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Glutamic Acid Decarboxylase Antibodies in Screening for Autoimmune Diabetes: Influence of Comorbidity, Age, and Sex on Specificity and Threshold Values,

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Antibodies against the 65-kDa isoform of glutamic acid decarboxylase (GAD_{65}) can be applied as a predictive tool for childhood type-1 diabetes *(1–6)* and to facilitate the differential diagnosis of diabetes in adults *(7–9)*. However, the sensitivity and specificity of GAD antibody screening have not been fully characterized, and the positive predictive value of screening varies from 20% to 70%, depending on the strategy applied and the population studied *(2, 3, 5–7, 9–12)*. The current study aims to identify factors that may lead to false-positive results in GAD antibody screening.

Previously, it has been demonstrated that the GAD antibody frequency in mixed connective tissue disease and stiff-man syndrome is increased, although not all patients who suffer from these diseases and are positive for GAD antibodies develop type-1 diabetes *(13, 14)*. GAD is expressed in the islets of Langerhans, neuronal tissue, the ovaries, and the testes *(15, 16)*. Similar to the above-mentioned examples, comorbidity involving these tissues may lead to GAD antibody formation but not to diabetes. Therefore, we compared the prevalence of GAD antibodies in patients with cystic fibrosis, epilepsy, Guillain-Barré syndrome, and premature ovarian failure to the prevalence in an unselected population of 1403 schoolchildren.

In addition, thresholds for positivity for GAD antibodies have generally been defined in children. These thresholds might not be applicable when testing for type-1 diabetes in adults. Therefore, we studied whether GAD antibody concentrations are correlated to age and sex, and whether adjustment of assay thresholds to include these variables may improve screening specificity.

The frequencies of positive results for GAD antibodies and the concentrations of GAD antibodies were established in a population of 1403 schoolchildren, ages 10–12 years, without chronic diseases *(17)*. During a 10-year follow-up, two of these children developed type-1 diabetes [ascertainment >96% *(18)*].

The influence of comorbidity on GAD antibody concentrations and the frequencies of positive results were studied in four patient populations. The subjects included 394 patients who participated in the Dutch study of epilepsy in childhood *(19, 20)*. These patients were eligible for the current study if the diagnosis was confirmed on the basis of electroencephalograms or therapy and sufficient serum for antibody analysis was available. Sera collected within 2 months after the presenting seizure (mean duration, 0.7 months; n = 228) and at the longest disease duration available of each patient (mean duration, 12.2 months; range, 2–50 months; n = 294) were analyzed separately. The diagnosis and development of diabetes during follow-up (5 years) were recorded from the medical records. Forty-three serum samples from 38 cystic fibrosis patients (collected in 1990–1992) were analyzed for GAD antibodies. In addition, we studied 30 patients with premature ovarian failure and 28 patients with Guillain-Barré syndrome (14 males; age range, 19–64 years). All patient sera were stored at −80 °C.

The influence of age and sex on GAD antibody concentrations and the frequencies of positive results were studied in 1287 individuals from the city of Zoetermeer, who participated in a study of cardiovascular risk factors. Sera were collected in 1976 and stored at −20 °C until testing. The population is described in detail elsewhere *(21)*. The demographic data of the populations are shown in Table 1.

The study protocols were approved by the appropriate medical ethics committees according to the Helsinki Declaration. Informed consent was obtained from all participants or their parents.

Sera were tested for GAD antibodies by radiobinding assay (RBA) *(13)* or immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the Triton X-100 fraction of [^{35}S]methionine-labeled fetal rat

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Table 1. Demographic data of populations tested for GAD antibodies.

Population	Schoolchildren	General population	Epilepsy, all	Epilepsy, onset	Epilepsy, long duration	Cystic fibrosis	Premature ovarian failure	Guillain-Barré syndrome
n	1403	1287	522	228	294	43	30	28
Age range (mean), years	10–12	6–86 (32)	6–19 (6.42)	0–14 (5.77)	1–19 (6.87)			19–64
Disease duration (mean), months	NA[a]	NA	0–50 (6.9)	0–1.5 (0.23)	2–50 (12)			
Median GAD index (range)	−0.01 (−0.05 to 1.53)	0.04[b] (−0.07 to 1.71)	0.01 (−0.1 to 1.45)	0.01 (−0.1 to 1.45)	0.02 (−0.1 to 0.28)	−0.05 (−0.08 to 0.21)		
Positive RBA (n), %	0.4 (5)	1.0 (13)	0.8 (4)	0.9 (2)	0.6 (2)	2.3 (1)	NT	NT
Positive cIMP, n	5	NT	4	2	2	NT	0	1
cIMP/RBA	RBA/cIMP	RBA	RBA/cIMP	RBA/cIMP	RBA/cIMP	RBA	cIMP	cIMP

[a] NA, not applicable; NT, not tested.
[b] $P < 0.001$ compared with the schoolchildren.

islets (a gift from Dr. T. Dyrberg and H. Richter-Olesen, the Hagedorn Research Institute, Gentofte, Denmark) and evaluated using autoradiography [conventional immunoprecipitation (cIMP)] *(22)* as indicated in Table 1.

