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Prevalence of Autoantibody-Negative Diabetes Is Not Rare at All Ages and Increases with Older Age and Obesity

Jian Wang, Dongmei Miao, Sunanda Babu, Jeesuk Yu, Jennifer Barker, Georgeanna Klingensmith, Marian Rewers, George S. Eisenbarth, and Liping Yu

Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Aurora, Colorado 80010

Objective: A significant percentage of nonautoimmune forms of diabetes presents among children in all age groups, with a remarkable increase with age.

Design: From October 1992 to October 2004, a total of 859 children less than 18 yr of age were newly diagnosed with diabetes at the Barbara Davis Center for Childhood Diabetes and had blood samples obtained within 2 wk of disease onset for analysis of antiislet autoantibodies to glutamic acid decarboxylase-65, insulinoma-associated antigen-2, insulin, and islet cell autoantibodies. The relationship of autoantibody positivity with human leukocyte antigen (HLA) class II, body mass index (BMI), glycosylated hemoglobin, age, and ethnicity was analyzed.

Results: Overall 19% (159 of 859) of these children with newly diagnosed diabetes were negative for all autoantibodies, and autoantibody negativity was significantly increased with age ($P < 0.01$). The Hispanic and Black subjects had significantly increased autoantibody negativity among older children with higher BMI than White subjects. The patients with the highest risk HLA genotype, DR3-DQ2/DR4-DQ8, were significantly less autoantibody negative ($P = 0.001$), whereas the HLA-protective allele, DQB1*0602, was significantly increased among the autoantibody-negative patients ($P < 0.0001$). Insulin autoantibodies were dramatically age dependent and were inversely correlated with age in both prevalence ($P < 0.0001$) and levels ($P < 0.0001$). Autoantibody positivity was inversely correlated with both BMI and age using multivariate analysis ($P < 0.0001$ and $P = 0.0078$, respectively).

Conclusions: A significant percentage of children newly diagnosed with diabetes are negative for all antiislet autoantibodies with a marked increase in obesity-associated autoantibody-negative diabetes after age 10, suggesting diabetes heterogeneity at all ages. (***J Clin Endocrinol Metab* 92: 88–92, 2007**)

DIABETES EVEN IN childhood results from multiple etiologies. Although destruction of β-cells in type 1A diabetes is considered T cell-mediated, antiislet autoantibodies are the best-characterized markers of β-cell autoimmunity and play important roles in the differential diagnosis of this form of diabetes and in predicting the risk of progression to overt disease (1–3). These antibodies are usually present years before the clinical onset of diabetes. Currently, three major recombinant autoantibody assays with biochemically defined autoantigens are available, including insulin, glutamic acid decarboxylase-65 (GAD65), and insulinoma-associated antigen-2/islet cell autoantibody 512 (IA-2/ICA512). It has been reported that approximately 4–7% of patients with newly diagnosed type 1 diabetes are autoantibody negative (2–4). As immune modulatory therapies are developed for autoimmune diabetes, a clear understanding of how best to identify children who might benefit from these therapies is increasingly important. In this study, we gathered data from children with newly diagnosed diabetes, aged 0 to 18 yr, at the Barbara Davis Center between October of 1992 and October of 2004 and analyzed their islet autoantibodies, human leukocyte antigen (HLA) genotypes, body mass index (BMI), and glycosylated hemoglobin ($HbA1_c$).

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Abbreviations: BMI, Body mass index; GAA, GAD autoantibody; GAD65, glutamic acid decarboxylase-65; $HbA1_c$, glycosylated hemoglobin; HLA, human leukocyte antigen; IA-2, insulinoma-associated antigen-2; IAA, insulin autoantibody; ICA, islet cell autoantibodies; mIAA, micro-IAA.

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Subjects and Methods

Subjects

The Barbara Davis Center cares for children with new onset diabetes (type 1 and type 2) with a special program for the type 2 children only after diagnosis at the children's hospital. A total of 859 children (441 males and 418 females) who were newly diagnosed with diabetes from October 1992 to October 2004 and had blood drawn within 2 wk of diagnosis were enrolled in this study. Patients included 685 (79.7%) White, 55 (6.4%) Hispanic, 37 (4.3%) Black, 35 (4.1%) others, and 47 (5.5%) unknown, with ages ranging from 1 month to 18 yr (mean, 10.7 yr; median, 10.9 yr), and ages between the three major ethnic groups (White, Hispanic, and Black) had no significant difference. Blood samples from these patients were collected within 2 wk of diabetes diagnosis. Subjects were tested for antiislet autoantibodies, BMI data were collected, and $HbA1_c$ was determined for the great majority of subjects at the time of diagnosis. In addition, 441 subjects aged 1 month to 18 yr with a mean age 10.7 yr and median age 10.8 were typed for HLA DQ and DRB1 on the basis of DNA sample availability.

