Autoantibodies to a 38-kDa Glycosylated Islet Cell Membrane–Associated Antigen in (Pre)type 1 Diabetes

Association with IA-2 and islet cell autoantibodies

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OBJECTIVE — To study the association of autoantibodies against a 38-kDa glycated islet cell membrane–associated (GLIMA) protein with (pre)type 1 diabetes, patient characteristics, and other immune and genetic markers of the disease and to evaluate the possible added value of GLIMA antibody determinations for disease prediction and classification.

RESEARCH DESIGN AND METHODS — Recent-onset type 1 diabetic patients (n = 100), prediabetic siblings (n = 23), and nondiabetic control subjects (n = 100) were consecutively recruited by the Belgian Diabetes Registry. GLIMA antibodies were determined by immunoprecipitation of radiolabeled islet cell proteins; islet cell antibodies (ICAs) were determined by indirect immunofluorescence; and insulin autoantibodies (IAAs), insulinoma-associated protein-2 antibodies (IA-2As), and GAD antibodies (GADAs) were determined by radioligand assays.

RESULTS — GLIMA antibodies were detected in 38% of type 1 diabetic patients and 35% of prediabetic siblings (during follow-up) vs. 0% in control subjects ($P < 0.001$). Their prevalence was lower than that of other antibodies and was significantly associated with high levels of IA-2A and ICA ($P < 0.0001$). In (pre)diabetes, GLIMA antibodies could only be demonstrated in sera positive for ≥1 other autoantibody.

CONCLUSIONS — GLIMA antibodies are strongly associated with type 1 diabetes and antibody markers of rapid progression to clinical onset but have a lower diagnostic sensitivity for the disease than IAA, ICA, IA-2A, or GADA. In its present form, the GLIMA antibody assay does not provide much additional information for prediction or classification of diabetes, compared with that obtained from the measurement of IA-2As alone or in combination with IAAs, ICAs, and GADAs.

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Abbreviations: GADA, GAD antibody; GLIMA, glycated islet cell membrane–associated protein; IA-2, insulinoma-associated protein-2; IA-2A, IA-2 antibody; IAA, insulin autoantibody; ICA, islet cell antibody; JDF U, Juvenile Diabetes Foundation units; TX-114, Triton X-114.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

The presence of antibodies against an as yet unidentified 38-kDa glycated islet cell membrane–associated (GLIMA) protein has been proposed as a new biological marker of preclinical and recent-onset type 1 diabetes (1,2). It remains an open question so far whether the detection of these autoantibodies provides extra information for prediction or classification of clinical diabetes in addition to that obtained from the presence and levels of established diabetes-associated antibody markers. These include islet cell autoantibodies (ICAs), insulin autoantibodies (IAAs), and antibodies against the 65-kDa isoform of GAD and insulinoma-associated protein-2 (IA-2), a protein tyrosine phosphatase with unknown function in the islet cells (3–5). Taking into account the striking age-dependent heterogeneity of type 1 diabetes in terms of incidence, male-to-female ratio, clinical presentation, and biological markers (3), we have determined GLIMA antibodies in parallel with IAA, ICA, GAD antibodies (GADAs), and IA-2 antibodies (IA-2As) in representative groups of recent-onset type 1 diabetic patients, prediabetic siblings of type 1 patients, and nondiabetic control subjects (0–39 years). This was done with the purpose of *1*) confirming the diabetes-association of GLIMA antibodies, *2*) assessing their possible association with demographic data (age and sex) and with established immune (IAA, ICA, GADA, and IA-2A) or genetic (*HLA DQ* and the 5′ flanking region of insulin gene [5′ *INS*]) markers of type 1 diabetes (6), and *3*) investigating whether the determination of GLIMA antibodies may help refine disease prediction and classification.

RESEARCH DESIGN AND METHODS

— Recent-onset type 1 diabetic patients (n = 100, 0–7 days of insulin treatment) and prediabetic siblings

(n = 23, 3–77 months before diagnosis) aged 0–39 years were consecutively recruited by the Belgian Diabetes Registry (3,7). Type 1 diabetes was diagnosed according to the criteria of the National Diabetes Data Group (8). Demographic and clinical data were obtained by a questionnaire completed by physicians participating in the Belgian Diabetes Registry. Nondiabetic control subjects (n = 100) were recruited among blood donors and laboratory personnel (aged 18–39 years) and among children or adolescents attending emergency departments of Brussels hospitals for minor local surgery (7).

