ZnT8 Autoantibodies Complement GAD and IA-2 Autoantibodies in the Identification and Characterization of Japanese Type 1 Diabetes

Eiji Kawasaki 1*, Yoichi Oikawa 2, 3, Akira Okada 4, Norio Kanatsuna 5, Tomoyuki Kawamura 6, Tadashi Kikuchi 7, Jungo Terasaki 5, Junnosuke Miura 8, Yoshihisa Ito 9, Toshiaki Hanafusa 10

1 Diabetes Center, Shin-Koga Hospital, Kurume, Japan
2 Department of Internal Medicine, Tokyo Saiseikai Central Hospital, Tokyo, Japan
3 Department of Endocrinology and Diabetes, School of Medicine, Saitama Medical University, Saitama, Japan
4 Okada Clinic, Fukuoka, Japan
5 Department of Internal Medicine (I), Osaka Medical College, Takatsuki, Japan

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/JDI.13251

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Running Title: ZnT8A in Japanese Type 1 Diabetes

Word Count: main text 3,119 words, abstract 250 words, 23 references, 3 table, 4 figures, 3 supplementary materials

Address for Correspondence:
Eiji Kawasaki, M.D., Ph.D.
Diabetes Center, Shin-Koga Hospital
120 Tenjin-Cho, Kurume 830-8577, Japan
TEL: 81-942-38-2222  FAX: 81-942-38-2248
E-mail: e-kawasaki@tenjinkai.or.jp
Abstract

Aim/Introduction: This study aimed to investigate the significance of zinc transporter 8 autoantibodies (ZnT8A) in identifying and characterizing autoimmune-mediated type 1 diabetes (T1D) in Japanese subjects.

Methods: ZnT8A were determined in 324 patients with T1D, 191 phenotypic type 2 diabetes (T2D), and 288 healthy control subjects using bridging-type enzyme-linked immunosorbent assay (ELISA) in addition to autoantibodies to glutamic acid decarboxylase (GADA) and insulinoma-associated antigen-2 (IA-2A).

Results: We set a cut-off value of 10.0U/mL, and twenty-five percent of the T1D patients had ZnT8A levels exceeding this level. The prevalence of ZnT8A was significantly higher in patients with acute-onset T1D than in those with slowly-progressive and fulminant T1D (P<0.05). ZnT8A were more frequent in patients aged ≤10 years but less frequent in patients with duration ≥5 years (P<0.05). ZnT8A were detected in 5.2% of phenotypic T2D patients, with 90% of these being ZnT8A-single-positive. Furthermore, the ZnT8A levels in phenotypic T2D (143.8±194.9U/mL) were significantly higher than those in T1D cohort (22.9±8.3U/mL, P<0.05). In the acute-onset and slowly-progressive T1D patients with duration ≤ 5 years, additional measurement of GADA significantly increased the disease sensitivity in patients under 10 years-old but not in patients aged >10 years (11.7% vs. 3.6%, P<0.05). Multivariate analysis revealed that ZnT8A positivity was independently associated with age at sampling and IA-2A positivity.

Conclusion: These results suggest that the bridging-type ZnT8A ELISA may provide a valuable additional marker for Japanese patients with T1D, which could, in turn, allow for an increase in the number of identifiable cases and differentiate clinical phenotypes.

Keywords: Type 1 diabetes, Type 2 diabetes, ZnT8 autoantibodies
Introduction

Anti-islet autoantibodies directed against insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma-associated antigen-2 (IA-2A), and the recently described zinc transporter 8 (ZnT8A) are all useful markers in diagnosing, predicting, and differentiating the clinical phenotypes of type 1 diabetes (T1D). Among these, GADA is the most frequently used for the diagnosis of autoimmune diabetes \(^1\). ZnT8 is a 369-amino acid polytopic transmembrane protein localized in the insulin secretory granule that transports zinc iron from the cytosol into the vesicles \(^2,3\). Previous studies have reported that ZnT8A were associated with acute-onset and childhood-onset patients with T1D and, therefore, are considered as more specific marker of autoimmune-mediated \(\beta\) cell destruction \(^3\). Despite this, the relevance of ZnT8A in patients with adult-onset T1D, especially in slowly-progressive and fulminant T1D, and T2D has yet to be clarified.