GAD autoantibody (GAA) and ICA512 autoantibody (ICA512AA) assays

GAA and ICA512AA were measured by a combined radiobinding assay as previously described (5). Briefly, labeled recombinant GAD65 and ICA512bdc were produced by *in vitro* transcription/translation with different labeling (^{3}H-GAD65 and ^{35}S-ICA512bdc). The radioassay was performed on a 96-well filtration plate (Fisher Scientific, Loughborough, UK), and radioactivity was counted on a TopCount 96-well plate

β-counter (PerkinElmer Life Sciences, Wilmington, DE). The levels of both antibodies were expressed as an index. The interassay coefficients of variation are 10 and 5% (n = 50) for GAA and ICA512AA, respectively. The upper limits of normal, nondiabetic sera (0.032 for GAA; 0.049 for ICA512AA) were established as the 99th percentile of 198 healthy controls. In the most recent (2005) Diabetes Autoantibody Standardization Program (DASP) workshop, the sensitivity and specificity were 76 and 99%, respectively, for GAA, and 64 and 100%, respectively, for ICA512AA. ICA512AA were also measured using an alternative construct, IA-2ic, kindly provided by Dr. Ezio Bonifacio (Diabetes Research Institute, Munich, Germany). In the 2005 DASP workshop, the sensitivity and specificity for IA-2ic were 68 and 100%, respectively.

Insulin autoantibody (mIAA) assay

IAA was measured by a micro-radiobinding assay [micro-IAA (mIAA)] as described previously (6). In brief, ^{125}I-human insulin (Amersham, Little Chalfont, UK) was incubated with patient serum with and without cold human insulin followed by precipitation with protein A/G Sepharose. An index was determined based on the difference in counts per minute between wells without and with cold insulin, with a positivity criterion of 0.010, which was the 99th percentile of 106 normal controls. The interassay coefficient of variation is 20% (n = 100) at low positive levels. In the most recent (2005) DASP workshop, the sensitivity and specificity for mIAA were 58 and 99%, respectively.

ICA measurement

ICA was measured at Dr. William Winter's laboratory in Gainesville, Florida, by indirect immunofluorescence using cryostat-cut frozen sections of human blood type O pancreas. The results were expressed in Juvenile Diabetes Foundation (JDF) units, and a value equal to or more than 10 JDF units was considered positive (7, 8).

HLA typing

HLA class II polymorphisms were typed in an unselected sample of 441 patients, based on the sample availability. Genomic DNA samples were obtained from peripheral white blood cells. HLA class II subtyping was performed by PCR amplification of the polymorphic exon 2 of the *HLA- DQA1-DQB1* gene and hybridization of amplified DNA using sequence-specific oligonucleotide probes (Applied Biosystems, Foster City, CA; and Dynal Biotech, Oslo, Norway). DRB1 was typed by sequencing of the PCR-amplified exon 2 with alleles called by Matchmaker (Celera Genomics, Rockville, MD).

Statistical analysis

Categorical variables were analyzed using Fisher's exact test. Continuous variables were compared using logistic regression test and Pearson correlation test. Cochran-Amitage trend test was used to test for an age trend and *t* test for comparison of mean value. BMI distribution was tested with Mann-Whitney *U* test. Statistical analyses were performed using Prism or SAS software (GraphPad Software Inc., San Diego, CA).

