

Predictive power of screening for antibodies against insulinoma-associated protein 2 beta (IA-2 β) and zinc transporter-8 to select first-degree relatives of type 1 diabetic patients with risk of rapid progression to clinical onset of the disease: implications for prevention trials

J. De Grijse · M. Asanghanwa · B. Nouthe ·
N. Albrecher · P. Goubert · I. Vermeulen ·
S. Van Der Meeren · K. Decochez · I. Weets ·
B. Keymeulen · V. Lampasona · J. Wenzlau ·
J. C. Hutton · D. Pipeleers · F. K. Gorus ·
the Belgian Diabetes Registry

Received: 8 June 2009 / Accepted: 5 November 2009 / Published online: 29 November 2009
© Springer-Verlag 2009

Abstract

Aims/hypothesis We investigated whether screening for insulinoma-associated protein (IA-2) beta (IA-2 β) autoantibodies (IA-2 β A) and zinc transporter-8 (ZnT8) autoantibodies (ZnT8A) improves identification of first-degree relatives of type 1 diabetic patients with a high 5-year disease risk, which to date has been based on assays for insulin autoantibodies (IAA), GAD autoantibodies (GADA) and IA-2 autoantibodies (IA-2A).

Methods IA-2 β A and ZnT8A (using a ZnT8 carboxy-terminal hybrid construct, CW-CR, carrying 325Arg and 325Trp) were determined by radiobinding assay in 409 IAA⁺, GADA⁺ and/or IA-2A⁺ siblings or offspring (<40 years) of type 1 diabetic patients consecutively recruited by the Belgian Diabetes Registry. The median (interquartile range) age of the first-degree relatives was 12 (6–19) years. **Results** Of the first-degree relatives, 24% were IA-2A⁺ ($n=97$), 14% ($n=59$) IA-2 β A⁺ and 20% ($n=80$) ZnT8A⁺.

The Diabetes Research Center, Brussels Free University–VUB is a Partner of the Juvenile Diabetes Research Foundation Center for Beta Cell Therapy in Diabetes.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-009-1618-y) contains supplementary material, including a list of the members of the Belgian Diabetes Registry, which is available to authorised users.

J. De Grijse · M. Asanghanwa · B. Nouthe · P. Goubert ·
I. Vermeulen · K. Decochez · I. Weets · B. Keymeulen ·
D. Pipeleers · F. K. Gorus (✉)
Diabetes Research Center, Brussels Free University–VUB,
Laarbeeklaan 103,
B-1090 Brussels, Belgium
e-mail: frans.gorus@uzbrussel.be

K. Decochez · B. Keymeulen
Department of Diabetology,
University Hospital Brussels Free University–UZ Brussel,
Brussels, Belgium

V. Lampasona
Center of Genomics, Bioinformatics and Biostatistics & Diabetes
Research Institute, San Raffaele Scientific Institute,
Milano, Italy

J. Wenzlau · J. C. Hutton
Barbara Davis Center for Childhood Diabetes,
University of Colorado at Denver,
Aurora, CO, USA

N. Albrecher · P. Goubert · S. Van Der Meeren · I. Weets ·
F. K. Gorus
Department of Clinical Chemistry and Radioimmunology,
University Hospital Brussels Free University–UZ Brussel,
Brussels, Belgium

IA-2 β A and ZnT8A were significantly ($p < 0.001$) associated with IA-2A and prediabetes ($n = 86$); in IA-2A⁻ first-degree relatives ($n = 312$) the presence of IA-2 β A and ZnT8A was associated with an increased progression rate to diabetes ($p < 0.001$). Positivity for IA-2A and/or ZnT8A emerged as the most sensitive combination of two markers to identify first-degree relatives with a 5-year progression rate to diabetes of 45% (survival analysis) and as strongest predictor of diabetes (Cox regression analysis). Omission of first-degree relatives protected by *HLA-DQ* genotypes or maternal diabetes reduced the group to be followed from $n = 409$ to $n = 246$ (40%) with minor loss in the number of prediabetic IA-2A⁺ or ZnT8A⁺ first-degree relatives identified ($n = 3$). **Conclusions/interpretation** IA-2A⁺ and/or ZnT8A⁺ first-degree relatives may be the participants of choice in future secondary prevention trials with immunointervention in relatives of type 1 diabetic patients.