For the RBA, all sera were analyzed in triplicate, and precipitated radioactivity was counted in a microbeta plate reader (EG&G Wallac). Internal reference sera were included in each plate for the epilepsy and cystic fibrosis patients and in each third plate for the schoolchildren and the general population. These internal reference sera were used to calculate an index (GAD index) for the purpose of comparison of experiments *(13)*. The GAD antibody concentration in the positive reference serum was in the linear range of the dilution curve, which allowed the GAD index to be interpreted semiquantitatively. The threshold for positivity was defined as the 99.5th centile of the population of schoolchildren (GAD index >0.21).

The statistical package SPSS for Windows (SPSS) was used for data analysis. The χ^2, Mann–Whitney, and Kruskal–Wallis tests were used to analyze differences between groups. Trends within groups were analyzed by the Spearman correlation test.

The GAD antibody frequencies of positive results and concentrations in the six populations studied are shown in Table 1. The positive individuals did not differ from the negative individuals in age or sex distribution in any of the populations studied. The GAD antibody concentrations and frequencies of positive results in the patient populations were not significantly increased compared with the schoolchildren.

In the epilepsy patients, the GAD antibody positive frequency shortly after the presenting seizure did not differ from the frequency at long disease duration, nor was there a correlation between antibody concentrations or positive frequencies and duration of epilepsy. Two children from the epilepsy cohort developed diabetes during follow-up. One was positive for GAD antibodies in a sample taken before diabetes onset (at the presenting seizure) and 7 months after onset of diabetes (13-month epilepsy duration). The other was negative for GAD antibodies in all serum samples analyzed. Two other patients were positive for GAD antibodies (in only one sample) but did not develop diabetes during follow-up.

One of the cystic fibrosis patients had a GAD index of 0.21, which is just at the defined threshold for positivity. A serum sample from this patient collected 3 months later was negative (GAD index, 0.16). Two out of five schoolchildren who were positive for GAD antibodies developed diabetes during follow-up.

As shown in Fig. 1, there was a slight but statistically significant correlation between age and GAD index (Spearman correlation coefficient, 0.161; $P < 0.001$). In addition, the GAD index in women was significantly higher than in men ($P = 0.009$; median GAD index, 0.043 and 0.037, respectively). The correlation between sex and GAD index did not explain the correlation with age and vice versa.

To study whether the observed correlation with age affected threshold definition, the general population was split into 10 similarly sized age groups (Fig. 1). The threshold for positivity was adjusted to the 99.5th centile of each age group and compared with the 99.5th centile threshold of the general population (0.68; see Fig. 1) and the threshold established in the schoolchildren. This yielded four additional positive individuals. One of these was negative at the initially applied threshold of 0.21. One individual who was positive at the general population threshold was negative when the age-adjusted thresholds were applied. Adjustment of thresholds for sex did not affect the antibody frequencies.

Strikingly, the GAD index was significantly higher in the general population than in the schoolchildren cohort. This difference remained present when outliers were discarded from both populations. To exclude that this observation was attributable to age effects, we selected all individuals 10–12 years of age from the general population and compared their GAD index to the GAD index in the schoolchildren cohort. The median GAD index in this selection of the general population (0.01) was still significantly higher than in the schoolchildren cohort (−0.04).