Results

The majority of the 859 cases were diagnosed between ages 9 and 14 yr, with this group accounting for 54% of all cases. Antiislet autoantibodies including mIAA, GAA, and ICA512AA were measured for all 859 enrolled children. All subjects who were ICA512AA negative using the ICA512bdc construct were retested with the IA-2ic construct, and the 167 individuals who were negative for all above autoantibody determinations were tested for cytoplasmic ICA. Of those negative for GAD65, ICA512bdc, and mIAA, 10 of 177 (5.6%) were IA-2ic positive, and of the 167 negative for all biochemical autoantibodies, eight (4.8%) were positive for ICA. In total, 159 of 859 (18.5%) of the children were negative for all tested antiislet autoantibodies, equally distributed in both genders (80 of 441 males, 79 of 418 females). The total percentage of all autoantibody-negative diabetic children in each age group and in each year is plotted in Fig. 1, A and B, respectively. Overall, there are a significant percentage of autoantibody-negative cases in each age group, even among those diagnosed under the age of 5 (9%), and the rate of autoantibody negativity did not change over the 12 yr of the study, even in the older adolescent group. Autoantibody negativity increased with age (logistic regression test with age 0 to 18 yr as continuous variables, $P = 0.0078$; and Cohran-Amitage trend test, $P < 0.0001$), especially after age 14, resulting in a total of 36% negative for islet autoantibodies. The autoantibody negativities have significant differences comparing White (101 of 685, 14.7%) with Hispanic [16 of 55 (29.1%), $P = 0.01$] or with Black [14 of 37 (37.8%), $P = 0.0008$] and no significant difference between Hispanic and Black. Additional analysis found that the difference of autoantibody negativity between White and Hispanic or Black mainly existed among the older children with age above 14 [White 30 of 116, Hispanic 8 of 14 ($P = 0.02$), and Black 7 of 10 ($P = 0.007$)], whereas there is no significant difference among younger children (White 71 of 569, Hispanic 8 of 41, and Black 7 of 27).

Among 700 children who were positive for antiislet autoantibodies, in total, 55% were GAA positive, 60% ICA512AA positive, and 40% mIAA positive. The presence of mIAA was dramatically age dependent as illustrated in Fig. 2, A and B, and was inversely correlated with the age of diabetes onset in both prevalence (logistic regression: $P < 0.0001$) and levels (Pearson correlation: $P < 0.0001$). The prevalence of mIAA positivity declined from 83% under age

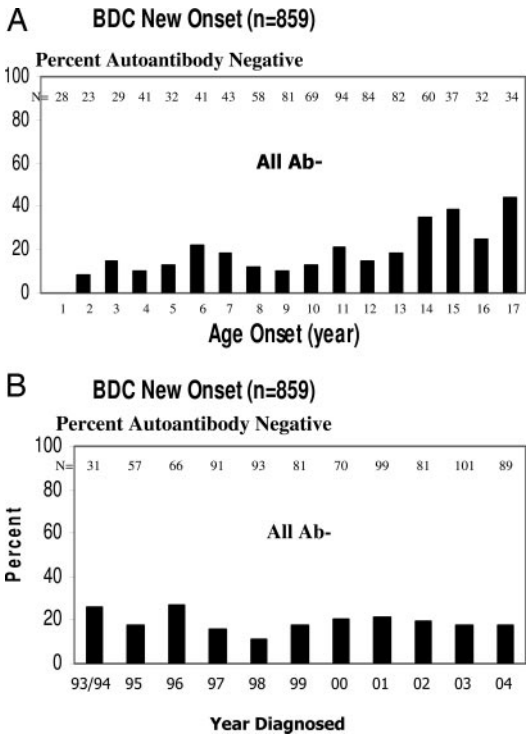

FIG. 1. The percentage of diabetic patients with all antiislet autoantibody-negative in each age group (A) and in each year diagnosed (B). BDC, Barbara Davis Center.