Keywords Autoantibodies · *HLA-DQ* · IA-2 · IA-2 β · Prediction · Type 1 diabetes · Zinc transporter-8

Abbreviations

BDR	Belgian Diabetes Registry
CW-CR	ZnT8 cDNA construct generated by fusion of CR and CW (zinc transporter-8 carboxy-terminal constructs carrying 325Arg and 325Trp, respectively)
GADA	GAD autoantibodies
IAA	Insulin autoantibodies
IA-2	Insulinoma-associated protein-2
IA-2A	IA-2 autoantibodies
IA-2 β	Insulinoma-associated protein 2 beta
IA-2 β A	IA-2 β autoantibodies
IQR	Interquartile range
ZnT8	Zinc transporter-8
ZnT8A	ZnT8 autoantibodies

Introduction

A short treatment with anti-CD3 antibodies at clinical onset of type 1 diabetes has been shown to preserve residual beta cell function for at least 18 months but only in patients with secretory function exceeding 25% of healthy controls as measured by hyperglycaemic clamp [1]. This opens perspectives for prevention trials in the late preclinical disease phase, where beta cell function is even better preserved than at diagnosis [2] and where anti-CD3 antibodies have also been shown to be effective in animal models [3]. However, such an undertaking requires effective identification of individuals at high risk of progression towards hyperglycaemia within a relatively short period,

e.g. 5 years, to ensure that potential benefits outbalance the risk of side effects; this would also allow conclusions within a reasonable time frame [4].

Until now, risk assessment for type 1 diabetes has mostly been based on screening for insulin autoantibodies (IAA), autoantibodies against the 65 kDa isoform of GAD, i.e. GAD autoantibodies (GADA) and insulinoma-associated protein-2 (IA-2) autoantibodies (IA-2A) [5–9]. The presence of IA-2A has been shown to predict impending clinical onset in first-degree relatives of type 1 diabetic patients [6, 9], but allows detection of only about two out of three prediabetic individuals [10]. More recently, antibodies against the related antigen IA-2 beta (IA-2 β , antibodies: IA-2 β A) were reported to identify a subgroup of first-degree relatives with higher progression rate to diabetes [11, 12]. In 2007, zinc transporter-8 (ZnT8) was identified as a novel diabetes autoantigen [13]. Autoantibodies against the carboxy-terminal intracellular domain of ZnT8 (ZnT8A) have been proposed as additional markers of rapid disease progression [13], but their epitope specificity has been shown to vary according to a non-synonymous single nucleotide polymorphism at codon 325 of *SLC30A8*, the gene encoding ZnT8 [14].

In preparation of secondary prevention trials with immunointervention, we investigated to what extent screening for individuals at high risk of diabetes based on positivity for IA-2A [6, 9] or the presence of multiple autoantibodies (IAA, GADA and/or IA-2A) [5–8] could be optimised in terms of sensitivity and progression rate to diabetes by measuring IA-2 β A and ZnT8A. A screening for both antibodies was conducted in first-degree relatives who tested positive for IAA, IA-2A or GADA. For the ZnT8A assay we used a radioligand derived from a hybrid ZnT8 cDNA construct generated by fusion of CR and CW (zinc transporter-8 carboxy-terminal constructs carrying 325Arg and 325Trp, respectively) (CW-CR), which is able to recognise antibodies directed against the wild type carboxyterminal domain of ZnT8 carrying 325Arg, as well as antibodies against a polymorphic variant carrying 325Trp [15]. The efficiency of antibody screening was compared before and after omission of relatives protected by *HLA-DQ* genotype or type of relationship to the diabetic proband [16].

Methods

Participants Between 30 August 1989 and 17 December 2006, the Belgian Diabetes Registry (BDR) consecutively recruited 6,432 siblings or offspring (under age 40 at entry) of type 1 diabetic probands according to previously defined criteria [10, 16]. The probands are considered representative of the Belgian population of type 1 diabetic patients

[17]. After obtaining written informed consent from each relative or their parents, a short questionnaire with demographic, familial and personal information was completed at each visit and blood samples were taken at entry and (as a rule) yearly thereafter. Only relatives ($n=5,635$) with two or more contacts during follow-up, the last being at diagnosis in case of prediabetes, were included in this study. This allows the clinical status of relatives at this last time point to be unambiguously ascertained. The study was conducted in accordance with the guidelines in the Declaration of Helsinki as revised in 2008 (www.wma.net/en/30publications/10policies/b3/index.html, accessed 1 June 2009) and approved by the Ethics Committees of the BDR and the participating university hospitals. Blood was sampled at random, divided into aliquots and stored at -80°C until analysed for diabetes-associated autoantibodies and *HLA-DQ* genotype. Relatives were screened for the presence of IAA, GADA and IA-2A and 409 individuals were positive for at least one of these antibodies at baseline. The median age (interquartile range [IQR]) of these 409 siblings was 12 (6–19) years at baseline; there were 210 males and 199 females (ratio 1.06), 228 siblings, 92 offspring of a diabetic father and 89 offspring of a diabetic mother. Of the 409 siblings, 42% carried susceptible *HLA-DQ* genotypes and 25% protective ones [18]. During follow-up (median [IQR]: 68 [36–103] months), 86 (21%) antibody-positive relatives developed diabetes; they were identified by BDR as previously described [16].

