Stratification of Type 1 Diabetes Risk on the Basis of Islet Autoantibody Characteristics

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Family history of type 1 diabetes and autoantibodies to the islet antigens insulin (IAA), glutamate decarboxylase (GADA), and the protein tyrosine phosphatase-like protein IA-2 (IA-2A) are strong predictors of type 1 diabetes, but the rate of progression to diabetes in multiple islet autoantibody-positive relatives varies widely. We asked whether detailed characterization of islet autoantibodies that included determination of titer, epitope specificity, and IgG subclass would improve diabetes prediction in a large cohort of autoantibody-positive relatives. The study shows a strong association between risk and high titer, broad antibody responses to IA-2 and insulin. The highest risks were associated with high-titer IA-2A and IAA, IgG2, IgG3, and/or IgG4 subclass of IA-2A and IAA, and antibodies to the IA-2–related molecule IA-2β. Using models based on these antibody characteristics, autoantibody-positive relatives can be classified into groups with risks of diabetes ranging from 7 to 89% within 5 years. *Diabetes* 53: 384–392, 2004

Autoantibodies to islet cell antigens such as insulin (IAA), the 65–kDa isoform of glutamate decarboxylase (GADA), and the protein tyrosine phosphatase (PTP)-like antigen IA-2 (IA-2A) are markers of the autoimmune process that precedes type 1 diabetes (1–9). At-risk relatives can be identified on the basis of positivity for these autoantibodies. Diabetes risk is highest in relatives with more than one islet autoantibody (4–11) or with high-titer islet cell antibodies (10,12), suggesting that the intensity of the humoral response may reflect the stage of β-cell destruction. It has however been shown that a proportion of relatives with multiple islet autoantibodies do not develop diabetes for many years (6,13), indicating that additional tests are necessary for accurate prediction of diabetes. IgG subclass and the epitope specificity of autoantibodies may reflect qualitative and quantitative differences in the autoimmune response (14–16), and their measurement could, therefore, improve our ability to predict diabetes. We have examined islet autoantibody titer, epitope specificity and IgG subclass in prospectively followed islet autoantibody-positive first-degree relatives of patients with type 1 diabetes, and determined how these can be used to stratify the likelihood of progression to clinical diabetes. The findings are consistent with the concept that high-titer multitarget responses signal late or aggressive preclinical diabetes and allow staging of diabetes risk on the basis of antibody measurements.

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BOX, Bart's Oxford; GADA, autoantibody to GAD; IA-2A, autoantibody to IA-2; IAA, autoantibody to insulin; PTP, protein tyrosine phosphatase; SDS, SD score.

RESEARCH DESIGN AND METHODS

Sera of all first-degree relatives of patients with type 1 diabetes from the Bart's Oxford (BOX) and the Munich family studies (13,17) were tested for IAA, GADA, and IA-2A. A total of 180 nondiabetic relatives (76 from BOX study and 104 from Munich family study) were selected on the basis of positivity for at least one of these antibodies on two or more occasions and whether a sufficient volume of the first positive sample was available for complete testing according to the study protocol. The study cohort consisted of 65 offspring (38 sons, 27 daughters), 68 siblings (32 brothers, 36 sisters), and 47 parents (21 fathers, 26 mothers) of the diabetic proband. The first autoantibody-positive serum sample from these relatives was used for all measurements performed in this study. The median age of relatives at the time of collection of the first available antibody-positive sample was 14.5 years (interquartile range 8.3–30.3 years). Subjects were prospectively monitored for the development of type 1 diabetes over a median follow-up period of 5.9 years (interquartile range 3.8–10.7 years) for a total of 1,248 subject years. Of the 180 autoantibody-positive relatives, 59 developed type 1 diabetes during follow-up (median time to diabetes 3.6 years; interquartile range 1.3–6.1 year). Diabetes was diagnosed using World Health Organization/American Diabetes Association criteria (18). Another 78 relatives with confirmed positive antibodies were not included due to insufficient serum sample. Twenty of these developed diabetes. An additional 136 relatives had islet autoantibodies in one sample but were either negative on follow-up samples (n = 70; none developed diabetes) or had no follow-up sample (n = 66; 13 developed diabetes) and were not included in the current study. Seven of 5,782 relatives who were islet autoantibody negative at screening developed diabetes during follow-up. The respective local ethical committees approved BOX and Munich family studies.

Islet autoantibody measurements. IAA, GADA, and IA-2A were measured by protein A/G radiobinding assays as previously described (7,19) using ^{125}I-labeled insulin and [^{35}S]methionine-labeled in vitro–translated recombinant human GAD65 and IA-2, respectively. Samples with antibody titers above the discriminatory range of the assays were titrated until they fell within this part of the standard curve and the units multiplied by the appropriate dilution factor. The thresholds for positivity in each assay corresponded to the 99th percentile of control subjects. These assays had sensitivities and specificities of 80 and 94% (GADA), 58 and 100% (IA-2A), and 30 and 98% (IAA) in the First DASP Assay Proficiency Evaluation (20).

GADA and IA-2A epitope specificity was determined by radiobinding assay of GAD65/67 chimeric proteins and IA-2/IA-2β fragments or chimeric proteins as previously described (21,22). Thresholds for positivity were defined as the upper limit of 50 control subject sera. GAD antibody epitope specificities were classified as GAD65–NH_2-terminal (residues 1–100), GAD65–MID (residues 235–442), GAD65–COOH-terminal (residues 436–585), and/or GAD67. IA-2/IA-2β antibody epitopes were classified as IA-2–JM (residues 605–682),

TABLE 1
Type 1 diabetes risk in relation to age, sex, relationship to proband, islet autoantibody number, and combinations in autoantibody-positive relatives: univariate analysis

Variable	*n*	Type 1 diabetes (*n*)	10-year diabetes risk (% ± SE)	HR (95% CI)	*P*
Age					0.06
<15 years	93	32	46 ± 7	1.7 (1.0–2.8)	
>15 years	87	27	32 ± 6	1*	
Sex					0.6
Male	91	28	34 ± 6	0.9 (0.5–1.5)	
Female	89	31	43 ± 6	1*	
Relation to proband					0.26
Offspring	65	22	47 ± 8	1.7 (0.9–3.4)	0.1
Sibling	68	22	37 ± 7	1.3 (0.7–2.6)	0.41
Parent	47	15	31 ± 7	1*	
Autoantibody number					0.0001
One	100	22	25 ± 5	1*	
Two	57	25	59 ± 9	3.1 (1.7–5.5)	<0.001
Three	23	12	69 ± 13	4.4 (2.1–9.0)	<0.001
Autoantibody combinations					0.0001
GADA alone	72	15	22 ± 6	1*	
IAA alone	19	3	21 ± 11	0.8 (0.2–2.7)	0.68
IA2A alone	9	4	47 ± 17	3.0 (1.0–9.2)	0.05
GADA, IAA	27	8	52 ± 17	2.1 (0.9–5.1)	0.09
GADA, IA2A	27	14	61 ± 12	4.0 (1.9–8.4)	<0.001
IAA, IA2A	3	3	100	13.3 (3.8–47.2)	<0.001
GADA, IAA, IA2	23	12	69 ± 13	4.8 (2.2–10.4)	<0.001

*Reference cell used in Cox's proportional hazard model.