Blood sampling

Blood was collected in K-EDTA tubes (Monovettes; Sarstedt, Essen, Belgium) for HLA-DQ and 5′ INS genotyping and in dry Monovettes (Sarstedt) for all other analytes. In diabetic subjects, blood was sampled before the start or within 7 days of insulin treatment. Serum and K-EDTA blood were transferred to aliquots and stored frozen at −80°C until further analysis.

Autoantibody assays

All antibody assays were performed by operators blinded for the identification and glycemic status of the study subjects. For the GLIMA antibody assay, we prepared Triton X-114 (TX-114) extracts of a subculture of RIN 5AH cells that were shown to have undetectable levels of GAD (9) and IA-2 by immunoprecipitation and Western blotting. The cells were cultured in the presence of ^{35}S methionine (L-[^{35}S]-methionine; Amersham Pharmacia Biotech, Uppsala, Sweden) for 7 h and harvested in HEPES-buffered saline containing protease inhibitors as previously described (9), and the cell pellet was stored frozen at −80°C until further processing. The cells were lysed in buffer containing 2% TX-114 for 2 h by repeated dispersion through a constricted pipette tip, followed by a 30-min centrifugation to remove insoluble material. Amphiphilic proteins were isolated by temperature-induced TX-114 phase separation (10). For the immunoprecipitation, an equivalent of 2.5×10^6 cpm of detergent-phase purified extract was incubated overnight with 5 μl of serum in a total volume of 25 μl. In blocking studies to investigate the possibility that epitopes for GLIMA antibodies are shared with IA-2, 5 μg of unlabelled recombinant IA-2 was added during the overnight incubation. Immune complexes were isolated using 5 μl of protein A-Sepharose per sample. Immunoprecipitates were evaluated by SDS-PAGE using 10% polyacrylamide gels and autoradiography. A sample was considered positive if a typical GLIMA38 band (1,2) was visible within 3 months of exposure to the X-ray film. Fluctuations in immune reactivity were evaluated by a semiquantitative analysis on a densitometric scan of the precipitated GLIMA38 protein. The intensity of GLIMA bands was related to that of a positive control serum obtained from a patient with recent-onset type 1 diabetes and was expressed as a GLIMA index (1).

ICAs were measured by indirect immunofluorescence using cryosections of human blood group O donor pancreata (11). IAAs, GADAs, and IA-2As were determined by liquid-phase radiobinding assays using, respectively, ^{125}I-labeled insulin, ^{35}S-labeled GAD65, and the ^{35}S-labeled intracellular domain of IA-2 (IA-2ic) as tracer (12). The cutoff values for ICAs (≥12 Juvenile Diabetes Foundation units [JDF U]), IAAs (≥0.6% tracer-bound), GADAs (≥2.6% tracer-bound), and IA-2As (≥0.4% tracer-bound) were established as the 99th percentile of 783 healthy control subjects after omission of outlying values (12). The IAA, ICA, IA-2A, and GADA assays repeatedly achieved 100% diagnostic sensitivity, specificity, consistency, and validity in serum exchange programs of the University of Florida and the Louisiana State University. In the combinatorial islet antibody workshop, assay sensitivity that was adjusted for 99% specificity amounted to 73, 36, and 85% for ICA, IAA, and GADA, respectively (IA-2A assay was not yet performed in 1995) (13).

HLA-DQ and 5′ INS genotyping

DNA-polymorphisms at the HLA-DQ gene and 5′ flanking region of the insulin gene were determined as previously described (14).

Statistical analysis

Differences between groups were assessed by two-tailed χ^2 test with Yates correction or Fisher's exact test, when appropriate, for categorical variables and with Mann-Whitney U test for continuous variables. Differences were considered significant at $P < 0.05$ or, in the case of k comparisons, at $P < 0.05/k$ (Bonferroni adjustment). Stepwise forward logistic regression was used for multivariate analysis. All statistical tests were performed by SPSS for Windows version 8.0 (SPSS, Chicago, IL) for personal computers.