Analytical methods IA-2A [9], GADA [19] and IAA [16] were determined at baseline by liquid-phase radiobinding assays and *HLA-DQ* polymorphisms by allele-specific oligonucleotide genotyping [18] as described previously. *HLA-DQ* genotypes were classified as susceptible, protective or neutral on the basis of data from the BDR [18]. Measurements of IA-2 β A and ZnT8A CW-CR [12, 14, 15] were also carried out at baseline by liquid-phase radiobinding assay using the same in-house protocol as for IA-2A and GADA, except that ^{35}S -labelled GAD or ^{35}S -labelled IA-2 (intracellular part) were replaced by ^{35}S -labelled IA-2 β (intracellular part) or ^{35}S -labelled ZnT8A (intracellular carboxy-terminal part derived from CW-CR constructs). All tracers were purified by ultrafiltration (Amicon Ultra-4 filter units; Millipore, Billerica, MA, USA) and antibody concentrations expressed as percentage of added tracer bound (10,000 cpm/tube). cDNAs for the preparation of radioligands by in vitro transcription-translation were kind gifts of Å. Lernmark (University of Washington, Seattle, WA, USA) for full length 65 kDa GAD, M. Christie (King's College School of Medicine and Dentistry, London, UK) for IA-2 (intracellular part), V. Lampasona (Istituto San Raffaele, Milano, Italy) for IA-2 β (intracellular part; amino acids

662–1033) and J. C. Hutton (Barbara Davis Center for Childhood Diabetes, Aurora, CO, USA) for the dimeric CW-CR ZnT8 construct containing both CR encoding the wild type amino acids 268–369 carrying 325Arg, and CW, a variant carboxy-terminal construct carrying 325Trp. In the Diabetes Antibodies Standardization Program 2009 Workshop diagnostic sensitivity and specificity were respectively 74% and 97% for GADA, 40% and 98% for IAA, 66% and 99% for IA-2A, 53% and 98% for IA-2 β A, and 68% and 100% for ZnT8A (CW-CR). Cut-off values for antibody positivity were determined as the 99th percentile of antibody levels in 761 non-diabetic controls and amounted to $\geq 0.6\%$ for IAA, $\geq 2.6\%$ for GADA, $\geq 0.44\%$ for IA-2A, $\geq 0.39\%$ for IA-2 β A and $\geq 1.20\%$ for ZnT8A. Between-day coefficients of variation determined for the Juvenile Diabetes Research Foundation standard serum were 9% for IAA ($n=413$), 10% for GADA ($n=427$), 11% for IA-2A ($n=474$), 10% for IA-2 β A ($n=156$) and 8% for ZnT8A ($n=115$).

Statistical analysis Statistical differences between groups were assessed by means of the Mann–Whitney *U* test for continuous variables and by the χ^2 test, using Yates' correction or Fisher's exact test for categorical variables. McNemar's test was used to assess differences between paired proportions. Kaplan–Meier analysis was used to estimate diabetes-free survival and survival curves were compared using the logrank test. Cox proportional hazards model, performed by forward stepwise method, was used to investigate the independent contributions of risk factors identified by univariate analysis, with calculation of 95% CIs on hazard ratios. In time-to-event analysis, follow-up started at entry and ended at the last contact with the relative or at clinical onset, whichever came first. Except for age and number of antibodies (between 1 and 5), all variables were introduced as categorical variables in Cox regression analysis. Univariate analyses (enter method) were first performed to identify which potential risk factors were significantly associated with progression to diabetes (Electronic supplementary material [ESM] Table 1). The significant variables selected in a first multivariate analysis (ESM Table 1) were then tested in a second model together with combinations of antibody positivity. All statistical tests were performed two-tailed by SPSS for Windows 16.0 (SPSS, Chicago, IL, USA), by EpiInfo version 6 (USD, Stone Mountain, GA, USA) or by GraphPad Prism version 4.00 for Windows (San Diego, CA, USA) and considered significant at $p < 0.05$.

Results

Progression to diabetes Of the 409 first-degree relatives selected on the basis of positivity for IAA, GADA and/or

Table 1 Prevalence of IA-2 β A and ZnT8A at baseline in 409 first-degree relatives according to antibody status for IAA, GADA, and IA-2A

Antibody pattern	<i>n</i>	IA-2 β A	ZnT8A
IAA ⁺ only	124	0 (0)	2 (2)
GADA ⁺ only	147	1 (1)	9 (6)
IA-2A ⁺ only	24	9 (38)	5 (21)
IAA ⁺ and GADA ⁺	41	2 (5)	8 (20)
IAA ⁺ and IA-2A ⁺	6	2 (33)	3 (50)
GADA ⁺ and IA-2A ⁺	33	21 (64)	27 (82)
IAA ⁺ and GADA ⁺ and IA-2A ⁺	34	24 (71)	26 (76)
All IA-2A ⁺	97	56 (58) ^a	61 (63) ^a
All IA-2A ⁻	312	3 (1)	19 (6)