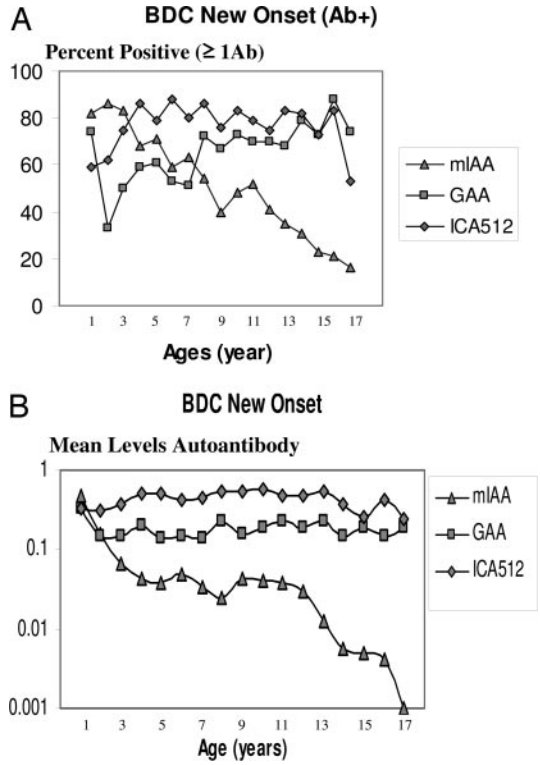

FIG. 2. Three antiislet autoantibodies (mIAA, GAA, and ICA512AA) tested in each age group are plotted by their prevalence (A) and by their mean index levels (B). BDC, Barbara Davis Center.

4 to only 16% at age 17, and mIAA mean levels from index 0.480 under age 2 to only 0.001 at age 17. In contrast, prevalence of GAA was positively correlated with the age of diabetes onset (logistic regression: $P = 0.03$), and the prevalence climbed from less than 40% (age 2) to above 80% as age-at-onset increased.

HLA class II alleles (DQA, DQB, and DRB) were subtyped in an unselected sample of 441 subjects based on the sample availability. The highest risk HLA genotype, DR3-DQ2/DR4-DQ8, was correlated with the presence of autoantibodies. Only 9% (9 of 105) of children who carried this highest risk HLA genotype were autoantibody-negative *vs.* 20% (66 of 336) among other HLA class II genotypes ($P = 0.007$). This 9%, however, is greater than the Denver population frequency of this highest risk genotype, 2.4% (750 of 31,778; $P < 0.0001$) (9), suggesting that a subset of these patients have type 1A but were missed with the autoantibody testing.

Furthermore, children with DR3-DQ2 and DR4-DQ8 comprised 26% [97 of 369 (HLA typed)] of children with positive biochemical autoantibodies, and the percentage increased with the number of autoantibodies (0Ab, 12%; 1Ab, 18%; 2Ab, 29%; 3Ab, 32%). In contrast, 63% (10 of 16) of children with the protective allele, DQB1*0602, were autoantibody negative ($P < 0.0001$). Thus, only 1.6% of autoantibody-positive children had DQB1*0602 *vs.* 13% among the autoantibody negatives.

The BMI was recorded at the time of diagnosis of clinical diabetes on 797 subjects. The mean BMI among the children with diabetes who were negative for autoantibodies was significantly higher than among children with diabetes who were positive for autoantibodies (23.3 *vs.* 18.9; $P < 0.0001$). Multivariate analysis of autoantibody positivity using logistic regression test with age and BMI as continuous variables demonstrated that both BMI and age were inversely correlated with autoantibody positivity ($P < 0.0001$ and $P = 0.0078$, respectively). The frequency analyses with Fisher's exact test at BMI SD score more than 1 SD above the mean (85 percentile), more than 1.5 SD (93 percentile), or more than 2 SD (96 percentile) were all shown negatively correlated with autoantibody positivity ($P < 0.0001$ for all three cutoffs). The negativity of autoantibodies was also significantly increased within three BMI cutoffs, 27.5% for BMI more than 1 SD, 36.6% for BMI more than 1.5 SD, and 43.4% for BMI more than 2 SD ($P = 0.01$). As illustrated in Fig. 3, the BMI showed a nearly "normal" distribution for autoantibody-positive children, but not for autoantibody-negative children. Nearly 20% of children with diabetes who were negative for autoantibodies were found to have a high BMI (> 30 kg/m^2) *vs.* only 3% ($P < 0.0001$) among diagnosed children who were positive for autoantibodies. More dramatically, among those diagnosed children with BMI greater than 30 kg/m^2, nearly 60% (27 of 46) were negative for all autoantibodies. To further analyze the BMI distribution, we found that there is clearly a difference between autoantibody-negative and autoantibody-positive children more than 10 yr or age (Fig. 4A), whereas the BMI distributions have no significant difference for both groups of children with autoantibody-negative and -positive among children younger than 10 yr of age (Fig. 4B). Comparing three ethnic groups, we found BMI in Hispanic (mean, 27.1; median, 23.0) and in Black (mean, 27.9; median, 27.5) were significantly higher than White (mean, 21.3; median, 19.5) among autoantibody-negative patients ($P = 0.005$ and $P = 0.002$, respectively), whereas among autoantibody-positive children BMI had no difference between White (mean, 18.9; median, 17.6) and Hispanic (mean, 18.4; median, 17.2) or Black (mean, 18.2; median, 18.6). Multivariate analysis of autoantibody positivity within White only confirmed that both BMI and age were significant inversely correlated with autoantibody positivity ($P = 0.02$ and $P = 0.01$, respectively).

