Reactivity to N-terminally truncated GAD\textsubscript{65}(96-585) identifies GAD autoantibodies that are more closely associated with diabetes progression in relatives of patients with type 1 diabetes

Short running title: Autoantibodies to N-terminally truncated GAD discriminate diabetes risk

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Word count: 2037
Figures: 4 Table: 1
ABSTRACT
Autoantibodies to glutamate decarboxylase (GADA) identify individuals at increased risk of type 1 diabetes, but many people currently found GADA positive are unlikely to develop clinical disease. More specific GADA assays are therefore needed. Recent international workshops have shown that reactivity of sera from healthy donors varies according to assay type, and indicated that use of N-terminally truncated GAD$_{65}$ radiolabels in GADA radiobinding assays is associated with higher specificity. To determine whether a radiobinding assay using radiolabeled GAD$_{65}$(96-585) identified individuals at higher diabetes risk, samples from recent-onset patients and GADA positive first-degree relatives participating in the Bart’s-Oxford type 1 diabetes family study were re-assayed with full-length or N-terminally truncated GAD using the NIDDK harmonized protocol. The sensitivity in patients was the same with both labels, but fewer relatives re-tested positive with truncated GAD. Among relatives who progressed to diabetes, similar proportions were GADA positive when tested with either label, but because of their higher specificity the cumulative risk of diabetes was higher in those with autoantibodies to GAD$_{65}$(96-585). Autoantibodies to GAD$_{65}$(96-585) in relatives are more closely associated with diabetes risk than those to full-length GAD, suggesting assays using N-terminally truncated GAD should be used to select participants for intervention trials.
Autoantibodies to glutamate decarboxylase (GADA) are the most widely used marker of type 1 diabetes. They are a mainstay of diabetes prediction and recruitment to therapeutic intervention trials as well as being used for disease characterization (1). However, many individuals found GADA positive with current assays are unlikely to progress to type 1 diabetes. Improved discrimination of diabetes risk can be achieved by testing for multiple islet autoantibodies, but the development of more disease-specific GADA assays that enable more efficient screening for type 1 diabetes is a high priority (2).

Recent international islet autoantibody workshops revealed systematic differences in reactivity between ELISAs and radiobinding assays (RBAs), which suggested that the performance of many RBAs may be improved by the use of the N-terminally truncated radiolabel $^{35}$S-GAD$_{65}$(96-585) (3). We therefore assessed the ability of a RBA using the truncated GAD label to identify patients with recent-onset type 1 diabetes and to discriminate diabetes progression in first-degree relatives (FDRs) of type 1 diabetes patients in comparison with an assay using full-length GAD$_{65}$.

**RESEARCH DESIGN AND METHODS**

*Patients with recent onset type 1 diabetes*

To determine the diabetes sensitivity of autoantibodies to the different GAD constructs, sera from 147 patients (89 male and 58 female, median age 11.6 years, range 1.3 to 21 years) with recent onset type 1 diabetes (median diabetes duration 1 day, range -7 to 90 days) were randomly selected from a well-characterized cohort (4) and assayed for GADA(1-585) and GADA(96-585).
First-degree relatives of type 1 diabetes patients previously screened for GADA.

Sera were available from 283 relatives (139 male and 144 female) previously found GADA positive with a local RBA using \(^{35}\text{S}\) labelled full-length GAD\(_{65}\), after screening of 4470 FDRs (2121 male and 2349 female) in the Bart’s-Oxford (BOX) family study (5). These GADA positive relatives were followed prospectively for disease development by annual questionnaire (median follow-up to last contact or diabetes diagnosis, 14 years; range 0.2–27.7 years; median age, 31.4 years; range 1.3–57.4 years). A subset of 459 (227 male and 232 female) of the first available samples from the 4187 BOX relatives who screened negative with the local GADA assay was randomly selected for re-assay for GADA(1-585) and GADA(96-585). This subset was enriched with 31 of 179 screen-negative relatives who progressed to diabetes (Table 1). Local radiobinding assays were used to measure autoantibodies to IA-2 and insulin (5) on all relatives and ZnT8A (6) on those relatives who initially screened positive for GADA or who had progressed to diabetes.

