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# **Autoantibodies to insulin, IA-2 and ZnT8 improve the prediction of early insulin requirement in adult-onset autoimmune diabetes**

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## **Abstract**

**Objective:** The aim of this study was to identify the predictive marker for early insulin requirement in adult-onset autoimmune diabetes in the Japanese populations.

**Design/Patients:** We analyzed insulin autoantibodies (IAA), IA-2 autoantibodies (IA-2icA), and ZnT8 autoantibodies (ZnT8A) by radioimmunoassay in 47 Japanese patients with adult-onset autoimmune diabetes who were identified by native GAD autoantibody (nGADA) screening in ~3,000 non-insulin-requiring diabetes and in 302 nGADA-negative type 2 diabetes. Furthermore, GAD65 autoantibody-specific epitopes were also analyzed using GAD65/GAD67 chimeric constructs.

**Results:** The prevalence of IAA, IA-2icA and ZnT8A in nGADA-positive patients was 26%, 15%, and 19%, respectively, which was significantly higher than that in nGADA-negative type 2 diabetes (2%, 2%, and 2%,  $P<0.0001$ ). Among nGADA-positive patients, 38% had one or more of IAA, IA-2icA, or ZnT8A, and 15% had two or more of these autoantibodies, compared with none of the nGADA-negative patients ( $P<0.0001$ ). Thirty-six % of nGADA-positive patients subsequently required insulin therapy, and high nGADA titer (log-rank  $P=0.003$ ), middle epitope recognition of GAD65A ( $P=0.002$ ), and the presence of one or more of IAA, IA-2icA, or ZnT8A ( $P=0.002$ ) at diagnosis marked the risk for early requirement of insulin therapy. Multivariate logistic regression analysis showed the multiple islet autoantibodies to be independently associated with the risk for insulin requirement (Odds ratio=13.77, 95%CI: 2.77-68.45,  $P=0.001$ ).

**Conclusions:** These results indicate that the determination of IAA, IA-2icA, and ZnT8A improves the prediction of a future insulin insufficiency in adult-onset autoimmune diabetes, which appears to be superior to GADA titer and GAD65A-specific epitopes.

## **Introduction**

Autoantibodies to GAD (GADA) identify the subset of adult-onset patients with type 2 diabetes who initially do not require insulin treatment but who may develop insulin dependency within a few years after diagnosis. This form of diabetes is variably referred to as latent autoimmune diabetes in adults (1), slowly progressive type 1 diabetes (2), or adult-onset autoimmune diabetes (3). Although the high titer of GADA has been reported as a predictive marker of insulin dependency, there are a certain number of patients with high titer of GADA who do not progressed to insulin dependency for many years, indicating that there are other markers which distinguish the non-progressors from progressors (2, 4). It has been reported that the determination of GADA epitopes in patients with type 2 diabetes helps to define type 1 diabetes phenotypes, and the presence of GADA binding to middle plus COOH-terminal epitope is strongly associated with a type 1 diabetes phenotype (5, 6). Japanese patients with slowly progressive type 1 diabetes with insulin treatment have GADA which recognize NH<sub>2</sub>- and COOH-terminal epitopes, suggesting that NH<sub>2</sub>-terminal epitope may be associated with the immunological characteristics of slowly progressive type 1 diabetes (2). Adult-onset autoimmune diabetes are solely identified by the detection of islet autoantibodies, with GADA being the antibody markers with the highest prevalence, followed by autoantibodies to IA-2 (IA-2A) and insulin (IAA), that are detected in 15-20% of GADA-positive cases (3, 7, 8), and their presence increases the relative risk of these patients to require insulin therapy (8, 9). Recently, the zinc transporter isoform 8 (ZnT8) has been identified as a novel autoantigen in type 1 diabetes, but the clinical relevance of ZnT8A in adult-onset autoimmune diabetes is uncertain. The aim of the present study was to identify the predictive marker for early insulin requirement in adult-onset autoimmune diabetes using IAA, IA-2A, and ZnT8A together with GAD65A-specific epitope recognition.

## **Materials and Methods**

**Subjects** All patients investigated were participants into the West Japan Study for GAD Autoantibody-Positive Diabetes, a prospective nationwide study in west part of Japan (Kyusyu,

Yamaguchi, and Osaka areas), conducted with the aim of assessing the predictive markers for early insulin requirement in adult-onset autoimmune diabetes (10). The following criteria were used for enrolment in the project: > 30 years of age at diagnosis, non-ketotic diabetes, no requirement for insulin treatment at the time of native GAD autoantibody (nGADA) screening, and an initial diagnosis of type 2 diabetes based on the criteria of the National Diabetes Data Group (11). Patients with other types of diabetes were excluded. Overall, 349 Japanese patients with initial non-insulin-requiring diabetes, including 47 nGADA-positive patients and 302 nGADA-negative patients, were studied. nGADA-positive patients were identified by nGADA screening in ~3,000 non-insulin-requiring diabetes recruited between April 1996 and December 1999, and were prospectively followed up to 9 years. nGADA-negative patients were randomly selected from nGADA-negative type 2 diabetes. The clinical characteristics of the subjects at the time of nGADA screening are shown in Table 1. All subjects were informed of the purpose of the study, and their consent was obtained. Protocols were approved by the ethics committee of the Nagasaki University. Sera were stored at -20°C until use.