IA-2-PTP–specific (unique to the IA-2 PTP domain and not shared with IA-2β PTP as determined by competition), IA-2/IA-2β-PTP–crossreactive (shared between IA-2 and IA-2β as determined by competition), and IA-2β-PTP–specific (unique to the IA-2β PTP domain and not shared with IA-2 PTP as determined by competition).

IgG subclasses and isotypes of IAA, GADA, and IA-2A were determined by radiobinding assays as previously described (23) using IgG subclass or isotype specific biotin-labeled mouse-anti-human monoclonal antibodies (Becton Dickinson, San Diego, CA) bound on Sepharose 4B streptavidin beads (Zymed, San Francisco, CA). The antibodies used were mouse monoclonal antibodies against human IgG1 (clone G17-1), IgG2 (clone G18-21), IgG3 (clone G18-3), IgG4 (clone JDC-14), IgM (clone G20-127), IgA (clone G20-359), and IgE (clone G7-26). Nonspecific binding was determined for each serum using anti-rat IgM monoclonal antibody (clone G53-238)-coated beads. The absence of cross-reactivity between subclasses was confirmed using high-titer human IgG1 monoclonal GADA and IA-2A antibodies. Results for IAA subclasses were expressed as nanounit of insulin bound per milliliter after subtraction of binding with the anti-rat IgM–coated beads. The cutoff for positivity for each IAA IgG subclass was 150 nU/ml (mean plus 3 SD of IAA-negative control subjects). GADA and IA-2A subclasses were expressed as difference (Δ) in counts per minute (IgG subclass or isotype specific cpm − anti-rat IgM cpm) and converted to an SD score (SDS). The cutoff for positivity for each GADA and IA-2A IgG subclass or isotype was 3 SDS, respectively.

Statistical analysis. Associations of variables with antibody titers were analyzed using the χ^2 test for trend. Time-to-event methods (life-table analysis and Cox proportional hazards model) were used to compare outcome (diabetes status) for participants with different covariate categories. The time between first antibody-positive sample and diagnosis of diabetes was defined as the time to event in relatives developing diabetes. Analyses considered censoring in relatives with diabetes-free status at the end of the follow-up period defined as date of last contact or date of entry into an intervention trial. Significance between groups was determined using the log rank test. Hazard ratios (HRs) were determined using Cox's proportional hazards model. The proportional hazards assumption in the Cox model was tested by including in the model the covariate in question and the interaction between time and that covariate. For all analyses, a two-tailed *P* value of 0.05 was considered significant. All statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0, Chicago, IL).

RESULTS

Characteristics of islet autoantibodies in first-degree relative cohort. GADA ≥99th centile were detected in 149 of the 180 relatives (83%), IA-2A in 62 (34%), and IAA in 72 (40%). Single autoantibodies were detected in 100 relatives (56%), two islet autoantibodies in 57 (32%), and three islet autoantibodies in 23 (13%) (Table 1). IgG1 was the most frequently detected subclass for each of the autoantibodies (Fig. 1*A*–*C*). IgG3 islet autoantibodies were rare, except for IAA where its prevalence was similar to that of IgG2 and IgG4. IgG subclasses other than IgG1 were found in 66 (44%) GADA-positive relatives, 29 (47%) IA-2A–positive relatives, and 39 (54%) IAA-positive relatives (Table 2). IgA and IgM autoantibodies were detected in 12–17% of relatives for each autoantibody. IgE autoantibodies were rare (<2%) in all groups.

Of the GADA-positive relatives, 115 (77%) had autoantibodies to multiple epitopes of the antigen, including 113 with antibodies to both GAD65–MID and GAD65–COOH-terminal epitopes (Fig. 1*D*). For IA-2A, 34 (55%) relatives had antibodies to multiple epitopes, and 30 (48%) relatives had antibodies that bound epitopes shared with IA-2β (Fig. 1*E*).

For each islet autoantibody, the detection of autoantibody IgG subclasses other than IgG1 were associated with high-titer antibodies, and for GADA and IA-2A, antibodies to multiple epitopes were associated with high-titer antibodies (Fig. 2), suggesting that multiple subclass usage and antibody epitope spreading were markers of pronounced high-titer humoral responses.

Association of islet autoantibody characteristics with type 1 diabetes risk: univariate analysis. The overall 5- and 10-year cumulative risks for disease were