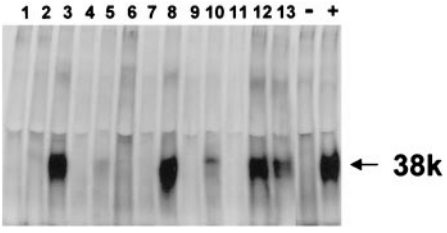

Figure 1—*Immunoprecipitation analysis of GLIMA antibodies in sera. Test sera from diabetic patients (1–13) and negative (–) or positive (+) control sera were analyzed for the ability to immunoprecipitate a 38,000-Mr (38k, arrow) protein from extracts of ^{35}S-methionine–labeled RIN m5AH cells. The figure illustrates a typical autoradiogram from the analysis. The samples that tested positive (as determined by densitometry from the original gels) are* lanes 3, 5 *(weak),* 8, 10, 12, *and* 13.

RESULTS — Examples of immunoprecipitation patterns observed in GLIMA antibody–positive and –negative sera are shown in Fig. 1. GLIMA antibodies were detected in 38 of 100 recent-onset type 1 diabetic patients (38%) but in none of the 100 nondiabetic control subjects (0%) ($P < 0.001$ vs. control subjects). Positivity for GLIMA antibodies was also found in 6 of 23 (26%) prediabetic siblings at first sampling ($P < 0.001$). Two more siblings became positive after initial sampling but before diabetes onset. Thus, GLIMA antibodies were observed at some point before diagnosis in 8 of 23 siblings (35%). In both patients and siblings at first sampling, GLIMA antibodies were less prevalent than GADAs (80 and 74%, respectively), ICAs (66 and 70%), IA-2As (60 and 61%), and IAAs (38 and 52%).

The occurrence of GLIMA antibodies was not dependent on sex, GADA, IAA, or certain genetic markers (*HLA-DQA1* 0501-DQB1* 0201* haplotype in patients and relatives; the *5′ INS I/I* risk genotype was only investigated in patients) (Table 1). At clinical onset, the prevalence of GLIMA antibodies tended to be higher in patients under the age of 15 years at diagnosis or in those carrying the *HLA-DQA1* 0301-DQB1* 0302* risk haplotype (P = 0.04) (Table 1). GLIMA antibodies were almost exclusively detected in IA-2A– or

Table 1—*Prevalence of antibodies against GLIMA in recent-onset type 1 diabetic patients and prediabetic siblings at first sampling*

Characteristics	Prevalence of GLIMA antibodies	
	Type 1 diabetic patients	Prediabetic siblings (first sample)
All subjects	38/100 (38)	6/23 (26)
Male	18/56 (32)	3/12 (25)
Female	20/44 (46)	3/11 (27)
Age (years)		
0–14	20/39 (51)	3/15 (20)
15–39	18/61 (30)*	3/8 (38)
ICA (JDF U)		
≥ 12	37/66 (56)	6/16 (38)
< 12	1/34 (3)†	0/7 (0)
GADA (%)		
≥2.6	30/80 (38)	5/17 (29)
<2.6	8/20 (40)	1/6 (17)
IA-2A (%)		
≥0.4	36/60 (60)	6/14 (43)
<0.4	2/40 (5)‡	0/9 (0)§
IAA (%)		
≥0.6	16/38 (42)	3/12 (25)
<0.6	22/62 (36)	3/11 (27)
IAA and/or ICA and/or GADA and/or IA-2A	38/92 (41)	6/22 (27)
No IAA, no ICA, no GADA, no IA-2A	0/8 (0)	0/1 (0)
HLA-DQAI*-DQBI*		
0301-0302	28/60 (47)	5/19 (26)
Non(0301-0302)	10/40 (25)‖	1/4 (25)
0501-0201	18/53 (34)	3/15 (20)
Non(0501-0201)	20/47 (43)	3/8 (38)
5′ INS		
I/I	26/65 (40)	not done
Non (I/I)	11/32 (34)	

Data are *n* (%). Fisher's exact test: *$P = 0.04$ vs. age 0–14 years, †$P < 0.001$ vs. ICA ≥12 JDF U; ‡$P < 0.001$, and §$P < 0.05$ vs. IA-2A ≥0.4%; ‖$P < 0.04$ vs. 0301-0302. Threshold for significance: $P < 0.05/19$ or $P < 0.003$ (Bonferroni correction for multiple comparisons).