There was no significant difference in $HbA1_c$ levels between the autoantibody-positive patients and the autoantibody-negative patients (11.17 *vs.* 11.26; $P = 0.259$), although the $HbA1_c$ levels were positively correlated with age of di-

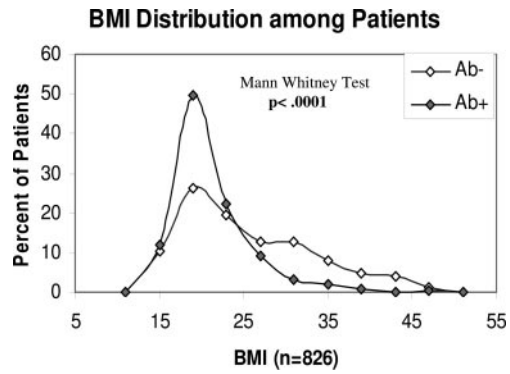

FIG. 3. BMI distribution in diabetic children was divided by negative and positive antiislet autoantibodies.

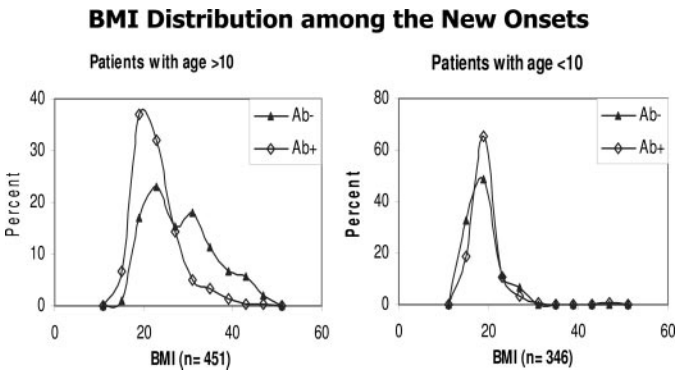

FIG. 4. BMI distribution was divided by autoantibody positivity among the diabetic children with age above 10 yr (A) and among the diabetic children with age less than 10 yr (B).

abetes onset in both autoantibody-positive (Pearson correlation: $P < 0.0001$) and autoantibody-negative patients (Pearson correlation: $P = 0.003$).

Discussion

Childhood diabetes can result from different disease processes. Next to type 1A diabetes, the second most common form of the disease is type 2 diabetes; other disorders are rare or uncommon, including type 1B, genetic defects including maturity-onset diabetes of the young, mitochondrial DNA disorders, and Kir6.2 mutations, as well as secondary and endocrine forms, and some rare diabetes syndromes. At present, we lack tools to definitively classify all forms of diabetes for individuals, given a lack of histological examination or effective imaging of the pancreas or "diagnostic" mutations for the great majority of individuals with either type 1A or type 2 diabetes.

Antiislet autoantibodies may be present years before the onset of type 1A diabetes and usually remain present during the prediabetic period (1, 2), although the titers may decline gradually with eventual lost in nearly half of patients after several years of the disease (10), and it might also be possible that a small proportion of cases could lose autoantibodies before diabetes onset. To identify diabetes associated with antiislet autoantibodies, we measured four currently available antiislet autoantibodies including IAA, GAA, ICA512AA, and ICA, including two constructs of ICA512 (ICA512bdc and IA-2ic). With our original three-autoantibody assays of mIAA, GAA, and ICA512AA (ICA512bdc construct only), the positivity of autoantibodies was 79.4% (689 of 868). By using an alternative ICA512 construct, IA-2ic (n = 177), followed by ICA testing (n = 167) for those negative subjects, we were able to identify 18 additional autoantibody-positive subjects, and thus the final percentage of autoantibody positivity among these new-onset children was 81.5%. To our surprise, nearly 20% of these pediatric cases were autoantibody-negative, including nearly 10% among even very young children, under age 5, and 44% at age 17. The rates of autoantibody negativity in Hispanic and Black were higher than in White, especially among the older children above age 14, although the number of Hispanic and Black patients in the study were limited. We believe that with improvements in present assays and the development of new autoantibody assays, more positive cases that are truly type 1A patients will be identified, especially for those nonobese children with high-risk HLA class II genotypes.

