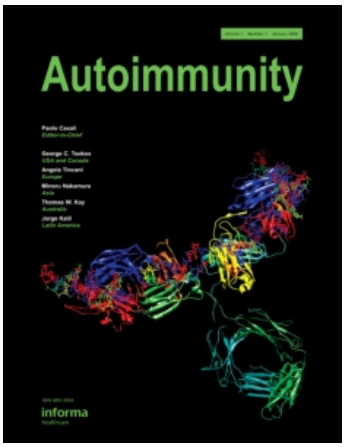

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Low agreement between radio binding assays in analyzing glutamic acid decarboxylase (GAD65Ab) autoantibodies in patients classified with type 2 diabetes

Bledar Daka [a]; Maria K. Svensson [b]; ke Lernmark [c]; Lucia Mincheva-Nilsson [d]; Göran Hallmans [a]; Olov Rolandsson [a]
[a] Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden [b] Department of Nephrology, Gothenburg University, Gothenburg, Sweden [c] Clinical Research Centre, University of Lund, Malmö, Sweden [d] Department of Clinical Microbiology, Umeå University, Umeå, Sweden

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Low agreement between radio binding assays in analyzing glutamic acid decarboxylase (GAD65Ab) autoantibodies in patients classified with type 2 diabetes

BLEDAR DAKA[1], MARIA K. SVENSSON[2], ÅKE LERNMARK[3], LUCIA MINCHEVA-NILSSON[4], GÖRAN HALLMANS[1], & OLOV ROLANDSSON[1]

[1]*Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden,* [2]*Department of Nephrology, Gothenburg University, Gothenburg, Sweden,* [3]*Clinical Research Centre, University of Lund, Malmö, Sweden, and* [4]*Department of Clinical Microbiology, Umeå University, Umeå, Sweden*

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Abstract
Autoantibodies against glutamic acid decarboxylase (GAD65Ab) are used in the classification of diabetes in adults. We assessed the concordance in GAD65 autoantibody levels within subjects between three different GAD65Ab radio binding assays (RBA). Plasma samples from 112 diabetes patients (median age 50 years) initially classified with type 2 diabetes was randomly selected from a local diabetes registry. Coded samples were analyzed with two RBA employing ^{35}S-labeled GAD65. The first used the pEx9 plasmid (pEx9 RBA), the second employed the pThGAD65 plasmid (pThGAD65 RBA) to label GAD65 by *in vitro* transcription translation. We also used a commercial kit employing plasmid pGAD17 labelled with ^{125}I (pGAD17 RBA). Subsequent analyses followed standard procedures. Two different cut-offs for GAD65Ab positivity were used in all three assays. We calculated the correlation, concordance, and agreement between the assays. The proportion of GAD65Ab positivity differed between assays when low cut-offs were used (pEx9 RBA 25%, pThGAD65 RBA 17.9%, and pGAD17 RBA 12.5%, respectively). When high cut-offs were applied, the concordance between the pEx9 RBA and the pThGAD65 RBA was 97.3 while their concordance to the pGAD17 RBA was lower (88.4 and 87.4, respectively). There was a low agreement between both pEx9 RBA and pGAD17 RBA (0.45, 95% CI 0.20–0.70) and between pThGAD65 RBA and pGAD17 RBA (0.43, 95% CI 0.18–0.68). We found discrepancies in determining the GAD65Ab positivity, which constitutes a problem when GAD65Ab are used clinically. Further methodological GAD65Ab assays studies are warranted.

Keywords: *Type 1 diabetes, type 2 diabetes, autoimmunity, immunological technique*

Abbreviations: *GAD65Ab, autoantibodies to the 65 kD glutamate decarboxylase isoform; RBA, radioligand binding assay; T1D, type 1 diabetes; T2D, type 2 diabetes; LADA, latent autoimmune diabetes in adults*

Introduction

The classification of diabetes is based on differences in etiology and pathogenesis of the disease [1]. Type 1 diabetes (T1D) is characterized by autoimmune destruction of beta cells in the pancreatic islets leading to insulin deficiency. In the majority of childhood-onset T1D this is combined with an autoantibody response to beta cell antigens which mark ongoing beta cell destruction. The most common autoantibodies include islet cell antibodies (ICA), insulin autoantibodies, protein tyrosine phosphatase-like protein autoantibodies (IA-2Ab) and glutamic acid decarboxylase 65 autoantibodies (GAD65Ab) [2].

T2D is caused by impairment of beta cell function combined with insulin resistance in peripheral target tissues. Both these defects may partly be inherited. There is also a close link between T2D, obesity and the metabolic syndrome [3]. Latent autoimmune diabetes in adults (LADA) is a subgroup of patients initially classified as T2D but where autoantibodies against beta cell antigens are found [4]. In UKPDS, the prevalence of LADA was estimated to ~10% of T2D patients and the presence of GAD65Ab

Correspondence: Dr O. Rolandsson, Department of Public Health and Clinical Medicine, Family Medicine, NUS, S-901 85 Umeå, Sweden. E-mail: olov.rolandsson@fammed.umu.se

predicted requirement of exogenous insulin treatment [5]. The measurement of autoantibodies is important in diabetes classification [6] and may have an impact on the choice of treatment [7].

GAD65Ab are commonly analyzed in radio binding assays (RBA) that display good sensitivity, specificity and concordance [8]. However, the concordance between assays identifying the same *individual* as GAD65Ab positive or negative has not been studied extensively.

The aim of this study was to assess the concordance within subjects between three different GAD65Ab RBA employing three different GAD65 reagents using plasma samples from a population with adult-onset diabetes initially classified as T2D.