ICA-positive patients ($P < 0.001$ vs. absence of IA-2A or ICA) or siblings ($P < 0.05$ vs. absence of IA-2A) (Table 1). In patients, ICA and IA-2A levels were significantly higher in the presence of GLIMA antibodies than in their absence ($P < 0.0001$) (Fig. 2). A similar nonsignificant tendency was noted in siblings (Fig. 2). When patients and prediabetic siblings were pooled, the difference in IA-2A and ICA levels according to GLIMA antibody status persisted ($P < 0.0001$), even when the analysis was restricted to IA-2A–positive or ICA-positive subjects (not shown). In both patients and siblings, GLIMA antibodies could not be detected in the combined absence of IAA, ICA, GADA, and IA-2A (Table 1).

Taking into account sex, age, presence of antibodies (IAA, ICA, GADA, and IA-2A), and *HLA-DQA1* -DQB1** risk haplotypes (*0301-0302* or *0501-0201*), multivariate analysis confirmed the preferential association of GLIMA antibodies with IA-2As ($P < 0.002$) and ICAs ($P < 0.01$) in patients. Taking into consideration log-transformed levels of IAA, ICA, GADA, and IA-2A instead of antibody prevalence, multivariate analysis revealed only an association between GLIMA antibody positivity and IA-2A levels ($P < 0.001$). Incubation of GLIMA antibody–positive sera with 5 μg recombinant human IA-2ic, conditions that effectively block antibody binding to IA-2 and—in many sera—IA-2β (15,16), failed to block antibody binding to GLIMA (Fig. 3) in the immunoprecipitation test, indicating that the association between antibodies to IA-2 and GLIMA is not the result of shared antigenic epitopes. GLIMA-positive and GLIMA-negative patients did not differ significantly in terms of age, prevalence of ketonuria, frequency or duration of typical clinical symptoms (polyuria, polydipsia, weight loss, and fatigue), random C-peptide levels, HbA_{1c} levels, and daily insulin dose (results not shown).

We found that 8 of 23 prediabetic siblings (35%) became positive for GLIMA antibodies at some time during the preclinical phase (Table 2). In none of these subjects were GLIMA antibodies detected before the appearance of other autoantibody markers. One prediabetic sibling seroconverted to GLIMA antibody positivity before clinical onset, but that subject remained IA-2A–negative (Table 2). The only prediabetic sibling found to be positive for ICAs but negative for the molecular autoantibodies (GADA, IA-2A, and IAA) remained negative for GLIMA antibodies (not shown).

CONCLUSIONS — Our results confirm that antibodies against a 38-kDa GLIMA protein mark the early phase of type 1 diabetes (1,2). In the present series of 100 patients and 23 prediabetic siblings consecutively recruited by the Belgian Diabetes Register, the prevalence of GLIMA antibodies was, respectively, 38 and 35% at some time point during follow-up, which is higher than the prevalences reported in previous publications (23% in patients, $P = 0.006$ by Fisher's exact test; 14% in first degree relatives, $P = 0.06$) (1,2). The higher sensitivity in our study was not obtained at the expense of a lower specificity because none of the 100 age-matched control subjects tested positive. Furthermore, the present study documents in both patients and prediabetic siblings a striking association of GLIMA antibody–positivity with the presence of IA-2A, a strong predictor of imminent clinical onset in prediabetes (17–19). Because the probability of progressing to clinical diabetes increases with ICA titer (20) and IA-2A levels (19) in first degree relatives, the association of these high antibody levels with GLIMA antibody positivity suggests that the appearance of this new marker signals future diabetes onset. Similar to IA-2A, GLIMA antibodies tended to be more frequent in patients with childhood-onset diabetes and in those carrying *HLA-DQA1* 0301-DQB1* 0302* (18), but after adjustment for the presence of IA-2A, these associ-