Values are *n* (%)

First-degree relatives positive for IAA, GADA or IA-2A

^a*p*<0.001 vs prevalence in absence of IA-2A

IA-2A, 86 (21%) developed diabetes after a follow-up time (IQR) of 39 (15–65) months. Progression occurred more frequently among siblings (55/228; 24%) or offspring of a diabetic father (24/92; 26%) than among offspring of a diabetic mother (7/89; 8%) (*p*=0.002). At entry, progressors were younger than non-progressors (median age [IQR]: 9 [5–16] years vs 12 [7–20] years; *p*=0.01) and were more

often carriers of susceptible *HLA-DQ* genotypes (72% vs 34%; *p*<0.001).

Antibody prevalence at baseline Among the 409 first-degree relatives, 50% were positive for IAA, 62% for GADA, 24% for IA-2A, 14% for IA-2 β A and 20% for ZnT8A. The prevalence of IA-2 β A and ZnT8A was significantly higher in IA-2A⁺ than in IA-2A⁻ first-degree relatives (Table 1) and in progressors than in non-progressors (IA-2 β A 47% vs 6%, *p*<0.001; ZnT8A 56% vs 10%, *p*<0.001).

Kaplan–Meier survival analysis Stratification of relatives according to the status of IA-2A, IA-2 β A or ZnT8A (ESM Fig. 1) showed that for each antibody, positivity was associated with a higher progression rate to diabetes than in their absence (*p*<0.001). The presence of IA-2 β A or ZnT8A significantly decreased diabetes-free survival in IA-2A⁻ (Fig. 1c, d), but not in IA-2A⁺ relatives (Fig. 1a, b). Combined screening for IA-2A and IA-2 β A, or IA-2A and ZnT8A identified respectively two or eight prediabetic relatives more (Fig. 1e, f) during the entire follow-up than did screening for IA-2A alone (Fig. 1a, b). Combined testing for IA-2A IA-2 β A and ZnT8A detected nine extra prediabetic relatives than testing for IA-2A alone with similarly high overall progression rate (not shown).

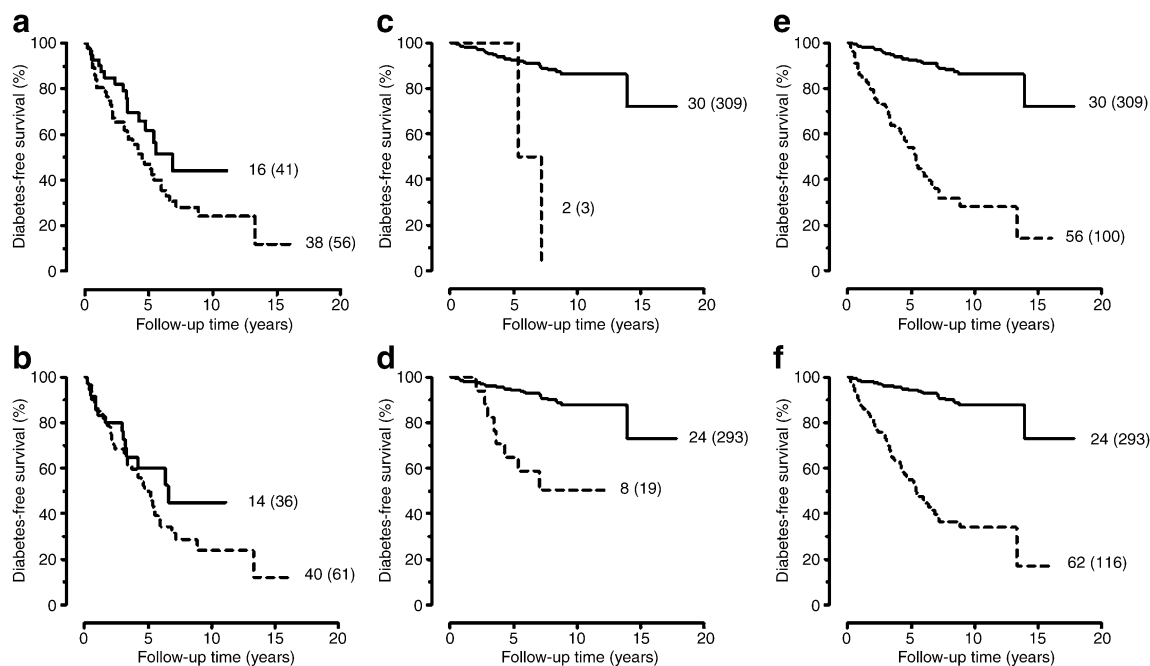


Fig. 1 Diabetes-free survival (%) in IA-2A-positive first-degree relatives at baseline sampling (*n*=97) stratified according to the presence or absence of IA-2 β A (a) and ZnT8A (b); in IA-2A-negative first-degree relatives at baseline sampling (*n*=312) stratified according to the presence or absence of IA-2 β A (c) and ZnT8A (d); and in all antibody-positive first-degree relatives (*n*=409) stratified according to the presence of IA-2A or IA-2 β A vs their joint absence (e) and the