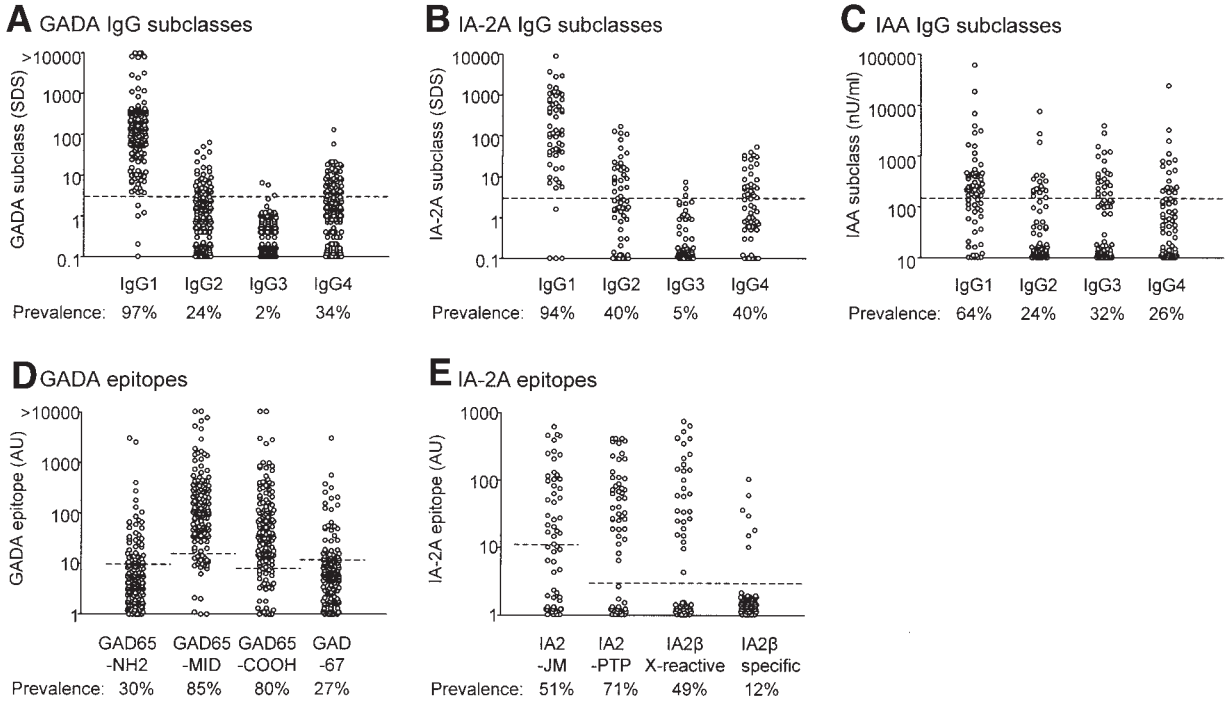

FIG. 1. Autoantibody subclass and epitope reactivities in 180 autoantibody-positive first-degree relatives of patients with type 1 diabetes. Titers of IgG subclasses were analyzed in GADA (n = 149; *A*), IA-2A (n = 62; *B*), and IAA (n = 72; *C*) positive subjects. Epitope reactivity was determined for GADA (*D*) and IA-2A (*E*) positive subjects. The dashed lines show thresholds for positivity.

21% (95% CI 14.8–27.2) and 39% (30.6–47.4), respectively. By univariate analysis, diabetes risk was significantly associated with the number and combinations of autoantibodies (Table 1), IA-2A titer and IAA titer (Table 2, Fig. 3), IA-2A IgG subclasses and IAA IgG subclasses (Tables 2 and 3), and IA-2A epitope reactivity (Tables 2 and 4).

Autoantibody number and combinations. Diabetes risk was significantly higher in relatives with two or more autoantibodies (61% by 10 years; 95% CI 46–76%) than in relatives with one autoantibody (25%; 15–35%; $P < 0.0001$) and, among single autoantibody-positive relatives, risk was significantly higher in relatives with IA-2A alone (47% by 10 years; 13–81%) than in those with GADA alone (22%; 11–33%; $P = 0.01$; Table 1).

Autoantibody titer. IA-2A–positive relatives with titers in the upper three quartiles (>27 units) had significantly higher diabetes risk (79% by 10 years; 95% CI 64–94%) than IA-2A–positive relatives with IA-2A titers in the lowest quartile (20%; 1–45%; $P = 0.002$), and IAA-positive relatives with IAA titers in the upper quartile (>9 units) had significantly higher risk (77% by 10 years; 53–99%) than IAA-positive relatives with IAA titers in the lower three quartiles (37%; 30–54%; $P = 0.002$; Table 2, Fig. 3). No relationship was found between GADA titers and risk of diabetes.

Autoantibody IgG subclasses. In the GADA-positive relatives, no relationship was found between GADA IgG subclasses and the risk of diabetes (Tables 2 and 3). In the IA-2A–positive relatives, risk was higher in those with IgG2, IgG3, or IgG4 IA-2A (100% by 10 years; 95% CI 85–100%) than those without these IgG subclasses (37%; 18–48%; $P = 0.0007$; Tables 2 and 3). In the IAA-positive relatives, risk was higher in those with IgG2, IgG3, or IgG4 IAA (68% by 10 years; 47–89%) than those without these IgG subclasses (28%; 10–46%; $P = 0.007$; Tables 2 and 3).

Autoantibody epitopes. No association was found between GADA epitope reactivity and the risk of diabetes (Tables 2 and 4). In the IA-2A–positive relatives, type 1 diabetes risk was higher in those with autoantibodies that bound IA-2β (86% by 10 years; 95% CI 70–99%) than IA-2β antibody-negative relatives (38%; 18–58%; $P = 0.008$; Tables 2 and 4), and in relatives with antibodies binding multiple epitopes (75% by 10 years; 57–93%) than single epitope reactivity (50%; 26–74%; $P < 0.05$; Table 2).

Association of islet autoantibody characteristics with type 1 diabetes risk: multivariate analysis. Covariates found to be significantly associated with type 1 diabetes in univariate analysis, namely autoantibody number, IA-2A titer, IAA titer, IA-2A subclass, IAA subclass, multiple IA-2 epitopes, and IA-2β positivity, were included in the Cox proportional hazards model (Table 5). IA-2A titer >25th centile of positives (adjusted HR 5.4; 95% CI 1–29), IA-2A IgG2, IgG3, or IgG4 subclass positive (3.3; 1.4–8.1), and IAA IgG2, IgG3, or IgG4 subclass positive (4.6; 1.5–14) significantly contributed to the proportional hazards model. The adjusted HR for the presence of multiple autoantibodies, IAA titer, multiple IA-2A epitopes, and IA-2β autoantibody status did not reach statistical significance.

Since testing for IgG subclasses is currently expensive and not in general use, the multivariate analysis was also performed without the IgG subclass covariates. In this model, IA-2A titer >25th centile of positives (adjusted HR 5.1; 95% CI 1.1–24.2) and IAA titer >75th centile of positives (2.4; 95% CI) significantly contributed to diabetes risk. Removal of the IgG subclass covariates from the model, however, significantly decreased the fit of the proportional hazards model (χ^2, 18.8; $P < 0.0001$).

Islet autoantibody models for type 1 diabetes risk stratification. Based on the univariate and multivariate

TABLE 2
Type 1 diabetes risk in relation to islet autoantibody titer, IgG subclass, and epitope reactivity in autoantibody-positive relatives: univariate analysis

Variable	*n*	Type 1 diabetes (*n*)	10-year diabetes risk (% ± SE)	HR (95% CI)	*P*
Autoantibody titer					
GADA					0.19
First quartile	37	11	35 ± 9	1*	
Second quartile	37	8	22 ± 9	0.8 (0.3–1.9)	0.55
Third quartile	37	17	52 ± 9	1.7 (0.8–3.6)	0.18
Fourth quartile	38	13	43 ± 10	1.6 (0.7–3.6)	0.26
IAA					0.01
First quartile	18	6	45 ± 16	1*	
Second quartile	18	4	30 ± 13	0.7 (0.2–2.4)	0.55
Third quartile	18	4	38 ± 17	0.8 (0.2–2.7)	0.68
Fourth quartile	18	12	77 ± 12	3.0 (1.1–8.1)	0.03
IA-2A					0.04
First quartile	15	3	20 ± 14	1*	
Second quartile	15	9	74 ± 15	6.0 (1.6–22.4)	0.008
Third quartile	16	12	84 ± 10	5.9 (1.7–21.1)	0.006
Fourth quartile	16	9	71 ± 15	4.8 (1.3–17.9)	0.02
Autoantibody IgG subclass					
GADA					0.36
IgG2, 3, and 4 negative	83	24	35 ± 7	1*	
IgG2, 3 or 4 positive	66	25	43 ± 7	1.3 (0.7–2.3)	
IAA					0.009
IgG2, 3, and 4 negative	33	7	28 ± 9	1*	
IgG2, 3, or 4 positive	39	19	68 ± 10	3.2 (1.2–7.6)	
IA-2A					0.0001
IgG2, 3, and 4 negative	33	11	37 ± 10	1*	
IgG2, 3, or 4 positive	29	22	100	4.9 (2.2–10.6)	
Autoantibody epitopes					
GADA					0.18
Single	34	9	32 ± 9	1*	
Multiple	115	40	41 ± 6	1.6 (0.8–3.4)	
IA-2A					0.048
Single	28	11	50 ± 12	1*	
Multiple	34	22	75 ± 9	2.1 (1.0–4.3)	
IA-2A					0.005
IA-2β negative	32	11	38 ± 10	1*	
IA-2β positive	30	22	86 ± 8	2.9 (1.4–6.0)	