Autoantibody assays

Sera from patients and relatives were assayed for GADA using the NIDDK harmonized assay protocol (7) with \(^{35}\text{S}\) methionine labeled antigens made by \textit{in vitro} transcription and translation of both N-terminally truncated GAD\(_{65}\)(96-585) and full-length GAD\(_{65}\) encoded in the pTNT plasmid vector (Promega, Madison, WI, USA) (3). To localize N-terminal epitopes more precisely, a subset of samples from the screen-positive FDRs was also re-assayed for GADA using \(^{35}\text{S}\) labeled GAD\(_{65}\)(46-585). A methionine residue was added to both truncated antigens to allow transcription. Samples were considered positive if \(\geq 97.5^\text{th}\) percentile of 222
healthy schoolchildren (8); 13.5 DK units/ml for GADA(1-585), 12.8 DK units/ml for GADA(96-585) and 25.4 DK units/ml for GADA(46-585). Using these thresholds, the sensitivity of the GADA(1-585) assay was 72% at a specificity of 92% and the sensitivity of the GADA(96-585) assay was 70% at 99% specificity in the Islet Autoantibody Standardization Program (IASP) 2013 workshop. The inter-assay CV of GADA(1-585) was 12.9% at 53 DK units/ml (n=32). The inter-assay CV of GADA(96-585) was 13.0% at 59 DK units/ml (n=32).

**HLA genotyping**

HLA class II genotyping was available on 209 (74%) of the 283 GADA positive relatives. HLA class II DRB1, DQA1, and DQB1 analysis was performed on blood and mouth swab DNA with sequence-specific primers as previously described (9). Haplotypes were established based on common patterns of linkage disequilibrium.

**Data analysis**

Categorical variables were compared using the $\chi^2$ test. Genetic risk was analyzed according to the high risk haplotypes DRB1*03-DQA1*0501-DQB1*0201 (DR3-DQ2) and DRB1*04-DQA1*0301-DQB1*0302 (DR4-DQ8) as well as the protective haplotype DRB1*02-DQB1*0602 (DR2-DQ6). Other haplotypes were classified as X. Survival analysis was carried out using Kaplan–Meier methods and log-rank testing (Mantel–Cox) used to compare survival between groups. For all analyses, a two-tailed $P$-value of 0.05 was considered significant. The area under the ROC curve (ROC-AUC) with 95% confidence interval (CI) was calculated assuming a nonparametric distribution of results using R software. Other
statistical analyses were performed using the Statistics Package for Social Sciences Version 21 (IBM, New York, USA).

RESULTS

Patients with recent onset type 1 diabetes

Using thresholds equivalent to the 97.5\textsuperscript{th} percentile in schoolchildren, the sensitivity of GADA(96-585) was identical to GADA(1-585) in patients with recent onset type 1 diabetes (Figure 1a). Of 147 patients, 117 (80\%) were positive for GADA(96-585), and 117 positive for GADA(1-585). One hundred and sixteen patients (79\%) had autoantibodies to both GAD constructs and there was excellent correlation between the levels of GADA (96-585) and GADA (1-585) (r = 0.99, p<0.001, Figure 2a). The ROC-AUCs based on the patients and schoolchildren were also very similar for GADA(1-585) and GADA(96-585); 0.94, 95\% CI: 0.91-0.97 and 0.93, 95\% CI: 0.9-0.96, respectively (Figure 3).