presence of IA-2A or ZnT8A vs their joint absence (f). Dashed lines, positivity; continuous lines, negativity. Number of events (total number of relatives at entry) are indicated next to each arm for each panel. a *p*=0.112 (logrank); (b) *p*=0.247 (logrank); (c) *p*=0.001 (logrank); (d) *p*<0.001 (logrank); (e) *p*<0.001 (logrank); (f) *p*<0.001 (logrank)

Table 2 Influence of baseline antibody status and protective factors on 5-year progression rate and number of prediabetic first-degree relatives (Kaplan–Meier analysis)

Antibody positivity per FDR group ^a	n	5-year progression to diabetes	
		% (95% CI)	n (%)
All			
IAA or GADA or IA-2A	409	17 (13–21)	61 (100)
IAA	205	17 (11–23)	32 (52)
GADA	255	21 (16–26)	47 (77)
IA-2A	97	47 (36–58)	41 (67) ^b
IA-2βA	59	51 (37–65)	28 (46)
IA-2A or IA-2βA	100	46 (35–57)	41 (67)
ZnT8A	80	47 (36–58)	35 (57)
IA-2A or ZnT8A	116	45 (35–55)	47 (77) ^b
IA-2βA or ZnT8A	94	46 (33–57)	40 (66)
IA-2A or IA-2βA or ZnT8A	118	45 (35–55)	47 (77)
IA-2A or GADA or ZnT8A	287	23 (18–28)	59 (97)
No protective <i>HLA-DQ</i>^c			
IAA or GADA or IA-2A	306	22 (17–27)	60 (98)
IA-2A	85	53 (42–64)	41 (67) ^d
IA-2A or IA-2βA	87	51 (40–62)	41 (67)
IA-2A or ZnT8A	103	48 (38–58)	46 (75) ^d
No offspring of diabetic mother			
IAA or GADA or IA-2A	320	21 (16–25)	56 (92)
IA-2A	83	53 (41–65)	39 (64) ^e
IA-2A or IA-2βA	86	51 (39–63)	39 (64)
IA-2A or ZnT8A	97	52 (41–63)	45 (74) ^e
No protective <i>HLA-DQ</i> and no offspring of diabetic mother			
IAA or GADA or IA-2A	246	25 (19–31)	55 (90)
IA-2A	75	57 (42–72)	39 (64) ^f
IA-2A or IA-2βA	77	55 (43–67)	39 (64)
IA-2A or ZnT8A	88	55 (44–66)	44 (72) ^f
Only carriers of <i>HLA-DQ2/DQ8</i>			
IAA or GADA or IA-2A	83	35 (24–46)	26 (43)
IA-2A	29	76 (59–93)	20 (33) ^g
IA-2A or IA-2βA	29	76 (59–93)	20 (33)
IA-2A or ZnT8A	34	73 (57–89)	23 (37) ^g

^a First-degree relatives (FDR) positive for IAA, GADA or IA-2A

^b $p=0.031$

^c As defined by Van der Auwera et al. [18]

^d $p=0.063$; ^e $p=0.031$; ^f $p=0.063$; and ^g $p=0.250$ by McNemar test

Table 2 shows that positivity for IA-2A, IA-2βA or ZnT8A tended to be associated with a higher 5-year progression rate to diabetes than positivity for IAA or GADA. Presence of IA-2A and/or ZnT8A identified a subgroup of first-degree relatives containing 77% of all individuals developing diabetes within 5 years, i.e. 10%

more than with IA-2A alone ($p=0.031$), and with an overall 5-year diabetes risk of 45%, which was similar to the risk observed for IA-2A alone or IA-2βA alone. Testing for IA-2βA in addition to IA-2A or to IA-2A and ZnT8A did not increase screening sensitivity, i.e. the additional IA-2βA-positive relatives did not develop diabetes within 5 years. Including GADA in the test panel improved the sensitivity of detecting prediabetes, but at the expense of an overall lower progression rate (Table 2).

Excluding first-degree relatives with protective *HLA-DQ* genotypes [18] or relatively protected offspring of diabetic mothers [20] reduced the antibody-positive group by 25% and 22%, respectively, but increased the 5-year progression rate of IA-2A⁺ or ZnT8A⁺ individuals by 3% and 7%, respectively (Table 2). Omission of both groups together increased 5-year risk by 10% as compared with that of the entire first-degree relatives group, while the group to be screened was reduced by 40%, without major decrease in the number of prediabetic first-degree relatives identified. In these subgroups of relatives, screening for IA-2A and ZnT8A (but not for IA-2A and IA-2βA) also tended to detect more individuals who developed diabetes within 5 years than screening for IA-2A alone ($p=0.063$ to 0.031), but significance was only reached in the group without offspring of a diabetic mother (Table 2). In carriers of *HLA-DQ2/DQ8*, positivity for IA-2A or ZnT8A defined a group with a 74% 5-year risk, representing 38% of progressors within 5 years (Table 2). In all subgroups, the fraction of rapid progressors identified by screening for IA-2A and ZnT8A was independent of age, but three out of four were between 5 and 30 years (results not shown).