**GADA screening** GADA radioimmunoassay used for screening of autoimmune diabetes was anti-GAD RIA kit using <sup>125</sup>I-labeled porcine brain native GAD (nGAD), which contains both GAD65 and GAD67 isoform (RIP anti-GAD Hoechst, Hoechst-Behring, Tokyo, Japan) as previously described (12). Sera were considered as nGADA-positive if they contained > 5 U/ml of autoantibody, which is 3SD above the mean value in 140 normal control subjects. In the fourth GAD antibody workshop this assay had 100% specificity and 100% sensitivity.

**GAD65A and GAD67A detection** GAD65A and GAD67A were detected by quantitative radioligand binding assay using full-length human islet GAD65 and GAD67 cDNA as previously described (13). “Positive” was based on the 99th percentile of sera from 204 healthy control subjects without family history of diabetes. The cut-off indices were an index of 0.028 for GAD65A and 0.071 for GAD67A. The inter-assay CV and intra-assay CV was 3.3% and 5.3% for GAD65A and 4.1% and 8.8% for

GAD67A. In the Diabetes Autoantibody Standardization Program 2005 (DASP 2005) the GAD65A assay had sensitivity of 74% and specificity of 98%.

**IA-2A detection** The cDNAs used for the detection of IA-2A were the complete cytoplasmic region of IA-2 (aa601-979, IA-2ic). IA-2icA were detected by radioligand binding assay as previously described (14). “Positive” for IA-2icA was based on the 99th percentile of sera from 204 healthy control subjects and the cut-off index was an index of 0.018. In the DASP 2005 the IA-2icA assay had sensitivity of 68% and specificity of 96%, respectively. Patients were also tested for IA-2<sub>256-760A</sub>, which has been recently reported as a sensitive marker for the detection of islet autoimmunity in adult-onset diabetes (7). The cut-off index for IA-2<sub>256-760A</sub> was an index of 0.097, which was based on the 99th percentile of sera from 102 healthy control subjects.

**IAA detection** IAA assay was carried out by a micro-IAA assay as previously described (15) with some modification. Briefly, in Tris-buffered saline/Tween 20 (TBST; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0% BSA, 0.15% Tween 20), <sup>125</sup>I-insulin (Amersham International, Buckinghamshire, UK) was incubated at 4°C overnight with 5 µl of serum (at a 1:25 dilution) with and without cold human insulin, respectively. After the incubation, 50µl of a 50% protein A/8% protein G-Sepharose 4FF (Pharmacia, Freiburg, Germany) in TBST was added to the reaction in a MultiScreen-DV 96-well filtration plate (Millipore, Burlington, MA, USA). After the incubation and washing with cold TBST (0.1% BSA), radioactivity was counted by β-counter in counts per minute (cpm). Based on the difference in cpm between wells without and with cold insulin, an index was determined, with a positivity criterion of 0.010 based on the 99th percentile of sera from healthy control subjects. The inter-assay CV and intra-assay CV was 6.8% and 1.4%, respectively. In the DASP 2005 this assay had sensitivity of 58% and specificity of 98%.

**ZnT8A detection** ZnT8A were determined by radioligand binding assay using human ZnT8 cDNA

as described previously (16). The human ZnT8 cDNA construct used in this study was a fusion of the cytoplasmic carboxy-terminal domains (aa268-369) of ZnT8 carrying either 325Trp (TGG) or 325Arg (CGG) with an immunoglobulin C $\gamma$ 3 hinge sequence with three Glycine (PSTPPGSSGGG) as linker peptide (pJH4.1). The cut-off index for ZnT8A was an index of 0.007, which was based on the 99th percentile of sera from 139 healthy control subjects. The inter-assay CV and intra-assay CV was 9.6% and 4.6%, respectively.

**GAD65A-specific epitope analysis** Reactivity to conformational epitopes of GAD65 was determined by radioligand binding assays using *in vitro* transcribed and translated <sup>35</sup>S-GAD65/GAD67 chimeric fusion proteins with unlabeled recombinant GAD67 protein as previously described (13). To evaluate the immunoreactivity to the NH<sub>2</sub>-terminal region of GAD65, we used the chimeric constructs designated as GAD65<sub>1-245</sub>/GAD67<sub>253-594</sub>, GAD65<sub>1-360</sub>/GAD67<sub>369-594</sub> and GAD65<sub>1-245</sub>/GAD67<sub>253-451</sub>/GAD65<sub>443-585</sub>. The middle region epitope was evaluated by GAD67<sub>1-253</sub>/GAD65<sub>245-360</sub>/GAD67<sub>369-594</sub>, GAD67<sub>1-253</sub>/GAD65<sub>245-585</sub>, and GAD65<sub>1-360</sub>/GAD67<sub>369-594</sub> constructs. The COOH-terminal epitope was evaluated by GAD67<sub>1-451</sub>/GAD65<sub>443-585</sub>, GAD65<sub>1-245</sub>/GAD67<sub>253-451</sub>/GAD65<sub>443-585</sub> and GAD67<sub>1-253</sub>/GAD65<sub>245-585</sub> constructs. To absorb the reactivity to GAD67 epitopes sera were preincubated with the excess amount (~30 $\mu$ g) of bacterially-produced and purified unlabeled recombinant GAD67 followed by the addition of *in vitro* translated <sup>35</sup>S-labeled each chimeric protein. Autoantibody levels were expressed as indices using a same positive and negative control serum for all chimeric molecules. The cut-off indices, defined as the 99th percentile of 102 healthy control sera, were an index of 0.036, 0.041, 0.020, 0.024, 0.001, and 0.003 for GAD65<sub>1-245</sub>/GAD67<sub>253-594</sub>, GAD67<sub>1-253</sub>/GAD65<sub>245-585</sub>, GAD67<sub>1-451</sub>/GAD65<sub>443-585</sub>, GAD65<sub>1-360</sub>/GAD67<sub>369-594</sub>, GAD67<sub>1-253</sub>/GAD65<sub>245-360</sub>/GAD67<sub>369-594</sub>, and GAD65<sub>1-245</sub>/GAD67<sub>253-451</sub>/GAD65<sub>443-585</sub>, respectively. To correct for the inter-assay variation, all GADA-positive samples were analyzed in a single assay. The intra-assay CV was 9.0% for GAD65<sub>1-245</sub>/GAD67<sub>253-594</sub>, 7.5% for GAD67<sub>1-253</sub>/GAD65<sub>245-585</sub>, 14.7% for GAD67<sub>1-451</sub>/GAD65<sub>443-585</sub>,