Furthermore, several studies have identified that the major epitopes for ZnT8A are localized in the carboxy-terminal 102 amino acids (aa 268-369) that lie within the cytoplasmic domain. Moreover, residue at position 325 is a major determinant that is controlled by the single nucleotide polymorphism (SNP) rs13266634 (Arg325Trp) in the ZnT8 gene, \(SLC30A8\) \(^4,5\).

In a recent report conducted by our team, we detailed the development and the characterization of bridging-type enzyme-linked sorbent assay (ELISA) with dimeric carboxy-terminal ZnT8 domains carrying either 325Trp or 325Arg as antigens \(^6\). While this assay achieved high sensitivity and specificity in the Islet Autoantibody Standardization Program (IASP) or Diabetes Antibody Standardization Program (DASP) \(^7\), its clinical significance in the Japanese populations remains ambiguous, as the serum samples used in these programs are derived from Caucasians. Therefore, this study aims to investigate the significance of ZnT8A on the identification and characterization of autoimmune-mediated T1D in the Japanese population.
Materials and Methods

Participants

In the present cross-sectional, observational, and collaborative study, we recruited 870 subjects, all of whom were of Japanese origin, including 364 patients with T1D, 191 with clinically diagnosed type 2 diabetes (T2D), and 288 healthy control subjects, from 7 hospitals in 3 major cities (Fukuoka, Osaka, and Tokyo). Sera used in the study were stored at -20°C until application. Among the 364 patients with T1D, 40 patients complicated by autoimmune thyroid disease at the time of serum sampling were excluded from this study, while the remaining 324 patients with T1D were analyzed. These patients were then classified into three subtypes according to the criteria of the Japan Diabetes Society; 240 acute-onset, 25 fulminant, and 59 slowly-progressive T1D. A diagnosis of slowly progressive T1D was made if patients showed positive for GADA and/or ICA at any time during the disease course irrespective of anti-islet autoantibody status at time of study. The clinical and immunological profiles of all subjects are summarized in Table 1.

Autoantibody Assays

ZnT8A, GADA, and IA-2A were determined using bivalent ELISA kits (RSR Ltd., Cardiff, UK) using biotinylated ZnT8, GAD65 and IA-2, respectively, as described previously. All of these determinations were based on the sandwich type principle with dimeric carboxy-terminal domains of ZnT8 (aa275-369) carrying either 325Trp or 325Arg, full-length GAD65, and intracellular domain of IA-2 (aa604-979) as antigens. In short, the serum samples and unlabeled recombinant antigens (ZnT8, GAD65, or IA-2) coated onto the ELISA plate were incubated in the well. After washing the wells, a corresponding biotinylated antigen was added to each well, with
any unbound biotinylated antigens then being removed by washing. Subsequently, streptavidin–peroxidase conjugate was added, and after the addition of tetramethylbenzidine, the absorbance of the plate wells was read at 405 and 450 nm using an ELISA plate reader. The results were then read from a calibration curve constructed in the same run as the calibrators, and expressed in U/ml. The cut-off value for the GADA was 5.0 U/ml, and 0.6 U/ml for the IA-2A, respectively \(^{12,13}\).

Statistical analysis

The results are expressed as mean ± standard deviation or median (range). Autoantibody prevalence was compared using the Chi-square test, Fisher’s exact test, and Cochran-Armitage’s test where appropriate, and differences in non-parametric data were tested using the Mann-Whitney U test. With the results of curve fitting using linear and nonlinear regression, the correlation between the duration of diabetes and ZnT8A level was analyzed using the exponential regression curve (Table S1). A P-value <0.05 was considered statistically significant. Patient-only logistic regression analysis was performed to test for the association of ZnT8A positivity using gender, duration, age at sampling, GADA positivity, and IA-2A positivity as variables. The optimum cut-off point for age at sampling was determined based on the receiver operating characteristic curve, and statistical analysis was carried out using both StatView statistical software (version 5.0; SAS Institute, Cary, NC, USA) and SigmaPlot software (version 14.0, Systat Software Inc., San Jose, CA, USA).