Cox regression analysis The significant variables selected in a first model (see Methods and ESM Table 1) were tested in a second model additionally including combinations of antibodies. Positivity for *HLA-DQ2/DQ8* and IA-2A or ZnT8A were selected as independent predictors of diabetes while having a type 1 diabetic mother proved protective (Table 3).

Discussion

The present study is the first to examine the added value of measuring both IA-2βA and ZnT8A for prediction of impending diabetes in siblings or offspring of type 1 diabetic patients. It confirms the association of IA-2A [6, 9], IA-2βA [12, 21] and ZnT8A [13, 22] with rapid disease progression and demonstrates that IA-2A and ZnT8A represent the most sensitive combination of two markers to identify relatives with a high progression rate.

Table 3 Cox regression analysis in 409 first-degree relatives

Variable	Univariate analysis (enter method)		Multivariate analysis (forward stepwise method)	
	<i>p</i> value	HR for diabetes (95% CI)	<i>p</i> value	HR for diabetes (95% CI)
Model 2 ^a				
IA-2A ⁺	<0.001	8.1 (5.2–12.7)	NS	NS
<i>HLA-DQ2/DQ8</i>	<0.001	2.7 (1.8–4.3)	0.008	1.9 (1.2–2.9)
Offspring of diabetic mother ^b	<0.001	0.2 (0.1–0.5)	0.002	0.3 (0.1–0.6)
IA-2A ⁺ or IA-2βA ⁺	<0.001	8.6 (5.5–13.5)	NS	NS
IA-2A ⁺ or ZnT8A ⁺	<0.001	9.3 (5.8–14.9)	<0.001	5.2 (2.2–12.3)

First-degree relatives positive for IAA, GADA or IA-2A

For details of Cox regression analysis (Model 1), see ESM Table 1. The dataset was complete for all variables

^a For the basis for selection of variables at each step of building the models, see the Methods.

^b Sibling or offspring of diabetic father=0, offspring of diabetic mother=1

Exclusion of relatives protected by *HLA-DQ* genotype [18] or by maternal diabetes [20] reduced the group to be followed by 40% without major loss in screening sensitivity. These findings are useful for screening programmes aiming to enrol high-risk individuals in immunointervention trials.

The strengths of this study are its registry-based nature, the use of a sensitive ZnT8A assay, the broad age range tested and the confirmation of glycaemic status at the last follow-up point for each relative. Criticisms might be that few relatives were followed for more than 10 years and that we did not test all relatives for IA-2βA and ZnT8A. However, the low prevalence of both antibodies in IAA⁻, GADA⁻ and IA-2A⁻ first-degree relatives (0/441 in our own first-degree relatives, not shown; 0.2% for ZnT8A⁺ in a previous publication [22]) and of solitary ZnT8A⁺ in new-onset type 1 diabetes (4% of all patients in a previous study [13]; 2% in >400 patients of the BDR; M. Asanghanwa, unpublished results) supports the validity of our results for all first-degree relatives. Our baseline results cannot rule out the possibility that more first-degree relatives could have developed IA-2βA and/or ZnT8A at a later time point prior to clinical onset.

IA-2A, IA-2βA and ZnT8A target intracellular domains of antigens anchored in the membrane of beta cell secretory vesicles [23–25]. These domains may only become visible to the immune system at the outer cell surface in the case of beta cell damage or dysfunction [13, 26, 27]. These antibodies appear generally later than IAA or GADA in prediabetes [13, 28–30]. Our finding that IA-2A, IA-2βA and ZnT8A tend to cluster in first-degree relatives and predict, alone or in combination, rapid progression to diabetes is in line with the above observations, indicating that they preferentially mark the later stages of the preclinical phase of type 1 diabetes.