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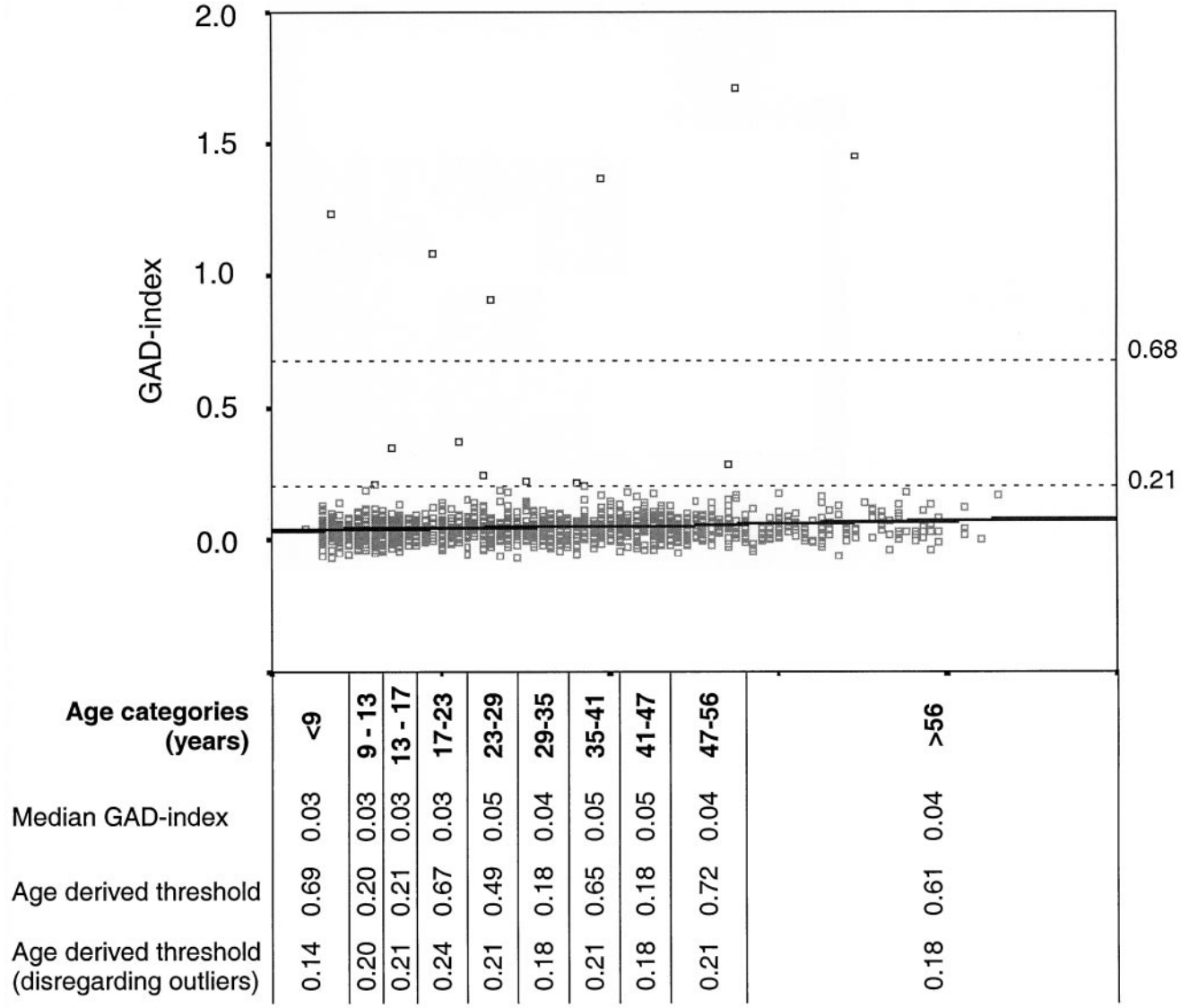

Fig. 1. Correlation between age and GAD antibody concentrations in 1287 individuals from the general population.
Spearman correlation coefficient, 0.161; $P < 0.001$. *Dotted lines* indicate the applied threshold (0.21) and the population-adjusted threshold (0.68).

None of the diseases involving tissues expressing GAD evaluated in the current study caused a significant increase of GAD antibody concentrations or frequencies of positive results. One might argue that the use of different methods for antibody detection in the patients and reference populations weakens the comparative analysis. However, by evaluation of all epilepsy patients and the schoolchildren who had GAD antibody concentrations higher than the mean + 1 SD in cIMP, we demonstrated that cIMP is at least as sensitive or possibly more sensitive than the RBA (data not shown). In the current study, we hypothesized that the GAD antibody frequency of positive results in neuroendocrine patients was higher because of non-diabetes-associated formation of GAD antibodies. Using the highly sensitive immunoprecipitation technique in the patient populations would only magnify this effect.

One of 28 (3.6%) Guillain-Barré syndrome patients was positive for GAD antibodies. These data clearly suggest that GAD antibodies are not part of the Guillain-Barré syndrome, but additional studies are needed to draw definite conclusions.

We observed a slight, but statistically significant, positive correlation between age and GAD index, but this correlation was not reflected in threshold definition. Exclusion of outliers from the distribution did not alter these perspectives. Although we observed a difference in the mean GAD index between males and females, this did not

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affect threshold definition. These results are in concordance with the observations from a previous study in a substantially smaller population *(23)* and indicate that adjustment of assay thresholds for age or sex is not indicated.

The GAD index in the general population was significantly increased compared with the schoolchildren. We concluded that this difference was attributable to differences in age between the populations. In addition, both populations were tested in one experiment (using one batch of tracer, control sera, and protein A-Sepharose), thus excluding technical variation of the methods used. A noticeable difference between both populations was serum storage. The general population sera were stored at −20 °C for 16 years in tubes with snap caps. When defrosted, some of these sera contained precipitates, which were removed by centrifugation before GAD antibody analysis. The sera of the schoolchildren cohort, on the contrary, were stored at −80 °C for 10 years in tubes with screw caps and had never been defrosted before the GAD antibody analysis. It is possible that the storage conditions of the samples from the general population led to the observed increased antibody concentrations in the general population sera. This observation implies that storage conditions should be monitored carefully to exclude such technical problems in future studies. Because all sera from the general population were stored in one freezer in identical tubes for the same period of time, it is not likely that the storage conditions affected the analysis of age and sex effects in the general population. In addition, positive samples did not differ in macroscopic aspects from negative samples.

This study demonstrates that age- and sex-nondefined populations can be used for definition of reference values for GAD antibodies and that comorbidity involving tissues that express GAD needs not be taken into account when screening for GAD antibodies. Because storage conditions may significantly affect the outcome of GAD antibody tests, these need to be monitored meticulously in collaborative studies.

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