*Reference cell used in Cox's proportional hazard model.

analyses, four models were selected, and their ability to stratify diabetes risk compared (Fig. 4, Table 6). Model 1 was based on antibody number with classification of relatives into one islet autoantibody (A), any two autoantibodies (B), and all three autoantibodies (C). Model 2 included all antibody characteristics that contributed significantly to the proportional hazards model. Relatives were classified into those having none (A), one (B), two (C), or all three (D) of the significant diabetes risk covariates (IA-2A titer >25th centile of positives; IgG2 or IgG4 IA-2A subclass positive; and IgG2, IgG3, or IgG4 IAA subclass positive). Model 3 was based on the multivariate analysis that excluded IgG subclasses with classification of relatives into those with none (A), one (B), or both (C) of the significant diabetes risk covariates (IA-2A titer >25th centile of positives and IAA titer >75th centile of positives). Model 4 was based on IA-2A and IA-2β autoantibody measurement with relatives classified into IA-2A

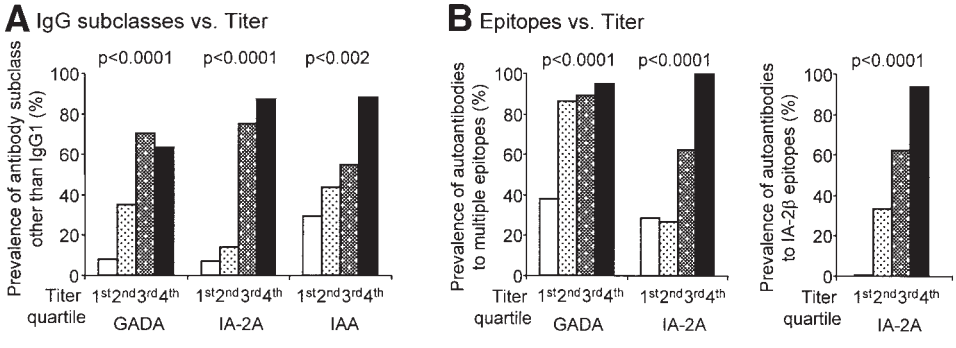

FIG. 2. Relationship between islet autoantibody titer and IgG subclass and eptiope reactivity of islet autoantibodies. For each autoantibody, the prevalences of IgG subclasses other than IgG1 (*A*) and the prevalences of autoantibodies to multiple epitopes or IA-2β epitopes (*B*) were significantly associated with autoantibody titer, which is represented as quartiles of positive titers from lowest (1st) to highest (4th) quartile. For each autoantibody, only positive samples were considered for determining quartiles.

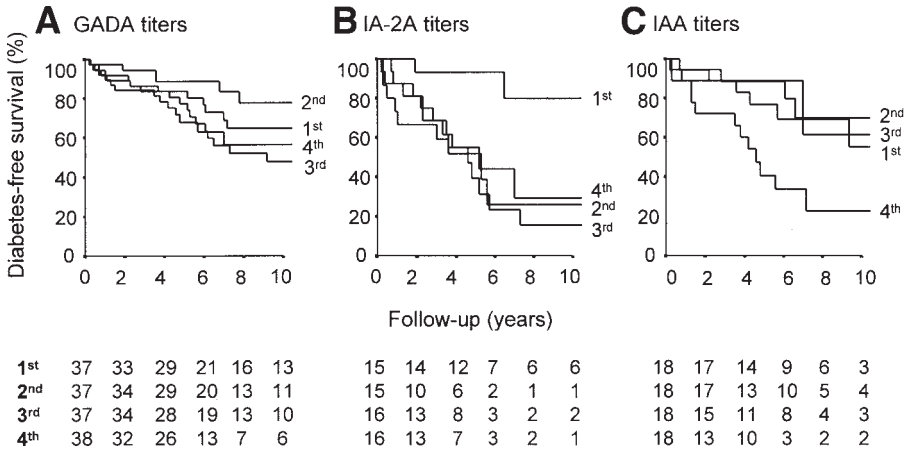

FIG. 3. The cumulative risk of diabetes in relatives in relation to autoantibody titer for GADA (*A*), IA-2A (*B*), and IAA (*C*). Titers for each antibody are stratified as quartiles from lowest (1st) to highest (4th). For each autoantibody, only positive samples were considered for determining quartiles. Increased diabetes risk was observed in relatives with IA-2A titers above the 25th centile and for IAA titers above the 75th centile (see also Table 2). No significant association was found between diabetes risk and GADA titers.

negative (A), IA-2A positive and IA-2β autoantibody negative (B), and IA-2β autoantibody positive (C). Each of these models was able to stratify diabetes risk in the entire cohort (all $P < 0.0001$) (Fig. 4*A*). Models 2, 3, and 4 significantly stratified diabetes risk both within the relatives who had single islet autoantibodies (Fig. 4*B*) and the relatives who had multiple islet autoantibodies (Fig. 4*C*). Addition of models 2, 3, or 4 to model 1 in Cox's proportional hazards model significantly improved diabetes risk stratification over that provided by antibody number alone (model 2, $P < 0.0001$; model 3, $P < 0.0001$; model 4, $P < 0.0001$). Models 2, 3, and 4 could identify autoantibody-positive relatives with a 5-year diabetes risk that was >50%. Model 2 provided the widest risk discrimination (5- and 10-year risks: 7 and 20% in category A, 19 and 47% in category B, 54 and 85% in category C, and 89 and 100% in category D*)* (Table 6).