Results

Establishment of the cut-off value for ZnT8A in the Japanese population

Table S2 summarizes the results of ZnT8A in the 288 healthy control subjects in comparison to those obtained from the manufacturer (RSR Ltd., n= 297). The mean and median level of
ZnT8A in the 288 healthy control subjects was 3.8 ± 2.3 (mean ± SD) U/mL and 3.7 (range 0.0 – 28.0) U/mL, respectively. Although the mean and median levels were higher than those in the RSR normal control subjects, mean ± 3SD (10.7 U/mL) and 99th percentile (9.6 U/mL) were lower than those in the RSR controls due to the smaller SD value (2.3 U/mL). Consequently, we set the cut-off value for the Japanese population at 10.0 U/mL. By this criterion, three of the 288 (1.0 %) healthy control subjects had positive ZnT8A titers, of 11.3, 13.4 and 28.0 U/mL. However, none of these subjects were found to be positive for GADA or IA-2A.

ZnT8A positivity and the distribution of ZnT8A levels

Figure 1 illustrates the distribution of ZnT8A levels in patients with T1D, phenotypic T2D, and healthy control subjects. The prevalence of ZnT8A in the T1D patients was 24.7% (80 of 324) and median level of ZnT8A in the autoantibody-positive subjects was 111.5 U/mL (range 10.7-1871.5 U/mL). Furthermore, the prevalence and median level of ZnT8A were 28.8% (69 of 240) and 118.0 U/mL (range 10.7-1871.5 U/mL) for acute-onset T1D, 15.3% (9 of 59) and 84.9 U/mL (range 13.7-876.9 U/mL) for slowly-progressive T1D. Only two of twenty-five (8.0%) patients with fulminant T1D were positive for ZnT8A, with their levels being 11.7 U/mL and 14.0 U/mL. Our study revealed that the prevalence of ZnT8A in acute-onset T1D patients to be significantly higher than in slowly-progressive or fulminant T1D patients (P<0.05, Figure 2). Additionally, ZnT8A was also detected in 10 of the 191 (5.2%) patients with phenotypic T2D, where the median level was 54.8 U/mL (range 11.3-607.5 U/mL). Considering the cut-off value suggested by the manufacturer (< 15 U/mL), the prevalence of ZnT8A was 26.7% (64 of 240) for acute-onset T1D, 0% (0 of 25) for fulminant T1D, 13.6% (8 of 59) for slowly-progressive T1D, and 4.7% (9 of 191) for T2D. Among these 9 cases positive for ZnT8A between 10 and 15 U/mL, three were not associated with any other autoantibodies.
**Association between ZnT8A and duration of T1D**

The three-hundred and twenty-four patients in this study with acute-onset and slowly-progressive T1D, whose duration of diabetes was available, were divided into three groups using the tertile of diabetes duration: T1 (0-3 years, n=114), T2 (4-15 years, n=112), and T3 (16-58 years, n=98). Subsequently, we investigated the association between the prevalence of ZnT8A and duration of diabetes (Figure 3). As a result, we found the prevalence of ZnT8A decreased across the three tertiles according to diabetes duration (37.7%, 28.6%, and 15.3%; \( P_{\text{trend}}<0.001 \)) (Figure 3A). Furthermore, as expected, we observed a significant negative correlation between ZnT8A level and disease duration in ZnT8A-positive patients (\( r=-0.350, P<0.001 \)) (Figure 3B).

**Combinatorial Analysis of ZnT8A, GADA, and IA-2A**

Combinatorial analysis of GADA and IA-2A in addition to ZnT8A revealed that 75.0% (180 of 240) of patients with acute-onset, 81.4% (48 of 59) of slowly-progressive, 16.0% (4 of 25) of fulminant T1D, and 11.5% (22 of 191) of phenotypic T2D were positive for at least one of these autoantibodies (Figure 4). Furthermore, among patients who showed negative for GADA and IA-2A, 9.1% (6 of 66) of patients with acute-onset, 8.3% (1 of 12) of slowly-progressive, 4.5% (1 of 22) of fulminant, and 5.1% (9 of 178) of phenotypic T2D were positive for ZnT8A. Rather unexpectedly, in patients with ZnT8A-single positive, the mean ZnT8A level in the phenotypic T2D patients (143.8±194.9 U/mL) was significantly higher than any in the T1D cohort (22.9±83 U/mL) (\( P<0.05 \), Figure S1).