4.6% for GAD65<sub>1-360</sub>/GAD67<sub>369-594</sub>, 2.3% for GAD67<sub>1-253</sub>/GAD65<sub>245-360</sub>/GAD67<sub>369-594</sub>, and 4.6% for GAD65<sub>1-245</sub>/GAD67<sub>253-451</sub>/GAD65<sub>443-585</sub>, respectively.

**Statistical analysis** Statistical analysis was performed using StatView statistical software (version 5.0, SAS Institute, Cary, NC). Results were expressed as mean±SD unless otherwise indicated. Autoantibody prevalence was compared using the Chi-square test, Fisher's exact test, and Cochran-Armitage's test where appropriate. Differences in nonparametric data were tested by Mann-Whitney *U* test or Kruskal-Wallis test. Kaplan–Meier analysis of time to starting insulin therapy in relation to autoantibody status with a log-rank test was performed. Relative risks (RR) were calculated from frequency tables comparing multiple autoantibody-positive groups with those negative for all three autoantibodies in nGADA-positive diabetes. Multivariate logistic regression analysis was performed to assess the importance of high titer of nGADA, the recognition of middle GAD65A epitope and the presence of multiple islet autoantibodies to the requirement for insulin in nGADA-positive patients. All variables were entered simultaneously into the model. A *P* value less than 0.05 was considered statistically significant.

## **Results**

### ***Clinical characteristics and prevalences of IAA, IA-2icA, and ZnT8A in nGADA-positive and -negative adult-onset diabetes***

Clinical characteristics at the time of nGADA screening and the prevalence of IAA, IA-2icA, and ZnT8A in nGADA-positive and -negative patients are shown in Table 1. The adult-onset patients with nGADA were significantly younger age at diagnosis and lower BMI than nGADA-negative patients with type 2 diabetes ( $P<0.05$ , Table 1). The prevalence of IAA, IA-2icA, and ZnT8A in nGADA-positive patients were 26%, 15% and 19%, respectively, and were significantly higher than those in nGADA-negative patients ( $P<0.0001$ , Table 1). Among nGADA-positive patients, 30 (64%) patients resulted positive for nGADA alone and 17 (36%) for nGADA with one or more of IAA,



IA-2icA, or ZnT8A (Table 1 and Figure 1). However, none of the nGADA-negative patients had a positive result for more than one of these autoantibodies (Figure 1).

#### ***GAD65A-specific epitope reactivity in nGADA-positive patients***

A total of 16/47 (34%) nGADA-positive patients had a GAD67A index above the 99th percentile of healthy controls. Since construction of the chimeras for expression of GAD65 epitopes incorporated regions of GAD67, all samples positive for GAD67A were preincubated with the excess amount of recombinant GAD67 protein before the GAD65A-specific epitope analyses. The most frequent epitope pattern was reactivity to both the COOH-terminal (amino acids 443-585) and middle regions (amino acids 245-360) (30/47, 64%) and 7/30 (23%) patients had also reactivity to NH<sub>2</sub>-terminal region (amino acids 1-245). One (2%) and 13 (28%) of 47 nGADA-positive patients recognized middle and COOH-terminal region alone, respectively. No reactivity to any of the epitopes of GAD65 could be detected in 3 patients.

nGADA titers were associated with the recognition of GAD65A-specific epitopes. The median titer of nGADA in patients reacted with middle (168.0 U/ml) and NH<sub>2</sub>-terminal (300.0 U/ml) epitope region was significantly higher than in patients without reactivity (10.0 U/ml,  $P<0.0001$  and 38.0 U/ml,  $P<0.05$ , respectively) (Supplementary Table 1). Of note, all of patients reacted with NH<sub>2</sub>-terminal epitope had nGADA titer above the 50th percentile of nGADA-positive patients (43.0 U/ml). Furthermore, the number of epitopes was also associated with nGADA titer, i.e. patients with all 3 epitopes had highest nGADA titer (Supplementary Table 2). However, there were no associations between GAD65A-epitope recognition and clinical phenotypes among nGADA-positive patients. Moreover, no significant differences were seen in the frequencies of IAA and/or IA-2icA and/or ZnT8A between patients with and without NH<sub>2</sub>-terminal, middle or COOH-terminal region reactivity as well as number of epitopes (Supplementary Table 1 and Supplementary Table 2).