DISCUSSION

Autoantibodies to islet autoantigens are the most widely used markers of pre–type 1 diabetes, and risk of diabetes in relatives can be defined on the basis of the number of islet autoantibodies detected, together with first-phase insulin response to intravenous glucose (24). We have shown that diabetes risk can be further stratified if other islet autoantibody characteristics including antibody titer, IgG subclass, and/or positivity against IA-2β are also taken into consideration. Risk of type 1 diabetes was increased in relatives with titers of IA-2A and IAA above a threshold that was substantially higher than the 99th centile of control subjects, but risk was not related to GADA titer. The breadth of the humoral response as measured by IgG subclass usage and epitope reactivity was directly related to antibody titer, and for IA-2A and IAA these parameters also correlated with risk. IA-2A were particularly strong predictors of type 1 diabetes in this cohort and indicated high risk even in the absence of antibodies to GAD or insulin. On the basis of these findings we have developed a strategy that, using autoantibody testing alone, is able to identify a subgroup of relatives with an 89% risk of diabetes within 5 years and a subgroup of relatives with islet autoantibodies whose risk was <10%.

The particular strengths of this study are the relatively large number of islet autoantibody-positive relatives fol-

TABLE 3
Combinations of islet autoantibody IgG subclasses and risk for type 1 diabetes

Subclass combination				GAD antibodies		IA-2 antibodies		Insulin antibodies	
IgG1	IgG2	IgG3	IgG4	*n** (type 1 diabetes)	10-year risk†	*n** (type 1 diabetes)	10-year risk†	*n** (type 1 diabetes)	10-year risk†
+	−	−	−	79 (23)	34%	29 (11)	54%	19 (4)	33%
+	+	+	+	3 (3)		2 (1)		5 (3)	
+	+	+	−	0		1 (1)		1 (0)	
+	+	−	−	15 (11)	44%	3 (2)	100%‡	4 (2)	67%‡
+	−	+	+	0		0		4 (3)	
+	+	−	+	17 (2)		19 (15)		4 (3)	
+	−	−	+	30 (9)		4 (3)		2 (0)	
+	−	+	−	0		0		7 (2)	
−	+	+	+	0		0		0	
−	−	+	+	0		0		0	
−	+	+	−	0		0		1 (0)	67%§
−	+	−	+	0		0		0	
−	+	−	−	1 (0)		0		2 (1)	
−	−	+	−	0		0		5 (3)	
−	−	−	+	0		0		4 (2)	
−	−	−	−	4 (1)		4 (0)		14 (3)	24%

*Number of relatives positive for that autoantibody IgG subclass combination; †risk of diabetes calculated using life-table analysis (risks are not shown for cells containing less than five relatives); ‡$P < 0.005$ vs. IgG1 only; §$P = 0.01$ vs. IgG1 only, log rank test.

TABLE 4
Combinations of islet autoantibody epitopes and risk for type 1 diabetes

GAD antibodies						IA-2 antibodies				
Epitope combination*				n† (type 1 diabetes cases)	10-year risk‡	Epitope combination*			n† (type 1 diabetes cases)	10-year risk‡
MID	COOH	NH2	67			IA-2β	PTP	JM		
+	+	+	+	24 (8)	39%	+	+	+	13 (8)	81%
+	+	+	−	12 (6)	26%	+	+	−	12 (10)	100%§
+	+	−	+	15 (5)	56%	+	−	+	1 (1)	
+	+	−	−	62 (21)	45%	+	−	−	4 (3)	
+	−	+	−	1 (0)		−	+	+	8 (3)	34%
+	−	−	−	13 (3)	27%	−	+	−	10 (4)	44%
−	+	+	+	1 (0)		−	−	+	9 (2)	41%
−	+	−	−	5 (2)	48%					
−	−	+	−	6 (1)	20%					
−	−	−	−	10 (3)	33%	−	−	−	4 (1)	

*For GAD antibodies, MID refers to epitopes within GAD65 amino acids 235–442, COOH refers to epitopes within GAD65 amino acids 436–585, NH2 refers to epitopes within GAD65 amino acids 1–100, and GAD67 refers to epitopes found in GAD67. For IA-2 antibodies, IA-2β refers to epitopes found in the PTP region of IA-2β and IA-2, PTP refers to epitopes found in the PTP region of IA-2 and not IA-2β, and JM refers to epitopes within the IA-2 juxtamembrane region amino acids 601–682 (combinations with no relatives are not shown). †n refers to the number of relatives positive for that epitope combination; ‡risk of diabetes calculated using life-table analysis (risks are not shown for cells containing less than five relatives); §$P = 0.009$ vs. PTP and/or JM-positive/IA-2β–negative groups, log rank test.

lowed, the duration of follow-up, and the extensive autoantibody characteristics examined. The number and combinations of islet autoantibodies found in the study cohort were similar to those in biochemical islet autoantibody-positive relatives identified in the Diabetes Prevention Trial 1 (DPT-1) clinical trial (9), suggesting that the findings are unlikely to be biased by inappropriate selection. Limitations of the study include the lack of metabolic data, which means that we have not been able to compare the models based on autoantibodies alone with others that include both autoantibody and metabolic testing, and the complexity of the analyses, which could result in false-positive or false-negative associations. Previous studies have found that some family members with all four islet autoantibodies (ICA, GADA, IA-2A, and IAA) appear relatively protected from developing diabetes (6,13), thereby limiting the use of autoantibody testing alone for accurate risk assessment. We also found that up to 30% of relatives positive for three autoantibodies are likely to be diabetes-free for at least 10 years. Although confidence intervals were wide, the study has been able to identify a subset of relatives with extremely high risk (all developed type 1 diabetes within 6 years of follow-up). Metabolic testing is unlikely to have improved risk stratification in this group, though we cannot exclude the possibility that it would have been useful in the remaining relatives at lower risk.

A general inverse relationship between antibody level and potentially β-cell–destructive T-cell responses, particularly to GAD, has been suggested (25). We have, however, previously shown that ICA titer was directly related to risk of type 1 diabetes (12) and have now found that high titers of IA-2A and IAA, though not GADA, are also associated with increased risk. Together with the observation that titer is related to IgG subclass usage and epitope reactivity, these findings do not support the hypothesis that a strong humoral response to autoantigens marks a protective nondestructive Th2 response. IA-2A were particularly strong predictors of diabetes in this cohort, and as in other studies (26,27), were highly specific indicators of risk even in the absence of autoantibodies to GAD or insulin. In contrast, GADA and IAA are found in other diseases and, in the absence of IA-2A, are associated with a relatively low risk or slow progression to diabetes (4,28). IA-2A may develop later than IAA and GADA in the preclinical disease (7,29). A marked rise in IA-2A titer is seen in some relatives close to diabetes onset (P.A., unpublished observation) and, in the model of islet transplantation where alloislets are placed into patients with long-standing type 1 diabetes, IA-2A are activated only when there is clear evidence of alloimmunity (30), whereas GADA can be immediately activated upon exposure to islet mass without evidence of alloreactivity. These observations support the hypothesis that IA-2A are markers of active β-cell destruction and suggest that autoimmunity against IA-2