 Relatives who previously screened GADA positive

Of 283 relatives previously found positive for GADA using the local RBA, 259 (92\%) were positive on re-assay using the harmonized assay protocol with \textsuperscript{35}S-GAD\textsubscript{65}(1-585), 206 (73\%) with \textsuperscript{35}S-GAD\textsubscript{65}(96-585) and 195 (69\%) with both labels (Figure 1b). Of 70 relatives who progressed to diabetes, 66 (94\%) were positive for GADA(1-585), 63 (90\%) positive for GADA(96-585) and 61 (87\%) positive for both specificities. Of 76 relatives previously found positive for GADA who had at least one additional islet autoantibody, 73 (96\%) were positive for GADA(1-585), 70 (92\%) were positive for GADA(96-585) and 69 were positive for both specificities. Of these multiple antibody positive relatives, 39 (53\%) with GADA(1-
585) and 38 (54%) with GADA(96-585) developed diabetes. Of 207 relatives with no additional autoantibodies, 187 (90%) were positive for GADA(1-585) compared to 136 (66%) for GADA(96-585) (p<0.001). There was a good correlation between the levels of GADA(1-585) and GADA(96-585) in sera from the GADA positive relatives (r=0.96, p<0.001) (Figure 2b). Of 64 relatives positive for GADA(1-585), but negative for GADA(96-585), 38 (59%) had GADA(1-585) levels of less than 50 DK units/ml, a level found in 27 of the 147 (18%) recent-onset patients. Deletion of amino acids 46 to 95 of GAD65 was important in improving specificity with little loss of sensitivity; of the 64 samples with GADA(1-585) alone, 49 (77%) were positive for GADA(46-585) of whom only 4 (8%) developed diabetes.

Kaplan-Meier survival analysis showed that positivity for GADA(96-585) identified relatives positive for autoantibodies to full-length GAD who were at increased risk of diabetes progression (Figure 4, p<0.001). Of the 11 relatives who re-screened positive only for GADA(96-585), one had additional autoantibodies (IAA and IA2A), but was lost to follow-up after 4 years, while two others developed diabetes after 5 and 6 years of follow-up. Of 160 relatives carrying at least one HLA risk haplotype who re-screened positive for GADA(1-585), 129 (81%) were positive for GADA(96-585), compared with 18 of 35 (51%) with no HLA risk haplotype (p<0.001). Furthermore, GADA(96-585) were less common in GADA positive relatives carrying protective haplotypes; of 13 relatives carrying HLA-DQ6, 12 were positive for GADA(1-585), but only 3 were positive for GADA(96-585) (p=0.001).

*Relatives who previously screened GADA negative*
Of the 428 relatives who remained non-diabetic during follow-up who previously screened negative with the original GADA assay, 7 (1.6%) were positive for GADA(1-585) alone, 2 (0.5%) were positive for GADA(96-585) alone and 4 (0.9%) positive for both. Of the 31 relatives who progressed to diabetes, but screened negative for GADA with the original assay, none were positive for GADA(1-585) and one was positive for GADA(96-585).

DISCUSSION

GADA measured using N-terminally truncated antigen achieved the same sensitivity in recent onset patients as the assay using full-length GAD$_{65}$, while those relatives having autoantibodies to GAD$_{65}(96-585)$ were at higher risk of progression than those with autoantibodies to full-length GAD alone. Survival analysis showed that very few GADA positive relatives without autoantibodies to GAD$_{65}(96-585)$ progressed to diabetes within 20 years. Furthermore, only a minority of GADA positive relatives who carried protective HLA haplotypes were found positive when using the N-terminally truncated label.

Birth cohort studies of relatives of type 1 diabetes patients have shown that autoantibody epitope reactivity typically spreads from the C-terminal and middle (PLP) regions to the N-terminal domains of the molecule (10; 11). Autoantibodies to the N-terminal region normally constitute a relatively minor component of GAD autoreactivity and in isolation have little association with progression to diabetes (12). Our data would support this observation, since most samples from relatives who progressed showed similar antibody binding and levels with full-length GAD$_{65}$ and GAD$_{65}(96-585)$ (Figure 2b). Furthermore, the majority (59%) of relatives found positive for GADA(1-585), but negative for GADA(96-585) had relatively
low levels of GADA (<50 DK units/ml), consistent with a less vigorous autoimmune response in these individuals. This explains why sensitivity in recent onset patients was maintained, but most relatives carrying protective haplotypes and only a small proportion of relatives with multiple islet autoantibodies were found negative when GADA were measured using the truncated construct.