### ***Relationship of humoral autoreactivity to disease progression***

Seventeen of 47 (36%) nGADA-positive patients subsequently required insulin therapy during the follow up period (median 2.0 yrs, range: 1.0-9.0 yrs). “Insulin requirement” was defined as the clinical need to commence insulin therapy in patients whose glycemic control became unacceptable despite oral hypoglycemic agents. As expected, high titer of nGADA was associated with the requirement of insulin therapy (log-rank  $P=0.003$ , Figure 2) (10). Furthermore, the middle epitope recognition of GAD65A marked the risk for requirement of insulin therapy (log-rank  $P=0.002$ , Supplementary Figure 1). However, the presence or absence of NH<sub>2</sub>- or COOH-terminal epitope was not associated with early insulin requirement (data not shown).

The prevalence of nGADA-positive patients who had been started insulin therapy during the follow up was 75% (9/12), 71% (5/7), or 67% (6/9) in IAA-, IA-2icA-, or ZnT8A-positive patients, respectively. The presence of IAA was marginally associated with the early requirement of insulin therapy in nGADA-positive patients by Kaplan-Meier analysis (log-rank  $P=0.04$ ). However, the presence of either IA-2icA or ZnT8A was not associated with increased risk of disease progression (data not shown). Given the combined analysis of IAA, IA-2icA, and ZnT8A, the risk of early insulin requirement in patients with one or more of these autoantibodies significantly increased versus patients with nGADA alone (log-rank  $P=0.002$ , Figure 3). The prevalence of nGADA-positive patients who had been started insulin therapy was 17% (5/30) in patients with nGADA alone, compared to 70% (7/10), 67% (2/3), or 75% (3/4) in patients with one, two, or three of IAA, IA-2icA, and ZnT8A, respectively. IA-2<sub>256-760</sub>A were detected in 16/47 (34%) nGADA-positive patients, a higher percentage versus IA-2icA (15%,  $P<0.05$ ) as recently reported (7), but were not associated with the risk for insulin requirement (data not shown). The prevalence of IA-2<sub>256-760</sub>A in nGADA-negative patients was 10.9% (33/302), which is significantly lower than that in nGADA-positive patients ( $P<0.0001$ ).

Next, we analyzed the relationship of the risk of early insulin requirement to nGADA titer, the recognition of GAD65A-specific epitopes, and the presence of multiple islet autoantibodies (Table 2). The prevalence of insulin requiring diabetes in patients with multiple islet autoantibodies (IAA, IA-2icA, ZnT8A) was significantly higher than that in nGADA single-positive patients both in high nGADA titer group (RR 3.81, 95%CI 1.30-11.13,  $P=0.004$ ) and in middle GAD65A epitope-positive group (RR 3.56, 95%CI 1.21-10.48,  $P=0.007$ ). Multivariate logistic regression analysis of the likelihood of requiring insulin showed that only the presence of multiple islet autoantibodies was significant predictor (OR 13.8, 95%CI 2.77-68.45,  $P=0.001$ ) in nGADA-positive patients (Table 3).

## **Discussion**

This study shows that 1) the presence of two or more of IAA, IA-2icA, and ZnT8A is highly specific for nGADA-positive patients, 2) the middle and NH<sub>2</sub>-terminal epitope are associated with high nGADA titer, and 3) the presence of one or more of IAA, IA-2icA, or ZnT8A marks the risk for insulin requirement in adult-onset autoimmune diabetes.

Among 47 patients with nGADA, 15-25% had positive results for IAA, IA-2icA, or ZnT8A, and 36% for one or more of these autoantibodies. However, none of the nGADA-negative patients had a positive result for more than one of these autoantibodies. These results indicate that the presence of two or more of IAA, IA-2icA, and ZnT8A is highly specific and these autoantibody screening will be helpful only among those nGADA-positive patients, suggesting that GADA may still be the first screening tool and the other autoantibodies will be the second tool. Furthermore, this study demonstrated that IAA and ZnT8A, reported as autoantibodies associated with childhood onset type 1 diabetes, are also useful markers to be analyzed in adult-onset autoimmune diabetes initially diagnosed as having type 2 diabetes.

Anti-islet autoantibodies including GADA are polyclonal and determination of conformational

epitopes of GAD65A has been suggested as a method for differentiation of disease and/or progression in adult-onset diabetes (2, 5). Autoantibody epitopes on GAD65 engage conformational determinants that are widely distributed over the linear sequence of the three domains, NH<sub>2</sub>-terminal, middle, and COOH-terminal domains (17-19). We demonstrated that major GAD65A epitopes in adult-onset autoimmune diabetes locate in the middle and COOH-terminal domain and the reactivity to NH<sub>2</sub>-terminal epitope is essentially associated with higher titer of nGADA. Our findings contrasted with some previous studies, in which NH<sub>2</sub>-terminal epitope was associated with non-diabetic subjects, but not in patients with type 1 diabetes, in a Caucasian population (4, 17-20). The discrepancy between these findings and those of the present study could be related to the elimination of GAD67A-positive sera (4), determination of GAD65A epitopes without preabsorbing the GAD67A reactivity (4, 5) in the previous studies, or different genetic background. Indeed, none of GAD67A-negative sera bound to NH<sub>2</sub>-terminal epitope in our study. However, our data are consistent with the evidences that GAD65/67 reactive sera have broader epitope reactivity than GAD65-specific sera (20), the presence of NH<sub>2</sub>-terminal GAD65A was strongly associated with that of GAD67A and high levels of GAD65A (5), and NH<sub>2</sub>-terminal GAD65A-positive sera were also positive for both GAD65A middle- and COOH-terminal epitope (5).