TABLE 5
Type 1 diabetes risk in autoantibody-positive relatives: multivariate analysis

Islet autoantibody characteristics significant in univariate analysis	n	Type 1 diabetes (n)	Adjusted HR (95% CI)	P
Two or more antibodies	80	37	1.6 (0.6–3.8)	0.32
IA-2A high titer	47	30	5.4 (1–29)	0.05
IAA high titer	18	12	1.0 (0.4–2.7)	0.97
IA-2A subclass	29	22	3.3 (1.4–8.1)	0.008
IAA subclass	39	19	4.6 (1.5–14)	0.007
Multiple IA-2A epitopes	34	22	1.2 (0.5–3)	0.70
IA-2β antibody positive	30	22	1.1 (0.4–3.2)	0.83

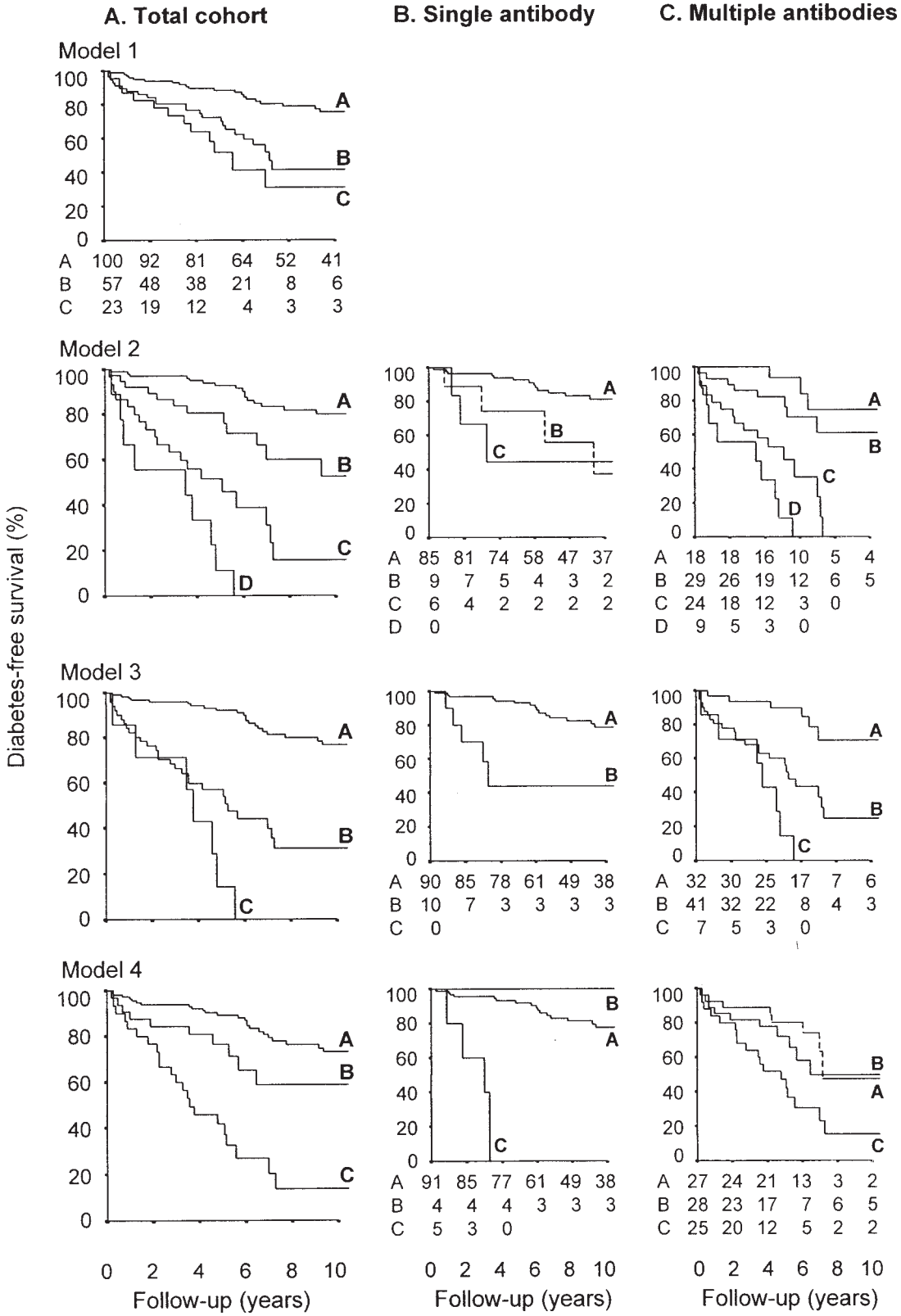

FIG. 4. The cumulative risk of diabetes in autoantibody-positive relatives classified on the basis of islet autoantibody characteristics. Stratification is shown for the total cohort (*A*), for the 100 relatives with one islet autoantibody (*B*), and for the 80 relatives with two or more islet autoantibodies (*C*). Model 1 shows stratification of relatives by the number of islet autoantibodies (A = one autoantibody, B = any two autoantibodies, C = all three autoantibodies). Model 2 shows stratification of relatives by their autoantibody titer and subclass (A = none, B = one, C = two, and D = all three of high-titer IA-2A, positivity for IgG2 or IgG4 IA-2A, and positivity for IgG2, IgG3 or IgG4 IAA). Model 3 shows stratification of relatives by their autoantibody titer (A = none, B = one, C = both of high-titer IA-2A or high-titer IAA). Model 4 shows stratification of relatives by their IA-2A and IA-2β autoantibody status (A = IA-2A negative, B = IA-2A positive/IA-2β autoantibody negative, C = IA-2β autoantibody positive).

TABLE 6
Performance of type 1 diabetes risk stratification models

Risk stratification model*	n	Type 1 diabetes	Diabetes risk	
			5 years	10 years
Model 1				
Category A	100	22 (37%)	12% (6–18)	25% (15–35)
Category B	57	25 (42%)	30% (18–42)	59% (41–77)
Category C	23	12 (20%)	48% (26–70)	69% (43–95)
Model 2				
Category A	103	19 (32%)	7% (2–12)	20% (11–29)
Category B	38	12 (20%)	19% (6–32)	47% (25–69)
Category C	30	19 (32%)	54% (34–74)	85% (66–100)
Category D	9	9 (15%)	89% (68–100)	100%
Model 3				
Category A	122	24 (41%)	8% (3–13)	23% (14–32)
Category B	51	28 (47%)	43% (28–58)	69% (52–86)
Category C	7	7 (12%)	86% (60–100)	100%
Model 4				
Category A	118	26 (44%)	11% (5–17)	27% (17–37)
Category B	32	11 (19%)	24% (8–40)	41% (20–62)
Category C	30	22 (37%)	63% (45–81)	86% (70–100)

Data are n (%) and % (95% CI). *Model 1—antibody number: category A = one autoantibody, category B = any two autoantibodies, category C = all three autoantibodies (IAA, IA-2A, GADA); Model 2—antibody titer (IA-2A) and subclass (IA-2A and IAA): category A = none, category B = one, category C = two, category D = all three of high-titer IA-2A, positivity for IgG2 or IgG4 IA-2A, and positivity for IgG2, IgG3 or IgG4 IAA; Model 3—antibody titer (IA-2A and IAA): category A = no high-titer IA-2A or high-titer IAA, category B = high-titer IA-2A or high-titer IAA, category C = high-titer IA-2A and high-titer IAA; Model 4—IA-2A epitopes: category A = IA-2A negative, category B = IA-2A positive but IA-2β autoantibody negative, category C = IA-2β autoantibody positive.

participates in advancing β-cell destruction to clinical disease or has characteristics that promote the process.