Several groups have investigated the effect of N-terminal truncations of GAD$_{65}$ on the disease sensitivity of GADA. Deletion of the first 194 amino acids did not cause decreased binding by 8 prediabetic/diabetic sera (13), while in agreement with our findings an assay using $^{125}$I labeled GAD$_{65}$(46-585) performed similarly to an assay using $^{35}$S labeled full-length GAD$_{65}$ (14). However, to our knowledge, this is the first study to show improved discrimination of diabetes progression using an N-terminally truncated GAD$_{65}$ label. The use of truncated antigens is established practice for the measurement of autoantibodies to islet antigen-2 (IA-2) and zinc transporter 8 (ZnT8), since the main diabetes-relevant epitopes are located in the intracellular portion of IA-2 and carboxy terminal region of ZnT8 (15; 16). If confirmed in other populations, including young children, our finding suggests that screening strategies to identify individuals at high risk of diabetes should use GADA RBAs based on N-terminally truncated protein.

Although fewer autoantibodies to disease irrelevant GAD$_{65}$ epitopes were detected using the N-terminally truncated label, the Kaplan-Meier survival curve suggests that less than half of GADA(96-585) positive relatives will develop diabetes within 25 years (Figure 4). N-terminally truncated GADA associated with autoantibodies to other islet antigens, but could not discriminate risk of progression within multiple antibody positive relatives. Further
improvements in assay specificity are therefore desirable. This may be achieved by more radical N-terminal deletions, if additional diabetes irrelevant epitopes are disrupted without affecting binding to diabetes relevant epitopes. Our addition of an N-terminal methionine to GADA(96-585) to allow protein expression is unlikely to have affected antibody binding as it is neither highly charged nor bulky. Inclusion of affinity measurements may also help to identify GADA positive individuals at increased risk of diabetes progression (12; 17). The potential for truncated GAD_{65} labels to identify patients with slow onset autoimmune diabetes in adults with a clinical presentation of type 2 diabetes, also needs to be investigated. A previous study using GAD_{65}/GAD_{67} chimeras rather than truncated GAD_{65} found no difference in the time to insulin requirement between those patients with or without N-terminal autoantibodies (18).

Radiobinding assays are still widely used for prediction and characterization of type 1 diabetes despite the advent of high quality alternative assay formats such as the bridging ELISA (19) and electrochemiluminescence assay (20). Advantages of RBAs include their relatively low cost, high sensitivity, good flexibility, small serum volume requirement, and proven track-record in diabetes prediction as well as the wide availability of equipment and reagents. However, a major shortcoming of GADA RBAs has been their relative lack of specificity. We have demonstrated that use of an N-terminally truncated GAD_{65} label can improve the disease specificity of the GADA assay without loss of sensitivity in patients and identify GADA positive relatives at higher risk of progression. As recruitment of high-risk relatives to therapeutic intervention trials normally includes initial testing for GADA, these findings strongly suggest that adoption of autoantibody assays using N-terminally truncated GAD_{65} would greatly improve screening efficiency for future studies aimed at preventing type 1 diabetes.
AUTHOR CONTRIBUTIONS

A.J.K.W., V.L. and P.A. researched data; contributed to the discussion; and wrote the manuscript. R.W., C.B., and K.M.G. researched data, and reviewed/edited the manuscript. P.J.B researched data; contributed to the discussion; reviewed/edited the manuscript; and coordinated the BOX study. A.J.K.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

ACKNOWLEDGEMENTS

The Islet Autoantibody Standardization Program contributed to the costs of this study. The BOX study is supported by Diabetes UK. V.L. and C.B were supported by the Italian Ministry of Research “Ivascomar project, Cluster Tecnologico Nazionale Scienze della Vita ALISEI’ and by the Associazione Italiana per la Ricerca sul Cancro “AIRC bando 5 × 1000 N_12182” grants.

The authors know of no conflicts of interest relevant to this article.

Parts of this study were presented in abstract form at the 49th European Association for the Study of Diabetes meeting, Barcelona, Spain, 23-27 September 2013 and at the 13th International Congress of the Immunology of Diabetes Society, Mantra Lorne, Victoria, Australia, 7-11 December 2013.