Identification of better predictive markers associated with disease progression in patients with adult-onset autoimmune diabetes could assist with maintenance of good glycemic control and hence avoidance of diabetic complications. It has been reported that high titer of GADA and GAD65A epitope act as predictive markers of future insulin insufficiency in adult-onset autoimmune diabetes (5, 10, 21, 22). Here we demonstrated that the presence of middle-epitope GAD65A and multiple islet autoantibodies is associated with early insulin requirement (Figure 3 and Supplementary Figure 1). Furthermore, our study showed that > 90% of patients with middle-epitope GAD65A have high titer of GADA (Supplementary Table 1), which may explain the previously identified association between the GADA titer and future insulin insufficiency. The additional determination of IAA, IA-2icA, and

ZnT8A might discriminate the higher risk patients for early requirement of insulin therapy independent of high GADA titer or middle GAD65A epitope. However, the number of patients with GADA studied is relatively small, which could bring on the low statistical power to assess the risk for insulin requirement especially in IA-2icA and ZnT8A-positive subjects. Therefore, further studies using larger number of subjects are required to establish the strategy for the prediction of future beta cell failure in adult-onset autoimmune diabetes.

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## Figure Legends

**Figure 1** The frequencies of IAA, IA-2icA, and ZnT8A in nGADA-positive and -negative adult-onset diabetes initially diagnosed as having type 2 diabetes.

**Figure 2** Kaplan-Meier plot of the proportion of nGADA-positive patients requiring insulin therapy classified according to the titer of nGADA.

High GADA titer, nGADA  $\geq$  20U/ml; Low GADA titer, nGADA < 20U/ml

*P* value was evaluated by a log-rank test.

**Figure 3** Kaplan-Meier plots of the proportion of nGADA-positive patients requiring insulin therapy classified according to the simultaneous presence of one or more of IAA, IA-2icA, or ZnT8A.

GADA alone, nGADA single positive; Multiple Abs, IAA and/or IA-2icA and/or ZnT8A positive in addition to nGADA

*P* value was evaluated by a log-rank test.

## **Figure Legend**

**Supplementary Figure 1 Kaplan-Meier plot of the proportion of nGADA-positive patients requiring insulin therapy classified according to the GAD65 autoantibody epitope recognition.**

Middle epitope -, patients without reactivity to middle region of GAD65; Middle epitope +, patients with reactivity to middle region of GAD65

*P* value was evaluated by a log-rank test.

**Table 1 Clinical characteristics of adult-onset diabetes**

	nGADA-positive diabetic patients	nGADA-negative type 2 diabetes	<i>P</i> value
n	47	302	
Male, n (%)	22 (47%)	139 (46%)	N.S.
Age at diagnosis (years)	48.1 ± 12.5	53.6 ± 11.0	<0.005
Duration of diabetes (years)	4.9 ± 5.8	8.0 ± 6.8	<0.001
Body mass index (kg/m <sup>2</sup> )	22.0 ± 4.1	23.0 ± 3.4	<0.05
HbA <sub>1C</sub> (%)	8.1 ± 2.2	8.1 ± 7.5	N.S.
IAA, n (%)	12 (26%)	5 (2%)	<0.0001
IA-2icA, n (%)	7 (15%)	5 (2%)	<0.0001
ZnT8A, n (%)	9 (19%)	6 (2%)	<0.0001
≥ 1 autoantibodies <sup>a</sup> , n (%)	17 (36%)	16 (5%)	<0.0001
≥ 2 autoantibodies, n (%)	7 (15%)	0 (0%)	<0.0001
3 autoantibodies, n (%)	4 (9%)	0 (0%)	<0.0001

Data are means ± SD or n (%). N.S., not significant

<sup>a</sup> Positive for one or more of IAA, IA-2icA, or ZnT8A

**Table 2 Prevalence of insulin requiring diabetes in patients with high nGADA titer or GAD65A middle epitope subdivided for presence of IAA, IA-2icA and ZnT8A**

	Insulin requiring diabetes, n (%)		RR	95%CI	P value
	Multiple islet autoantibody-positive	nGADA single-positive			
Total	12/17 (71%)	5/30 (17%)	4.24	1.80-9.98	0.0002
High GADA titer	10/13 (77%)	4/17 (24%)	3.81	1.30-11.13	0.004
Middle GAD65A epitope-positive	10/13 (77%)	5/18 (28%)	3.56	1.21-10.48	0.007

High GADA titer, nGADA  $\geq$  20U/ml

Multiple islet autoantibody-positive, positive for one or more of IAA, IA-2icA, or ZnT8A in addition to nGADA

**Table 3 Multivariate logistic regression analysis for the association of islet autoantibody status with early insulin requirement among nGADA-positive patients.**

Variable	Insulin requirement		
	OR	95% CI	<i>P</i> value
High GADA titer	0.61	0.05-6.91	0.652
Middle GAD65A epitope-positive	12.03	0.64-224.9	0.096
Multiple islet autoantibody-positive	13.77	2.77-68.45	0.001

All variables were entered simultaneously into the model.