The associations between diabetes risk and autoantibody characteristics such as subclass and epitope reactivity have varied between studies (15,16,23,31–39). Most have, however, examined these characteristics only for single antibodies without considering the other islet autoantibodies present or antibody titer. In this cohort, IA-2A and IAA IgG subclass and IA-2A epitope reactivity were found to modify risk. Although other diabetes-associated characteristics were strongly associated with high-titer antibodies, multivariate analysis indicated that IgG subclass status significantly improved risk estimation based on IA-2A and was superior to titer for IAA. The reason for this additional benefit is unclear. These characteristics may reflect specific mechanisms underlying the pathogenesis of type 1 diabetes or may simply improve the accuracy of measurement of antibody titer, which may appear artifactually low if antibodies bind to multiple epitopes. We favor the hypothesis that titer is the primary marker of diabetes risk and that multiple IgG subclasses and IA-2β positivity act as confirmatory markers of high titer responses.

The size of our study has allowed us to consider a number of models combining different antibody characteristics. The most effective model included quantification of IA-2A and measurement of IA-2A and IAA IgG subclasses. The feasibility of applying such a model to clinical trials depends on practical considerations such as the ability to measure these characteristics reproducibly. Preliminary studies suggest that quantification of IA-2A is relatively concordant between laboratories (20) and initial assessment of GADA IgG subclass measurement indicated that some, though not all, laboratories have sensitive, specific, and concordant assays (E.B., unpublished observation). The strength of the associations between type 1 diabetes risk and IA-2A titer, IA-2A IgG subclass, and IAA IgG subclass suggest that efforts at standardized and reliable measurement are worthwhile. An alternative, less effective, but relatively simple model was based on only IA-2A and IA-2β antibody testing, and even a single measurement of IA-2β antibodies was an effective test to identify those at highest risk. The number of islet autoantibodies did not significantly improve the Cox models based on antibody titer, subclasses, and/or epitopes (data not shown). Nevertheless, the risk assessment models were effective in stratifying risk both in relatives who were positive for a single islet autoantibody marker and in relatives who had multiple islet autoantibodies, suggesting that other variations of these models that also include the number of islet autoantibodies might be effective in stratifying diabetes risk.

We have shown that the combination of autoantibody titer, subclass, and/or epitope reactivity may improve type 1 diabetes risk stratification, which could be effectively stratified on the basis of these characteristics in a single sample. These observations need to be validated in other large cohorts but, if replicated, they have the potential to simplify screening and recruitment for clinical trials.