After this, we analyzed the relationship between age at sampling and the prevalence of GADA and/or ZnT8A in the 118 patients with acute-onset and slowly-progressive T1D within 5 years of...
At this point, the prevalence of ZnT8A was found to be significantly higher in patients aged \( \leq 10 \) years (52.9\%) than in the other age groups (\( P<0.05 \)). Moreover, an additional measurement of GADA increased diagnostic sensitivity in patients aged \( \leq 10 \) years (11.7\%, 4 of 34) in comparison to patients aged \( \geq 11 \) years (3.6\%, 3 of 84). Besides this, we also evaluated the relationship between the presence of ZnT8A and clinical and immunological parameters through multiple logistic regression analysis (Table 3). In doing so, we found the optimal cut-off point for age at sampling, based on ROC curve, to be 11 years (sensitivity 46.3 \%, specificity 77.9 \%). There were no associations regarding the presence of ZnT8A with gender, duration, or the presence of GADA. However, the ZnT8A positivity was associated with a younger age of onset (OR 2.64, 95 %CI 1.00-6.97, \( P = 0.049 \)) and the presence of IA-2A (OR 2.97, 95 %CI 1.24-7.12, \( P = 0.015 \)).

**Discussion**

In this study we have evidenced five key factors: The first being twenty-five percent of patients with T1D and 5.2\% of phenotypic T2D had ZnT8A levels exceeding the cut-off level. Second, there was a higher prevalence of ZnT8A in both patients with acute-onset T1D and those aged \( \leq 10 \) years. Third, a significant inverse correlation between ZnT8A levels and disease duration was observed in ZnT8A-positive patients. Fourth, the levels of ZnT8A in patients with phenotypic T2D was significantly higher than in those with T1D. Fifth, ZnT8A positivity was independently associated with age at time of sampling and IA-2A positivity.

As the bridging ELISA for ZnT8A used in this study would be easy to implement and has proven to achieve a high degree of sensitivity and specificity in the recent proficiency evaluations by the IASP, this assay should be deemed a useful diagnostic method for determining T1D. Although the provided cut-off value for this kit (ElisaRSR™ ZnT8 Ab) by the manufacturer is <

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15 U/mL, we have established a distinctive cut-off value of < 10 U/mL for Japanese populations based on the 99th percentile and 3SD above the mean value obtained through the 288 healthy Japanese control subjects. Although the exact reasons for the cut-off difference between Japanese and Caucasians are unknown, one possibility may be attributed to the effect of ethnicity. Seeing as the incidence of T1D in Caucasians is much higher than Japanese, the frequency of incidental recruitment of anti-islet autoantibody-positive healthy individual might also be higher in Caucasoid population. With this newly determined cut-off value, the prevalence of ZnT8A in the 324 patients with T1D was 24.7%, which was lower than in previous reports 6, 14, 15). Since the higher prevalence of ZnT8A in acute-onset T1D patients in comparison to the slowly-progressive form observed in this study was in accordance with previous reports, the possible reason for this discrepancy could be due to the differences in age, the duration of diabetes, or the proportion of the subtype of T1D 16). Kawasaki and colleagues previously reported that none of the patients with fulminant T1D showed positive for ZnT8A using radioligand binding assay 16). Although we were able to detect two ZnT8A-positive patients with fulminant T1D in our current study, the level of ZnT8A in these patients was 11.7 U/mL and 14.0 U/mL, which is under the cut-off value provided by the manufacturer, indicating that there is fundamentally no humoral autoreactivity to the ZnT8 molecule in fulminant T1D. These results are consistent with previous studies which demonstrate that ZnT8A reflect an autoimmune-mediated destruction of β cells and that non-autoimmune mechanisms such as innate immunity following viral infection of β cells are the major causes of fulminant T1D.

The prevalence of ZnT8A in patients with T1D decrease across the three tertiles according to diabetes duration and that there was a negative correlation between the levels of ZnT8A and the duration of diabetes as was previously reported 17). In addition, we found the rate of ZnT8A positivity to be prevalent in young patients with T1D. In previous reports, ZnT8A positivity has
been shown to have a more rapid decline than GADA, while the C-peptide responses after onset of T1D decline at a rate paralleling the titers of ZnT8A\textsuperscript{18,19}, indicating a possible link between humoral autoreactivity to ZnT8 and a decline of β-cell mass. Although the association of ZnT8A with IA-2A has already been demonstrated to be stronger than that with GADA in new-onset patients with T1D\textsuperscript{2,20}, we found that this association remains strong in long-standing patients, as well. These findings could be related to the fact that both ZnT8 and IA-2 are transmembrane proteins located within the insulin secretory granules and the ability of autoantibodies to recognize the cytoplasmic domain of the molecule.