High GADA titer, nGADA $\geq$  20U/ml

Multiple islet autoantibody-positive, positive for one or more of IAA, IA-2icA, or ZnT8A in addition to nGADA

**Supplementary Table 1**

**Clinical characteristics of nGADA-positive patients subdivided for GAD65A epitopes**

	Middle epitope		COOH-terminal epitope		NH <sub>2</sub> -terminal epitope	
	positive	negative	positive	negative	positive	negative
Number	31	13	43	1	7	37
Male, n (%)	15 (48%)	7 (54%)	21 (49%)	1 (100%)	3 (43%)	19 (51%)
Age at onset (years)	47.9 ± 11.2	48.1 ± 16.3	48.1 ± 12.6	51.0	45.8 ± 14.2	48.3 ± 12.6
Body mass index (kg/m <sup>2</sup> )	22.1 ± 4.7	22.3 ± 3.3	22.0 ± 4.1	22.1	25.5 ± 7.2	21.7 ± 3.6
nGADA titer (U/ml)	168.0 (300.0-41.0) <sup>a</sup>	10.0 (17.0-5.0)	55.0 (300.0-17.0)	12.0	330.0 (731.5-175.0) <sup>b</sup>	38.0 (300.0-15.0)
High GADA titer, n (%)	28 (90%) <sup>a</sup>	2 (15%)	30 (70%)	0 (0%)	7 (100%) <sup>b</sup>	23 (62%)
IAA, n (%)	10 (32%)	2 (15%)	12 (28%)	0 (0%)	1 (14%)	11 (30%)
IA-2icA, n (%)	6 (19%)	1 (8%)	7 (19%)	0 (0%)	0 (0%)	7 (19%)
ZnT8A, n (%)	6 (19%)	3 (23%)	9 (21%)	0 (0%)	0 (0%)	9 (24%)
≥ 1 autoantibodies, n (%)	13 (42%)	4 (31%)	17 (40%)	0 (0%)	1 (14%)	16 (43%)

Data are n (%), means ± SD for age at onset and body mass index, or medians (interquartile range) for nGADA titer;

High GADA titer, nGADA ≥ 20U/ml; <sup>a</sup> *P*<0.0001 vs. Middle epitope-negative; <sup>b</sup> *P*<0.05 vs. NH<sub>2</sub>-terminal epitope-negative

**Supplementary Table 2****Anti-islet autoantibodies and insulin requirement in nGADA-positive patients subdivided for number of GAD65A epitopes**

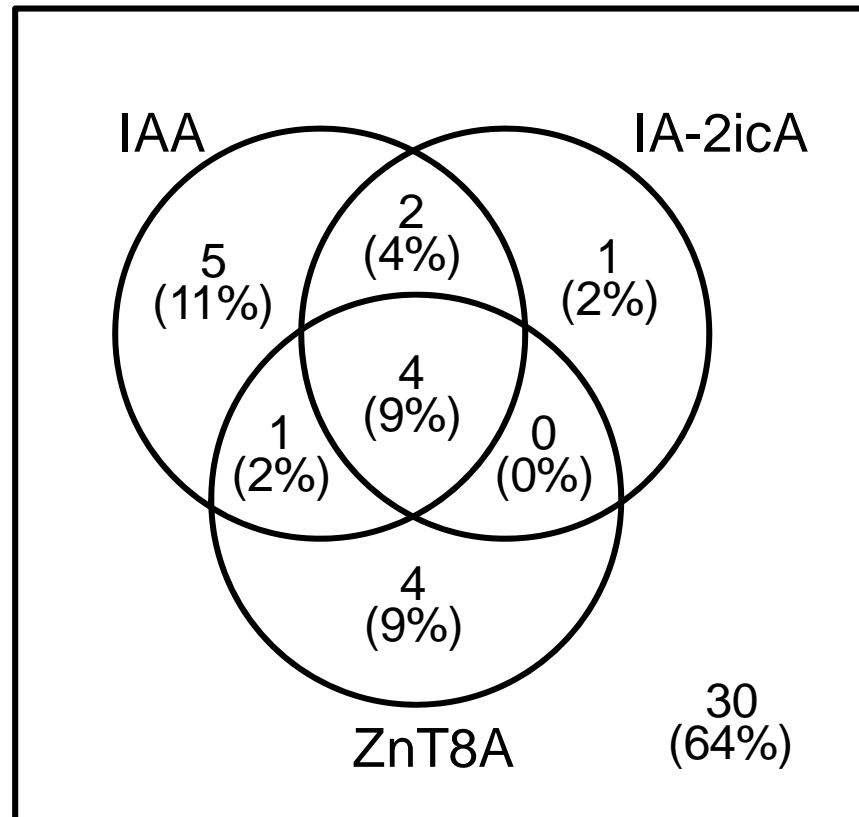
	COOH-terminal epitope alone	COOH-terminal and middle epitopes	NH <sub>2</sub> -terminal, COOH-terminal and middle epitopes
Number	13	23	7
nGADA titer (U/ml) <sup>a</sup>	10.0 (17.0-5.0)	150.0 (300.0-38.5)	300.0 (731.5-175.0)
GAD67A, n (%) <sup>b</sup>	1 (8%)	5 (22%)	7 (100%)
IAA, n (%)	2 (15%)	9 (39%)	1 (14%)
IA-2icA, n (%)	1 (8%)	6 (26%)	0 (0%)
ZnT8A, n (%)	3 (23%)	6 (26%)	0 (0%)
Insulin requirement, n (%)	2 (15%)	12 (52%)	3 (43%)

