 2 diabetes [47, 48]. The increase of the C allele in the young GADA-positive patients could thus be a corollary of the increase of the T allele in the young GADA-negative patients. Alternatively, the C allele could in some way be associated with autoimmunity.

The *TCF7L2* gene product is part of the Wnt signalling pathway, where after binding to beta-catenin, it activates transcription of a number of genes involved in cell proliferation [49, 50]. It is not known whether, like transcription factor 7, it influences Th1 responses through crosstalk with TGF- β signalling.

Our failure to find any difference in the frequency of the type 2 diabetes-associated T allele of rs7903146 in the *TCF7L2* gene between middle-aged GADA-positive and

GADA-negative patients, while finding a significantly higher frequency than in young GADA-positive patients, indicates that autoimmune diabetes in the middle-aged is different from autoimmune diabetes in young adults. These findings complement and add to our previous paper [18] showing that frequency of the *TCF7L2* variants markedly differs between young and middle-aged autoimmune diabetes, further supporting the view that LADA represents an admixture of type 1 and type 2 diabetes. It also shows that if the age at onset for definition of LADA is lowered below 35 years, the LADA group will include an increasing number of patients with classical type 1 diabetes.

In conclusion, our findings suggest that common variants in the *TCF7L2* gene can be used together with *HLA-DQB1* genotyping to distinguish between young adults (15–34 years) with antibody-positive and antibody-negative diabetes. This is not possible in middle-aged (40–59 years) diabetic patients, suggesting that middle-aged antibody-positive patients are different from young antibody-positive patients and that autoimmune diabetes in middle-aged patients shares genetic features (common variants in the *TCF7L2* gene) with type 2 diabetes.

Acknowledgements We thank all Swedish diabetologists and diabetes nurses who contributed to DISS. M. Svenson, A. Berglund, E. Laurila, M. Svensson, C. Rosborn, U. Gustavsson, A. Radelius, G. Gremesperger and J. Pilz are acknowledged for expert technical assistance. The Juvenile Diabetes Foundation Wallenberg Diabetes Research Program (K 98-99 JD-128 13), the Swedish Diabetes Association, the Swedish Medical Research Council (72X-14531), the Albert Pahlsson Foundation, Research Fund at Malmö University Hospital are acknowledged for support of DISS and Knut & Alice Wallenberg foundation for equipment availability.

Duality of interest L. Groop has been a consultant for and served on advisory boards for Lilly, Sanofi-Aventis, GSK, Novartis and Tethys Bioscience. All other authors declare that there is no duality of interest associated with this manuscript.

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