Herein, we have demonstrated the interaction between age at sampling and ZnT8A positivity in T1D patients with a duration of ≤ 5 years. Gomes et al. reported a negative association between levels of ZnT8A and age at sampling, but not with the age of onset for diabetes\textsuperscript{17}). These results suggest that age at sampling rather than age of onset may receive priority in determining ZnT8A in long-standing patients with T1D. In support of this, our multivariate analysis revealed that both younger age and IA-2A positivity were independently associated with ZnT8A positivity. Despite these findings, the association between age and ZnT8A positivity remains controversial as some studies have reported ZnT8A as being more frequent in older patients, while other studies based on Caucasian populations found no age-dependent difference\textsuperscript{6,18,20}). However, in support of our findings, a study involving Chinese patients with T1D reported a similar prevalence of ZnT8A in younger patients\textsuperscript{21}), suggesting that its association may differ across ethnic groups.

In patients with phenotypic T2D, the prevalence of ZnT8A was 5.2% (10 of 191) and 90% (9 of 10) of those were positive for ZnT8A alone. The median level of ZnT8A-positive T2D was 54.8 U/mL (range 11.3 – 607.5 U/mL) and 80% (8 of 10) of the patients had ZnT8A levels exceeding 10SD of the normal control subjects. Given these results, it is hard to regard the
elevation of ZnT8A in patients with phenotypic T2D as a non-specific reaction. While we believe these results to be robust, further studies are needed to characterize ZnT8A-single positive patients with phenotypic T2D and whether or not these patients have low-affinity autoantibodies, similar phenomenon like GADA and IAA (22, 23), show progressive decline of β-cell function similar to slowly-progressive T1D or have the distinct clinical features of GADA-single positive patients.

In summary, the current study revealed that in addition to GADA the bridging-type ZnT8A ELISA is a valuable marker for Japanese patients with T1D, and is likely to increase the number of cases identified while enabling clinical phenotypes to be differentiated in the Japanese population. Investigation into the clinical features and natural history of ZnT8A-single positive patients initially diagnosed as T2D should warrant accurate diagnosis and suspicion of immune-mediated T1D in the future.

**Ethics Statement**

In accordance with the Declaration of Helsinki, this study’s protocol has been approved by the ethics committee of each participating hospital and informed consent was obtained from all participants.

**Acknowledgments**

This research has not received any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

**Conflict of Interest**

The authors declare that they have no conflict of interest.
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Figure Legend

Figure 1  Distribution of ZnT8A levels in subjects used in this study
Acute-onset T1D (n=240): median 118.0 U/mL (range 10.7 - 1871.5 U/mL), slowly-progressive T1D (n=59): median 84.9 U/mL (range 13.7 - 876.9 U/mL), Fulminant T1D (n=25): 11.7 U/mL and 14.0 U/mL, Phenotypic T2D (n=191): median 54.8 U/mL (range 11.3 - 607.5 U/mL)

Figure 2  Prevalence of ZnT8A in subjects used in this study
The prevalence of ZnT8A in patients with acute-onset T1D was significantly higher than in patients with slowly-progressive or fulminant T1D (P<0.05)

Figure 3  Correlation of ZnT8A positivity (A) or levels (B) with duration of diabetes in patients with acute-onset and slowly-progressive T1D
T1; duration 0-3 years, T2; duration 4-15 years, T3; duration 16-58 years. The correlation between levels of ZnT8A and diabetes duration was analyzed in ZnT8A-positive subjects (n=90)

Figure 4  Combined analysis of ZnT8A with GADA and IA-2A
A. Acute-onset T1D (n=240), B. Slowly-progressive T1D (n=59), C. Fulminant T1D (n=25), D. Phenotypic T2D (n=191)

Figure S1  Distribution of ZnT8A in patients positive for ZnT8A alone
The level of ZnT8A in patients with phenotypic T2D was significantly higher than in patients with entire T1D (P<0.05)

Supplementary Table Legend
Table S1 Comparing the curve fitting data of the different models