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REFERENCES

1. Atkinson MA, Eisenbarth GS: Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 358:221–229, 2001
2. Gorsuch AN, Spencer KM, Lister J, McNally JM, Dean BM, Bottazzo GF, Cudworth AG: Evidence for a long prediabetic period in type I (insulin-dependent) diabetes mellitus. *Lancet* 2:1363–1365, 1981
3. Srikanta S, Ganda OP, Rabizadeh A, Soeldner JS, Eisenbarth GS: First-degree relatives of patients with type I diabetes mellitus: islet-cell antibodies and abnormal insulin secretion. *N Engl J Med* 313:461–464, 1985
4. Bingley PJ, Christie MR, Bonifacio E, Bonfanti R, Shattock M, Fonte MT, Bottazzo GF, Gale EA: Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. *Diabetes* 43:1304–1310, 1994
5. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, Chase HP, Eisenbarth GS: Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 45:926–933, 1996
6. Kulmala P, Savola K, Petersen JS, Vahasalo P, Karjalainen J, Lopponen T, Dyrberg T, Akerblom HK, Knip M: Prediction of insulin-dependent diabetes mellitus in siblings of children with diabetes: a population-based study: the Childhood Diabetes in Finland Study Group. *J Clin Invest* 101:327–336, 1998
7. Ziegler AG, Hummel M, Schenker M, Bonifacio E: Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study. *Diabetes* 48:460–468, 1999
8. LaGasse JM, Brantley MS, Leech NJ, Rowe RE, Monks S, Palmer JP, Nepom GT, McCulloch DK, Hagopian WA: Successful prospective prediction of type 1 diabetes in schoolchildren through multiple defined autoantibodies: an 8-year follow-up of the Washington State Diabetes Prediction Study. *Diabetes Care* 25:505–511, 2002
9. Krischer JP, Cuthbertson DD, Yu L, Orban T, Maclaren N, Jackson R, Winter WE, Schatz DA, Palmer JP, Eisenbarth GS: Screening strategies for the identification of multiple antibody-positive relatives of individuals with type 1 diabetes. *J Clin Endocrinol Metab* 88:103–108, 2003
10. Bingley PJ: Interactions of age, islet cell antibodies, insulin autoantibodies, and first-phase insulin response in predicting risk of progression to IDDM in ICA+ relatives: the ICARUS data set: Islet Cell Antibody Register Users Study. *Diabetes* 45:1720–1728, 1996
11. Maclaren N, Lan M, Coutant R, Schatz D, Silverstein J, Muir A, Clare-Salzer M, She JX, Malone J, Crockett S, Schwartz S, Quattrin T, DeSilva M, Vander Vegt P, Notkins A, Krischer J: Only multiple autoantibodies to islet cells (ICA), insulin, GAD65, IA-2 and IA-2beta predict immune-mediated (type 1) diabetes in relatives. *J Autoimmun* 12:279–287, 1999
12. Bonifacio E, Bingley PJ, Shattock M, Dean BM, Dunger D, Gale EA, Bottazzo GF: Quantification of islet-cell antibodies and prediction of insulin-dependent diabetes. *Lancet* 335:147–149, 1990
13. Gardner SG, Gale EA, Williams AJ, Gillespie KM, Lawrence KE, Bottazzo GF, Bingley PJ: Progression to diabetes in relatives with islet autoantibodies: is it inevitable? *Diabetes Care* 22:2049–2054, 1999
14. King CL, Nutman TB: IgE and IgG subclass regulation by IL-4 and IFN-gamma in human helminth infections: assessment by B cell precursor frequencies. *J Immunol* 151:458–465, 1993
15. Butler MH, Solimena M, Dirkx R Jr, Hayday A, De Camilli P: Identification of a dominant epitope of glutamic acid decarboxylase (GAD-65) recognized by autoantibodies in stiff-man syndrome. *J Exp Med* 178:2097–2106, 1993
16. Kim J, Namchuk M, Bugawan T, Fu Q, Jaffe M, Shi Y, Aanstoot HJ, Turck CW, Erlich H, Lennon V: Higher autoantibody levels and recognition of a linear NH2-terminal epitope in the autoantigen GAD65, distinguish stiff-man syndrome from insulin-dependent diabetes mellitus. *J Exp Med* 180:595–606, 1994
17. Dittler J, Seidel D, Schenker M, Ziegler AG: GADIA2-combi determination as first-line screening for improved prediction of type 1 diabetes in relatives. *Diabetes* 47:592–597, 1998
18. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 20:1183–1197, 1997
19. Naserke HE, Bonifacio E, Ziegler AG: Immunoglobulin G insulin autoantibodies in BABYDIAB offspring appear postnatally: sensitive early detection using a protein A/G-based radiobinding assay. *J Clin Endocrinol Metab* 84:1239–1243, 1999
20. Bingley PJ, Bonifacio E, Mueller PW: Diabetes antibody standardization program: first assay proficiency evaluation. *Diabetes* 52:1128–1136, 2003
21. Bonifacio E, Lampasona V, Bernasconi L, Ziegler AG: Maturation of the humoral autoimmune response to epitopes of GAD in preclinical childhood type 1 diabetes. *Diabetes* 49:202–208, 2000
22. Bonifacio E, Lampasona V, Bingley PJ: IA-2 (islet cell antigen 512) is the primary target of humoral autoimmunity against type 1 diabetes-associated tyrosine phosphatase autoantigens. *J Immunol* 161:2648–2654, 1998
23. Bonifacio E, Scirpoli M, Kredel K, Fuchtenbusch M, Ziegler AG: Early autoantibody responses in prediabetes are IgG1 dominated and suggest antigen-specific regulation. *J Immunol* 163:525–532, 1999
24. Bingley PJ, Bonifacio E, Ziegler AG, Schatz DA, Atkinson MA, Eisenbarth GS: Proposed guidelines on screening for risk of type 1 diabetes (Commentary). *Diabetes Care* 24:398, 2001
25. Harrison LC, Honeyman MC, DeAizpurua HJ, Schmidli RS, Colman PG, Tait BD, Cram DS: Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin-dependent diabetes. *Lancet* 341:1365–1369, 1993
26. Decochez K, De Leeuw IH, Keymeulen B, Mathieu C, Rottiers R, Weets I, Vandemeulebroucke E, Truyen I, Kaufman L, Schuit FC, Pipeleers DG, Gorus FK, Belgian Diabetes Registry: IA-2 autoantibodies predict impending type I diabetes in siblings of patients. *Diabetologia* 45:1658–1666, 2002
27. Christie MR, Genovese S, Cassidy D, Bosi E, Brown TJ, Lai M, Bonifacio E, Bottazzo GF: Antibodies to islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity. *Diabetes* 43:1254–1259, 1994
28. Ziegler AG, Ziegler R, Vardi P, Jackson RA, Soeldner JS, Eisenbarth GS: Life-table analysis of progression to diabetes of anti-insulin autoantibody-positive relatives of individuals with type I diabetes. *Diabetes* 38:1320–1325, 1989
29. Kimpimaki T, Kulmala P, Savola K, Kupila A, Korhonen S, Simell T, Ilonen J, Simell O, Knip M: Natural history of beta-cell autoimmunity in young children with increased genetic susceptibility to type 1 diabetes recruited from the general population. *J Clin Endocrinol Metab* 87:4572–4579, 2002
30. Bosi E, Braghi S, Maffi P, Scirpoli M, Bertuzzi F, Pozza G, Secchi A, Bonifacio E: Autoantibody response to islet transplantation in type 1 diabetes. *Diabetes* 50:2464–2471, 2001
31. Falorni A, Gambelunghe G, Forini F, Kassi G, Cosentino A, Candeloro P, Bolli GB, Brunetti P, Calcinaro F: Autoantibody recognition of COOH-terminal epitopes of GAD65 marks the risk for insulin requirement in adult-onset diabetes mellitus. *J Clin Endocrinol Metab* 85:309–316, 2000
32. Naserke HE, Ziegler AG, Lampasona V, Bonifacio E: Early development and spreading of autoantibodies to epitopes of IA-2 and their association with progression to type 1 diabetes. *J Immunol* 161:6963–6969, 1998
33. Miao D, Yu L, Tiberti C, Cuthbertson DD, Rewers M, di Mario U, Eisenbarth GS, Dotta F: ICA512(IA-2) epitope specific assays distinguish transient from diabetes associated autoantibodies. *J Autoimmun* 18:191–196, 2002
34. Couper JJ, Harrison LC, Aldis JJ, Colman PG, Honeyman MC, Ferrante A: IgG subclass antibodies to glutamic acid decarboxylase and risk for progression to clinical insulin-dependent diabetes. *Hum Immunol* 59:493–499, 1998
35. Petersen JS, Kulmala P, Clausen JT, Knip M, Dyrberg T: Progression to type 1 diabetes is associated with a change in the immunoglobulin isotype profile of autoantibodies to glutamic acid decarboxylase (GAD65): Childhood Diabetes in Finland Study Group. *Clin Immunol* 90:276–281, 1999
36. Seissler J, Eikamp K, Schott M, Scherbaum WA: IA-2 autoantibodies Restricted to the IgG4 subclass are associated with protection from type 1 diabetes. *Horm Metab Res* 34:186–191, 2002
37. Lohmann T, Hawa M, Leslie RD, Lane R, Picard J, Londei M: Immune reactivity to glutamic acid decarboxylase 65 in stiffman syndrome and type 1 diabetes mellitus. *Lancet* 356:31–35, 2000
38. Hawa MI, Fava D, Medici F, Deng YJ, Notkins AL, De Mattia G, Leslie RD: Antibodies to IA-2 and GAD65 in type 1 and type 2 diabetes: isotype restriction and polyclonality. *Diabetes Care* 23:228–233, 2000
39. Naserke HE, Bonifacio E, Ziegler AG: Prevalence, characteristics and diabetes risk associated with transient maternally acquired islet antibodies and persistent islet antibodies in offspring of parents with type 1 diabetes. *J Clin Endocrinol Metab* 86:4826–4833, 2001