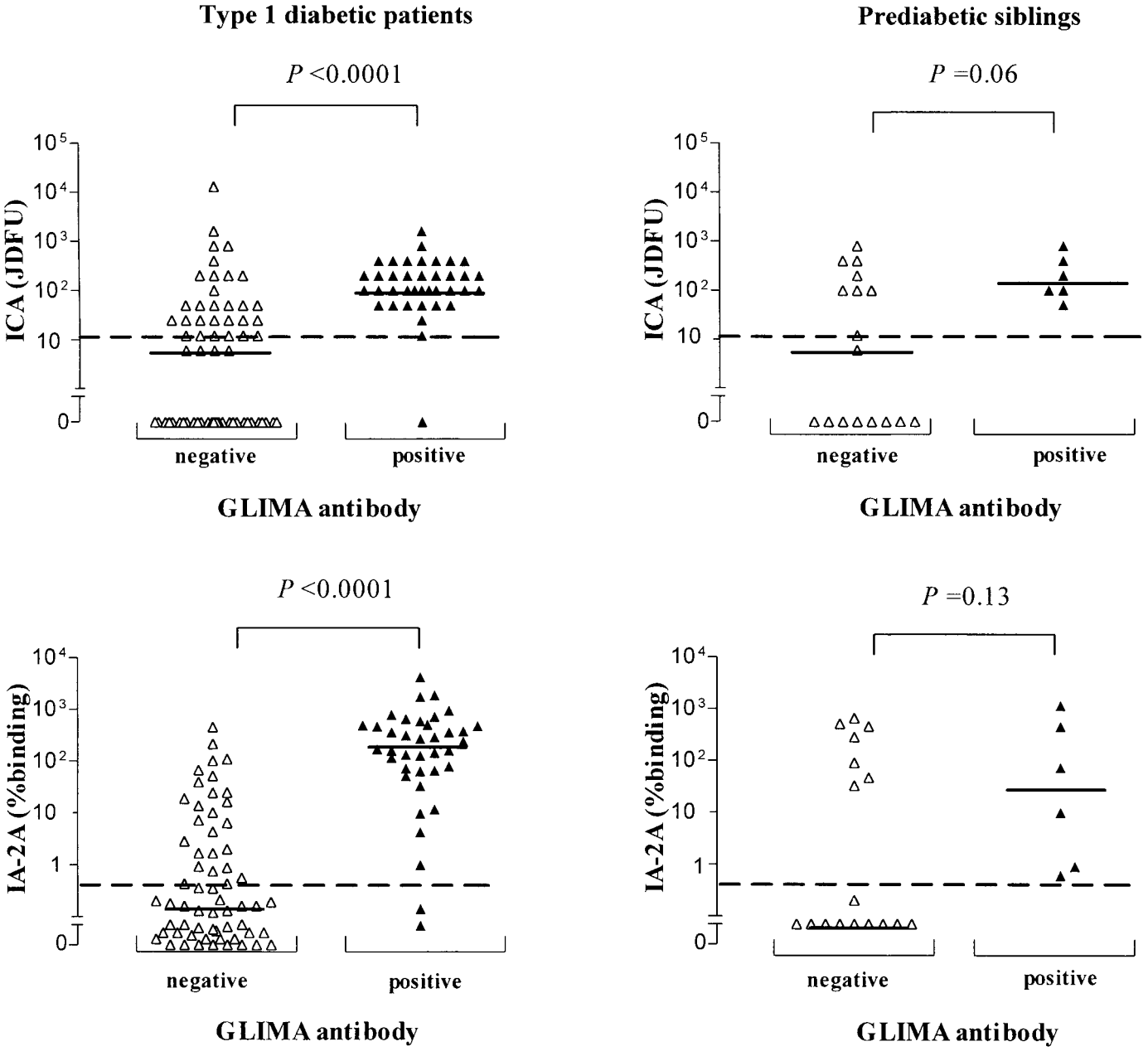

Figure 2— *Levels of IA-2A and ICA in recent-onset type 1 diabetic patients and in prediabetic siblings stratified according to the presence (▲) or absence (△) of GLIMA antibodies. The horizontal dotted line represents the cutoff for ICA positivity or IA2-A positivity, and the horizontal full line represents the median value for each subject group (*P *value by Mann-Whitney* U *test).*

ations disappeared in multivariate analysis. Despite the strong association between IA-2A and GLIMA antibodies, it appears that the 38-kDa GLIMA antigen is not structurally related to IA-2 because preincubation of GLIMA antibody–positive sera with recombinant human IA-2ic fails to block positivity in the immunoprecipitation assay. Moreover, several sera showed discordant results for both IA-2A and GLIMA antibody positivity.

Using available technology, the diagnostic sensitivity of GLIMA antibodies in (pre)diabetes is still lower than that for IAAs, ICAs, IA-2As, and GADAs. At variance with Roll et al. (2), we could not find examples of GLIMA antibodies appearing as first autoantibody during the prediabetic phase. In contrast to IA-2As and GADAs, which usually remain detectable for years after clinical onset (12), GLIMA antibodies were reported to disappear rapidly after diagnosis (1). Therefore, the time window during which GLIMA antibodies can be detected in (pre)diabetes may, on average, be shorter than the window for GADAs or IA-2As. In neither patients nor siblings could we detect one single sample with GLIMA antibody positivity in the combined absence of IAA, ICA, IA-2A, and GADA. The only ICA-positive but GADA- and IA-2A–negative prediabetic subject we identified remained GLIMA antibody–negative, indicating that GLIMA was not the islet antigen recognized by the patient's ICA (18).