Table S2 ZnT8 autoantibody levels in healthy controls
## Table 1 Clinical characteristics

|                  | Acute-onset T1D | Slowly-progressive T1D | Fulminant T1D | Phenotypic T2D | Healthy controls |
|------------------|-----------------|------------------------|---------------|----------------|-----------------|
| **Number**       | 240             | 59                     | 25            | 191            | 288             |
| **M : F**        | 1.0 : 1.4       | 1.0 : 1.1              | 1.0 : 0.8     | 1.0 : 0.6      | 1.0 : 1.0       |
| **Age (years)**  | 31.6±20.1       | 55.8±15.6              | 49.3±14.2     | 61.3±13.7      | 41.7±11.7       |
| **Age at diabetes onset (years)** | 20.3±17.4 | 43.6±14.4 | 42.4±14.3 | 46.9±14.0 | NA |
| **Duration (years)** | 11.4±11.9 | 12.2±10.8 | 7.6±6.3 | 13.7±10.2 | NA |

NA: not applicable

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Table 2 Relationship between age and the prevalence of GADA and ZnT8A in patients with acute- and slowly-progressive type 1 diabetes with duration less than 5 years

| Age     | n  | GADA                  | ZnT8A                  | GADA and/or ZnT8A |
|---------|----|-----------------------|------------------------|-------------------|
|         |    | n (%) | OR  | 95%CI | n (%) | OR  | 95%CI | n (%) | OR  | 95%CI |
| ≦ 10 years | 34 | 21 (61.8) | 1   | —     | 18 (52.9) | 1   | —     | 25 (73.5) | 1   | —     |
| 11~34 years | 43 | 31 (72.1) | 1.60 | 0.61-4.18 | 12 (27.9) | 0.34* | 0.13-0.89 | 33 (76.7) | 1.19 | 0.42-3.36 |
| ≧ 35 years | 41 | 32 (78.0) | 2.20 | 0.80-6.06 | 11 (26.8) | 0.33* | 0.12-0.86 | 33 (80.5) | 1.49 | 0.50-4.39 |

*P < 0.05
Table 3 Logistic regression analysis for the association of clinical and immunological parameters with ZnT8A positivity among acute-onset and slowly-progressive T1D with duration ≤ 5 years

| Variable                | OR   | 95%CI     | P value |
|-------------------------|------|-----------|---------|
| Gender (Female)         | 1.06 | 0.44-2.55 | 0.898   |
| Duration (years)        | 0.85 | 0.65-1.11 | 0.221   |
| Age at sampling (≤11 years) | 2.64 | 1.00-6.97 | 0.049   |
| GADA positive           | 2.25 | 0.75-6.76 | 0.149   |
| IA-2A positive          | 2.97 | 1.24-7.12 | 0.015   |
Figure 2

| Condition                  | Prevalence (%) |
|----------------------------|----------------|
| T1D, all (n=324)           | 24.7%          |
| Acute (n=240)              | 28.8%          |
| Slowly-progressive (n=59)  | 15.3%          |
| Fulminant (n=25)           | 8.0%           |
| Phenotypic T2D (n=191)     | 5.2%           |

\[ P < 0.05 \]

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Figure 3

A

Prevalence

P<0.001

37.7% 28.6% 15.3%

T1 (n=114) T2 (n=112) T3 (n=98)

B

y = 168.5 x 0.943 x
r = - 0.350, P<0.001
(n = 90)

ZnT8A (U/mL)

Duration (years)
Figure 4

A

Acute (n=240)

|        | GADA | IA-2A | ZnT8A |
|--------|------|-------|-------|
| Positive: | 56 (23%) | 38 (16%) | 17 (7%) |
| 19 (8%) | 32 (13%) | 12 (5%) | All negative |
| 6 (3%) | | | 60 (25%) |

B

Slowly-progressive (n=59)

|        | GADA | IA-2A | ZnT8A |
|--------|------|-------|-------|
| Positive: | 29 (49%) | 8 (14%) | 2 (3%) |
| 0 (0%) | 8 (14%) | 0 (0%) | All negative |
| 1 (2%) | | | 11 (19%) |

jadi_13251_f4ab.tif
Figure 4

C

Fulminant (n=25)

GADA

IA-2A

1 (4%)

0 (0%)

1 (4%)

0 (0%)

0 (0%)

1 (4%)

ZnT8A

All negative 21 (84%)

D

Phenotypic T2D (n=191)

GADA

IA-2A

7 (4%)

0 (0%)

5 (3%)

0 (0%)

0 (0%)

9 (5%)

ZnT8A

All negative 169 (88%)

jdi_13251_f4cd.tif

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