Data are n (%), or medians (interquartile range) for nGADA titer;

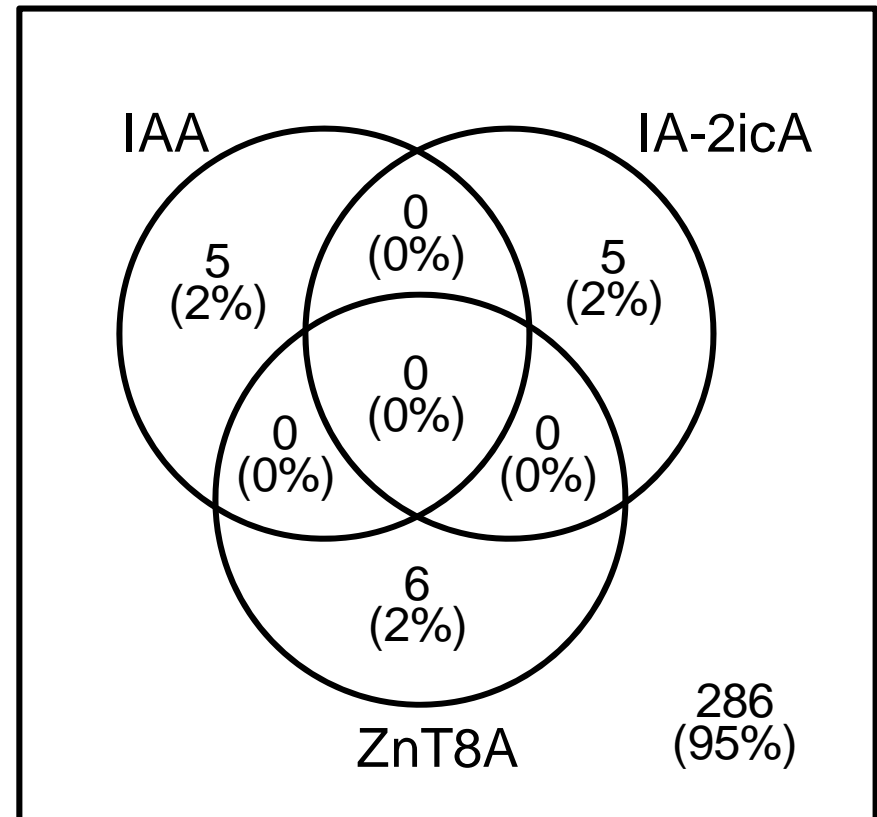
<sup>a</sup>  $P < 0.0001$  by Kruskal-Wallis test; <sup>b</sup>  $P < 0.0001$  by Cochran-Armitage's test

**Figure 1**

nGADA-positive (n=47)