Our studies confirm prior reported trends in autoantibody positivity with finer analysis of children by age (11). Among all antiislet autoantibodies, IAA is most dramatically age-dependent and is inversely correlated with age. In the present study, IAA positivity was 83% among very young children with diabetes onset under the age of 4 yr. The prevalence of IAA positivity rapidly declined with age to approximately 20% after age 15. In addition, IAA is usually the first detectable antiislet autoantibody (12, 13). GAA may appear first in some cases, whereas ICA512AA is found relatively late. Both IAA and GAA in the present study were highly prevalent among very young children under the age of 2 yr. GAA positivity decreased less than 50% after age 2, and, in contrast to IAA, GAA positivity gradually increased with age from 40% to near 80% as age of onset increased from 2 to 17 yr. In addition, GAA positivity was lower than ICA512AA in every single age group from 2 to 15 yr and accounts for most of the autoantibody positivity in the older age groups. This age-related reciprocal prevalence of three autoantibodies among children with type 1A diabetes provides evidence for the diversity of diabetes autoimmunity associated with age and might reflect underlying differential disease pathogenesis.

Compared with the background prevalence in the general population, the diabetes-susceptible HLA genotype of DR3-DQ2/DR4-DQ8 was 10-fold higher among the newly diagnosed children who were positive for the autoantibodies measured in this study: 26.2% (96 of 366) *vs.* only 2.4% in the general population (9). In contrast, the protective HLA allele of DQB1*0602 was 10-fold less prevalent among the children who were autoantibody-positive, only 1.6% (6 of 366) *vs.* 25% in the general population (14). In addition, the high-risk HLA genotype, DR3-DQ2/DR4-DQ8, appeared more often in younger children: 33% (20 of 61) among the children diagnosed under the age of five compared with 22% (85 of 380), whereas none of the children diagnosed under age 5 had the protective allele of DQB1*0602 although the protection is not absolute for older age. This situation is consistent with data indicating that the relative protection of DQB1*0602 against the development of type 1A decreases with increasing age of diagnosis (15, 16) and the frequency of high-risk HLA alleles decreases with increasing age at diagnosis (17, 18).

The present data indicate that nearly 20% of new onset childhood diabetes patients were negative for all four tested antiislet autoantibodies. The prevalence of islet autoantibody-negative diabetes increased significantly with age in all three ethnic groups and more significantly in Hispanic and Black individuals. The rate of autoantibody negativity was significantly higher in Hispanic and Black subjects among older children, and the BMI in Hispanic and Black among autoantibody-negative children were significantly higher than White. The current data are correlated with previous observations that Hispanic and African-American children have a greater tendency to develop type 2 diabetes (19), and the ethnicity-related clustering of type 2 diabetes is attributed to a greater degree of obesity and severity of insulin resistance (20–22). The prevalence of type 2 diabetes in children

is reported to be increasing, especially in children above 10 yr of age (23, 24). However, in this report, we found no increase in the percent of new onset children with antibody-negative diabetes from 1992 through 2004, although, among the newly diagnosed children with obesity (BMI SD score more than 2 SD above the mean), 43.3% were negative for all autoantibodies, and the majority of these cases were among children greater than age 10.

We conclude that a significant percentage of children with diabetes are negative for antiislet autoantibodies at the time of disease onset even among the very young. The prevalence of autoantibody positivity among newly diagnosed children was affected by the age of disease onset, HLA class II alleles, race, and BMI. A reciprocal prevalence of autoantibodies associated with ages suggests a potential mechanism of differential islet autoimmune pathogenesis at different age groups. A significant percentage of non-autoimmune forms of diabetes is likely to present among children in all age groups, with a remarkable increase after age 10.

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Address all correspondence and requests for reprints to: Liping Yu, M.D., Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, P.O. Box 6511, Mail Stop B140, Aurora, Colorado 80045-6511. E-mail: Liping.yu@uchsc.edu.

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Disclosure Statement: All authors have nothing to declare.

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