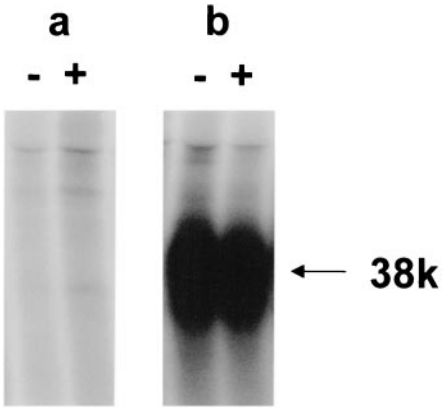

Figure 3— *Lack of cross-reactivity of antibodies to GLIMA and IA-2. Sera from a patient negative (a) or positive (b) for antibodies to GLIMA were incubated with extracts of ^{35}S-methionine–labeled RIN m5AH cells in the absence (–) or presence (+) of 5 μg of purified recombinant IA-2ic. The addition of IA-2ic failed to block antibody binding to the 38,000-Mr (38k, arrow) GLIMA.*

Finally, the 38-kDa autoantigen has not yet been identified and cloned. Hence, radiobinding assays using radioactively labeled recombinant human 38-kDa antigen are not available at the present time, rendering the measurement of GLIMA antibodies tedious and, at present, less suitable for high-throughput screening than IA-2A or GADA assays. The elaboration of a sensitive molecular binding assay for GLIMA antibodies should greatly facilitate the study of their precise relationship to IA-2A and GADA, and it should allow to better define their diagnostic performance for disease classification and prediction. Further characterization of the GLIMA antigen by both peptide mapping and the study of glycation patterns will provide a basis for its subsequent purification and identification (2).

In conclusion, the present data confirm the association of GLIMA antibodies with type 1 diabetes, but antibody prevalence was higher than that previously reported. They also document the strong association between GLIMA antibodies and high levels of IA-2As and ICAs, which are considered markers of rapid progression to clinical diabetes in risk groups. However, in its present form, the GLIMA antibody assay is less sensitive, less quantitative, and more time-consuming than the IA-2A assay, and thus it does not provide much additional information for the prediction or classification of diabetes compared with information obtained from the measurement of IA-2As alone or in combination with IAAs, ICAs, and/or GADAs.

Table 2—*Levels of diabetes associated antibodies in serum samples of siblings who tested GLIMA antibody–positive on at least one occasion during the preclinical phase of type 1 diabetes*

Sibling no.	Sex	Age (years)	Months before diagnosis	Autoantibodies				
				GLIMA antibodies	ICA (JDF U)	GADA (%)	IA-2A (%)	IAA (%)
1	female	2	−25	–	0	0.2	<0.1	0.3
		3	−5	**+**	**12**	**510.5**	<0.1	**1.3**
		5	12	**+**	**50**	**594.5**	0.1	**42.4**
2	male	4	−49	**+**	**100**	**12.9**	**0.9**	**7.2**
		8	−2	–	0	**33.7**	**0.9**	0.5
3	female	9	−19	**+**	**800**	**697.0**	**1,132.6**	**2.9**
		10	4	**+**	**800**	**195.6**	**527.0**	**24.2**
4	male	9	−77	–	0	**4.5**	<0.1	**1.6**
		10	−65	**+**	**400**	**9.1**	**5.8**	**2.0**
		15	0	**+**	**100**	**4.9**	**204.3**	**0.7**
5	male	12	−18	**+**	**50**	**226.3**	**0.6**	**1.3**
		13	−7	**+**	**50**	**175.1**	**0.6**	**0.8**
		14	11	–	**25**	**104.6**	0.2	**12.5**
6	female	15	−7	**+**	**200**	**46.6**	**445.7**	0.4
7	male	17	−25	**+**	**400**	1.9	**72.2**	0.5
		19	0	**+**	**100**	1.9	**120.3**	**1.0**
8	female	26	−14	**+**	**100**	**2,181.5**	**9.7**	0.4
		27	1	–	**100**	**484.0**	**1.0**	0.4

Results above the cutoff value for antibody positivity are indicated in bold.

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