Based on our results, screening for IA-2A and ZnT8A emerge as the most sensitive combination of two markers to identify rapid progressors. Regardless of age, it identified 77% of them, i.e. 10% more than by IA-2A alone, without loss in overall 5-year risk (≥45%). Risk stratification may further be improved by genotyping the ZnT8-encoding *SLC30A8* gene [22]. Unlike in the ENDIT study [12], IA-2βA did not improve risk assessment by IA-2A. This may relate to differences in inclusion criteria (registry-based vs preselection on basis of high-titre islet cell antigens) and the lower number of prediabetic participants in the present paper. Inclusion of functional tests may further improve screening specificity [2, 31]. Three out of four rapid progressors were between 5 and 30 years, which is the age group preferentially considered for participation in anti-CD3 trials [32]. Exclusion of genetically or maternally protected relatives increased screening efficiency for rapid progressors as the group to be followed was reduced by 40%, while 5-year risk increased to ≥55% for IA-2A⁺, IA-2βA⁺ or ZnT8A⁺ relatives with minor loss in sensitivity (<10%). There were too few progressors among offspring with a diabetic mother to establish their risk in case of positivity for IA-2A, IA-2βA, ZnT8A and susceptible genotypes, and so any decision on whether to exclude them or not would be ethically based only. Restricting follow-up to carriers of the *HLA-DQ2/DQ8* high-risk genotype identified a group with about 75% 5-year risk but at the expense of >50% loss of screening sensitivity. The choice of screening options should be investigated in further studies.

In conclusion, we propose that immunointervention trials in pre-type 1 diabetes should focus on first-degree relatives without protective factors and testing positive for IA-2A and/or ZnT8A.

Acknowledgements The present work was supported by grants from the Juvenile Diabetes Research Foundation (Center Grant 4-2005-1327), the Belgian Fund for Scientific Research (FWO-Vlaanderen: research grants G-0517-04, G-0311-07 and G-0374-08, and postdoctoral research fellowships to I. Weets, K. Decochez and B. Keymeulen), the Research Council of the Brussels Free University – VUB (OZR 1150 and OZR 1449) and the Scientific Fund Willy Gepts (University Hospital Brussels; projects 3-2005 and 3/22-2007). The Belgian Diabetes Registry is supported by the Belgian National Lottery and the Ministries of Public Health of the Flemish and French Communities of Belgium. We gratefully acknowledge the expert technical assistance of co-workers at the central unit and reference laboratory of the Belgian Diabetes Registry (V. Baeten, G. De Block, T. De Mesmaeker, L. De Pree, H. Dewinter, N. Diependaele, S. Exterbille, C. Groven, A. Ivens, D. Kesler, F. Lebleu, M. Lichtert, E. Quartier, G. Schoonjans, S. Vanderstraeten, U. Vandeveld, M. Van Molle and A. Walgraeve). We would also like to thank the different university teams of co-workers for their excellent assistance in collecting samples and organising the fieldwork in Antwerp (L. Van Gaal, C. De Block, J. Michiels, J. Van Elven and J. Vertommen), in Brussels (T. De Mesmaeker, S. Exterbille, P. Goubert, C. Groven, M. Lichtert, A. Walgraeve), in Ghent (J.-M. Kaufman, J. Ruige, A. Hutse, A. Rawoens) and in Leuven (C. Mathieu, M. Carpentier, A. Schoonis, H. Morobé). We sincerely thank all members of the Belgian Diabetes Registry who contributed to the recruitment of relatives for the present study.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