The authors are grateful to the diabetes team, pediatricians, physicians and families in the Oxford region for participating in the BOX study.
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Table 1. Characteristics of FDRs participating in the BOX family study whose samples were originally screened for GADA using a local assay and were re-assayed using the standard method with $^{35}\text{S}$-labeled GAD$_{65}$(1-585) and GAD$_{65}$(96-585). Additional autoantibodies from IA-2A, IAA and ZnT8A.

*not assayed for ZnT8A
Figure legends

Figure 1. Scheme showing the selection of samples and results for (a) healthy schoolchildren and patients with newly diagnosed type 1 diabetes or (b) BOX relatives, following re-assay with the harmonized protocol using either $^{35}$S labeled GAD$_{65}(1-585)$ or GAD$_{65}(96-585)$. The same proportion of patients was found positive for GADA(96-585) as for GADA(1-585). However, fewer relatives previously found GADA positive with the local assay were found positive on re-assay for GADA(96-585) than for GADA(1-585) (p<0.001).

Figure 2. Plot of GADA(96-585) against GADA(1-585) results for (a) 147 patients with recent-onset type 1 diabetes and (b) 283 FDRs from the BOX family study who previously screened GADA positive with a local RBA. Inset is an expanded plot of results for samples with GADA(1-585) levels up to 100 DK units/ml. Relatives who progressed to diabetes within 10 years are indicated with closed diamonds, those who progressed after 10 years with closed triangles and relatives who did not progress by open circles. Thresholds for the assays are given by the dotted lines and equivalent levels of both autoantibodies are indicated by the dashed lines. Correlation of GADA(96-585) with GADA(1-585) was excellent for patients ($y = 1.04x - 4.79, R = 0.99$) and very good for FDRs ($y = 1.16x - 14.84, R = 0.96$), although many sera from FDRs found positive for GADA(1-585) with levels up to 378 DK units/ml were found negative for GADA(96-585).

Figure 3. Receiver Operator Characteristics Curves for GADA(1-585) (bold dashed line) and GADA(96-585) (solid line) based on data from 147 patients with newly diagnosed type 1 diabetes and 222 healthy schoolchildren. The autoantibodies performed similarly; The area under the curve (AUC) was 0.94 for GADA(1-585) and 0.93 for GADA(96-585) (p=0.28).
The partial AUC (pAUC) calculates areas at specificities above 90% (within the grey box) and was 0.081 for GADA(1-585) and 0.080 for GADA(96-585) (p=0.69).

Figure 4. A Kaplan-Meier survival curve for FDRs positive for GADA(1-585) according to positivity for GADA(96-585). GADA(96-585) identified FDRs at increased risk of diabetes progression; few FDRs positive for GADA(1-585), but negative for GADA(96-585) developed diabetes within 20 years of follow-up.
2860 schoolchildren

Random selection of samples with serum available

222 schoolchildren

Assayed with harmonized assays

222

GADA (1-585)

4 (1.8%)

212 (95.5%)

GADA (96-585)

4 (1.8%)

2860 schoolchildren

613 new-onset patients

147 new-onset patients

GADA (1-585)

1 (0.7%)

29 (19.7%)

GADA (96-585)

116 (78.9%)

1 (0.7%)

613 new-onset patients
4470 BOX non-diabetic relatives

Tested for GADA using local assay

283 GADA +ve

283

4187 GADA -ve

Re-assayed with harmonized assays

283

459

GADA (1-585)

GADA (96-585)

GADA (1-585)

GADA (96-585)

64 (23.3%)

195 (68.9%)

11 (3.9%)

13 (4.6%)

7 (1.5%)

4 (0.9%)

3 (0.7%)

445 (96.9%)
GADA (96-585) +ve

GADA (96-585) -ve

p < 0.001

Cumulative Survival

Follow-up (years)

GADA (96-585) +ve  n  195  163  118  61  33  8
GADA (96-585) -ve  n  64  60  55  40  27  6