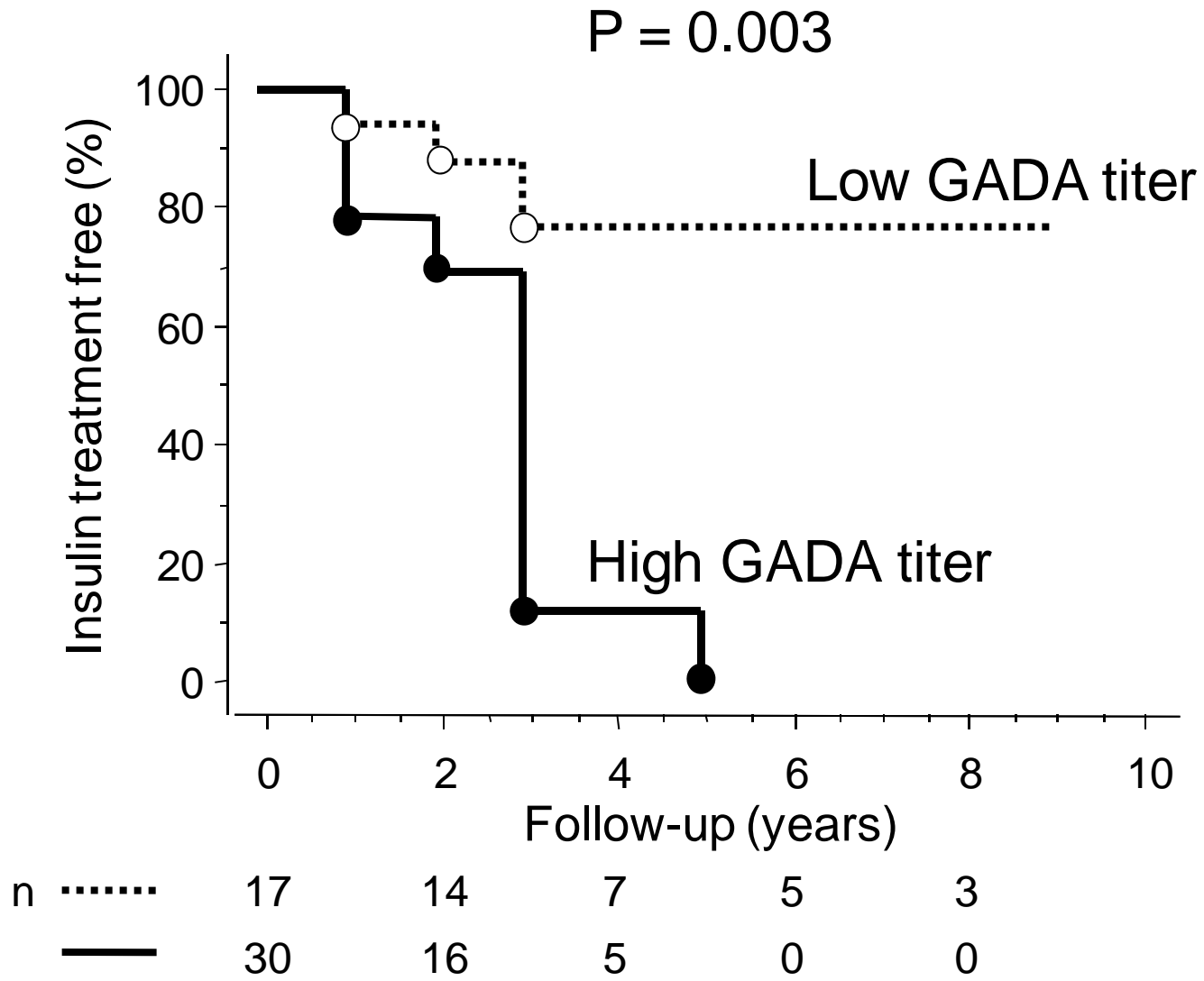


nGADA-negative (n=302)

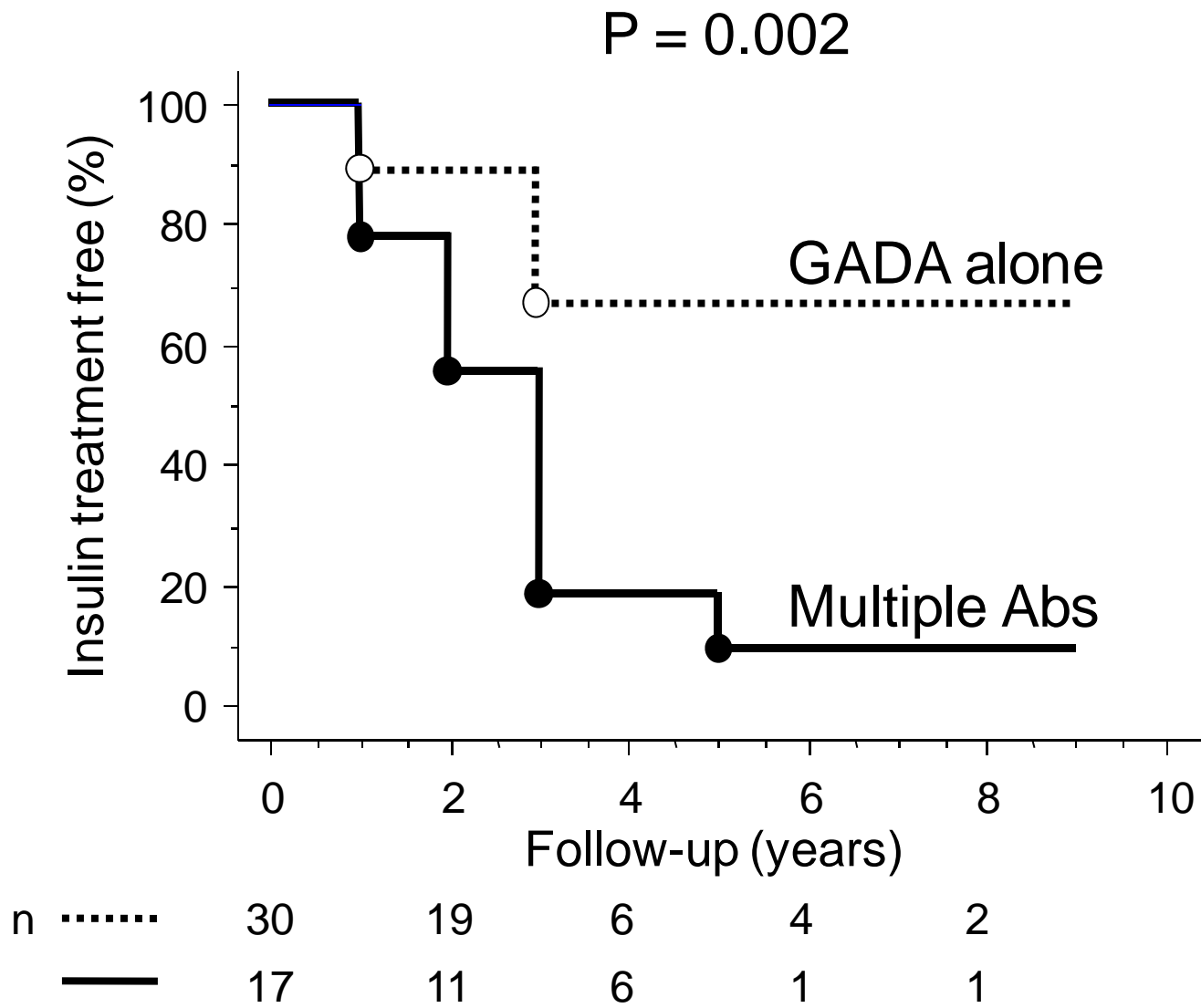




**Figure 2**



**Figure 3**



# Supplementary Figure 1