- Keymeulen B, Vandemeulebroucke E, Ziegler A et al (2005) Insulin needs following CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med* 352:2598–2608
- Vandemeulebroucke E, Keymeulen B, Decochez K et al (2009) Hyperglycaemic clamp test for diabetes risk assessment in IA-2 antibody-positive relatives of type 1 diabetic patients. *Diabetologia*. doi:10.1007/s00125-009-1569-3
- Chatenoud L, Thervet E, Primo J, Bach JF (1994) Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci USA* 91:123–127
- Gorus FK, Pipeleers DG (2001) Prospects for predicting and stopping the development of type 1 diabetes. *Best Pract Res Clin Endocrinol* 15:371–389
- Verge CF, Gianani R, Kawasaki E et al (1996) Prediction of type 1 diabetes in first-degree relatives using a combination of insulin, GAD and ICA 512bdc/IA-2 autoantibodies. *Diabetes* 45:926–933
- Kulmala P, Savola K, Petersen JS et al (1998) Prediction of insulin-dependent diabetes mellitus in siblings of children with diabetes. *J Clin Invest* 101:327–336
- Notkins AL, Lernmark Å (2001) Autoimmune type 1 diabetes: resolved and unresolved issues. *J Clin Invest* 108:1247–1252
- Bingley PJ, Gale EA (2006) Progression to type 1 diabetes in islet cell antibody-positive relatives in the European Nicotinamide Diabetes Intervention Trial: the role of additional immune, genetic and metabolic markers of risk. *Diabetologia* 49:881–890
- Gorus FK, Goubert P, Semakula C et al (1997) IA-2 autoantibodies complement GAD65-autoantibodies in new onset IDDM patients and help predict impending diabetes in their siblings. The Belgian Diabetes Registry. *Diabetologia* 40:95–99
- Decochez K, De Leeuw I, Keymeulen B et al (2002) IA-2 autoantibodies predict impending type 1 diabetes in siblings of patients. The Belgian Diabetes Registry. *Diabetologia* 45:1658–1666
- Achenbach P, Warncke K, Reiter J et al (2004) Stratification of type 1 diabetes risk on the basis of islet autoantibody characteristics. *Diabetes* 53:384–392
- Achenbach P, Bonifacio E, Williams AJ et al (2008) Autoantibodies to IA-2 beta improve diabetes risk assessment in high-risk relatives. *Diabetologia* 51:488–492
- Wenzlau JM, Juhl K, Yu L et al (2007) The cation efflux transporter ZnT8A (Sec 30A8) is a major autoantigen in human type 1 diabetes. *Proc Natl Acad Sci USA* 104:17040–17045
- Wenzlau JM, Liu Y, Yu L et al (2008) A common nonsynonymous single nucleotide polymorphism in the *SLC30A8* gene determines ZnT8 autoantibody specificity in type 1 diabetes. *Diabetes* 57:2693–2697
- Kawasaki E, Uga M, Nakamura K et al (2008) Association between anti-ZnT8 autoantibody specificities and *SLC30A8* Arg325Trp variant in Japanese patients with type 1 diabetes. *Diabetologia* 51:2299–2302
- Decochez K, Truyen I, Van der Auwera B et al (2005) Combined positivity for *HLA DQ2/DQ8* and IA-2 antibodies defines population at high risk of developing type 1 diabetes. *Diabetologia* 48:687–694
- Vandewalle CL, Coeckelberghs MI, De Leeuw IH et al (1997) Epidemiology, clinical aspects, and biology of IDDM patients under age 40 years. Comparison of data from Antwerp with complete ascertainment with data from Belgium with 40% ascertainment. *Diabetes Care* 20:1556–1561
- Van der Auwera BJ, Schuit FC, Weets I et al (2002) Relative and absolute HLA-DQA1-DQB1 linked risk for developing type 1 diabetes before 40 years of age in the Belgian population: implications for further prevention studies. *Hum Immunol* 63:40–50
- Vandewalle CL, Falorni A, Svanholm S et al (1995) High diagnostic sensitivity of glutamate decarboxylase autoantibodies in insulin-dependent diabetes mellitus with clinical onset between age 20 and 40 years. *J Clin Endocrinol Metab* 80:846–851
- Hämäläinen AM, Knip M (2002) Autoimmunity and familial risk of type 1 diabetes. *Curr Diab Rep* 2:347–353
- Christie MR, Genovese S, Cassidy D et al (1994) Antibodies to islet 37 k antigen, but not to glutamate decarboxylase discriminate rapid progression to IDDM in endocrine autoimmunity. *Diabetes* 43:1254–1259
- Achenbach P, Lampasona V, Landherr U et al (2009) Autoantibodies to zinc transporter 8 and *SLC30A8* genotype stratify type 1 diabetes risk. *Diabetologia* 52:1881–1888
- Solimena M, Dirx R Jr, Hermel JM et al (1996) ICA512, an autoantigen of type 1 diabetes, is an intrinsic membrane protein of neurosecretory granules. *EMBO J* 15:2102–2114
- Wasmeier C, Hutton JC (1996) Molecular cloning of phogrin, a protein-tyrosine phosphatase homolog localized to insulin secretory granule membranes. *J Biol Chem* 271:18161–18170
- Chimienti F, Devergnas S, Favier A, Seve M (2004) Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules. *Diabetes* 53:2330–2337
- Lampasona V, Bearzatto M, Genovese S et al (1996) Autoantibodies in insulin-dependent diabetes recognize distinct cytoplasmic domains of the protein tyrosine phosphatase-like IA-2 autoantigen. *J Immunol* 157:2707–2711
- Notkins AL, Zhang B, Matsumoto Y, Lan MS (1997) Comparison of IA-2 with IA-2beta and with six other members of the protein tyrosine phosphatase family: recognition of antigenic determinants by IDDM sera. *J Autoimmun* 10:245–250
- Yu L, Rewers M, Gianani R et al (1996) Antiislet autoantibodies usually develop sequentially rather than simultaneously. *J Clin Endocrinol Metab* 81:4264–4267

29. Leslie RDG, Delli Castelli M (2004) Age-dependent influences on the origins of autoimmune diabetes: evidence and implications. *Diabetes* 53:3033–3040
30. Achenbach P, Bonifacio E, Koczwara K, Ziegler AG (2005) Natural history of type 1 diabetes. *Diabetes* 54(Suppl 2):S25–S31
31. Truyen I, De Pauw P, Jørgensen P et al (2007) Proinsulin level and proinsulin-to-C-peptide ratio complement autoantibody measurement for predicting type 1 diabetes. *Diabetologia* 48:2322–2329
32. Herold KC, Hagopian W, Auger JA et al (2002) Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. *N Engl J Med* 346:1692–1698