Materials and methods

Subjects

Plasma samples from 112 patients (M/W 57/55, median age 50 years (min 30–max 60), mean BMI 30.2 ± 4.7) initially diagnosed with T2D by their treating physician were randomly selected from a local diabetes registry—the Diabetes Registry in the Västerbotten Intervention Program. The registry consists of subjects participating in a local health survey to which residents in the Västerbotten County, Sweden, were invited at 40, 50, and 60 years of age [9]. The subjects performed a WHO standardized oral glucose tolerance test (OGTT) and filled in a standardized questionnaire where they could self report if they were diagnosed with diabetes. They were also asked to donate a blood sample for research. The samples were stored at −80°C at the Medical Biobank at the Umeå University hospital, Umeå, Sweden. Subjects who were identified as having diabetes either by OGTT or the questionnaire were asked to participate in the diabetes registry. Information on the diagnosis of diabetes was retrieved from medical records and the T2D classification was validated according to WHO criteria [1]. The study was approved by the Ethics Committee, Umeå University, Umeå, Sweden.

Assay methods

Three different RBA analyses using different plasmids for generating recombinant GAD65 and different methods to label the protein with radioactivity were compared. The analyses were performed at two different laboratories each analyzing coded blood samples.

^{35}S-labeled GAD65 using pEx9

This assay is employing the pEx9 plasmid, which contains the full length coding region for human GAD65 cDNA [10]. The detail of the pEx9 plasmid is available at the R.H. Williams laboratory web site (http://depts.washington.edu/rhwlab/research.htm). The pEX9 plasmid containing the full length GAD65 cDNA has been made available to researchers as a modified pcDNAII vector (Invitrogen, Carlsbad, CA, USA) called pEx9 as described in detail [10]. The vector contains a modified Kozak consensus sequence to enhance translation efficiency when the plasmid is used to label the GAD65 by coupled *in vitro* transcription translation [10]. The recombinant GAD65 was labeled with 35S methionine as described [10].

The procedure of the assay is described in detail elsewhere [10,11] in which an over night incubation was used. Results are expressed as units per ml (U/ml) derived from standard curves of counts per minute for the WHO standard 97/550 [10]. The intra-assay CV% was determined for all samples including the seven standard curve samples and the four quality control samples. The inter-assay CV% was calculated from the four quality control samples used at a mean of 43, 54, 86, and 168 U/ml GAD65Ab and included in every run of the assay.

The intra- and inter-assay coefficients of variation were 7 and 11%, respectively. All samples with an intra-assay CV% greater than 20% were reanalyzed. In the Diabetes Antibody Standardization Program (DASP) 2005 workshop, the GAD65Ab analysis ranked at 76% sensitivity and 91% specificity [12]. The analysis was performed at the Lund University Clinical Research Centre, University Hospital MAS, Malmö, Sweden. The assay is referred to as "pEx9 RBA".

^{35}S-labeled GAD 65 using pThGAD65

Recently, we have recloned the human GAD65 cDNA into the pTnT^{TM} Vector (Promega PN L5610) that improves the translation performance beyond that of pEx9 and produces a protein that runs as a single band on an SDS polyacrylamide gel. The complete pThGAD65 cDNA sequence is available at http://depts.washington.edu/rhwlab/resMat/MatTransfer/pThGAD65seq.htm. The full length human GAD65 was labeled with 35S methionine as described [10]. The assay procedure is described elsewhere [11] in which an over night incubation was used. The intra-assay CV% was determined for all samples including the seven standard curve samples and the four quality control samples. The inter-assay CV% was calculated from the four quality control samples used at a mean of 43, 54, 86, and 168 U/ml GAD65Ab and included in every run of the assay. All samples with a CV% greater than 20% were reanalyzed. The intra- and inter-assay coefficients of variation were 6 and 9%, respectively. In the DASP 2007 workshop, the GAD65Ab analysis ranked at 82% sensitivity and 96% specificity. The analysis was performed at the Lund University Clinical Research Centre, University Hospital MAS, Malmö, Sweden. The assay is referred to as "pThGAD65 RBA".

^{125}I-labeled GAD65 using pGAD17

Finally, we used a kit from RSR Ltd (Cardiff, UK) using plasmid pGAD17 for expression of human GAD65 truncated at the NH2-terminal end by deleting amino acids 2–45 and labeled with ^{125}I [13]. Subsequent analyses followed standard procedures as recommended by the manufacturer including a incubation time of 2 h incubation of sera with labeled GAD65. The intra- and inter-assay coefficients of variation were 3.1 and 3.5%, respectively and all samples with an intra-assay CV% greater than 20% were reanalyzed. Sensitivity and specificity was 86 and 95%, respectively, in the 2007 DASP workshop. The analysis was performed at Clinical Immunology, Department of Clinical Microbiology, Umeå University Hospital, Umeå, Sweden. The assay is referred to as "pGAD17 RBA".

Displacement with cold rhGAD65

We performed a displacement study in order to study the possibility of unspecific binding in the assays influencing the lower GAD65Ab levels among GAD65Ab positive subjects. Plasma samples from five GAD65Ab positive subjects in the lower range (pGAD17 RBA 2.0–5.2 U/ml, pEx9 RBA 138–253 U/ml, and pThGAD65 RBA 147–252 U/ml, respectively) of GAD65Ab were used. Each sample previously analyzed for GAD65Ab was also analyzed in the presence of 3.36 μg/ml recombinant human (rh) GAD65 (kindly donated by Diamyd Medical AB, Stockholm, Sweden) to determine the level of unspecific binding.

Cut-offs

Both laboratories used two different GAD65Ab levels for cut-off. The cut-off for pEx9 RBA and pThGAD65 RBA were derived from evaluation of cumulative plots of GAD65Ab levels in 400 healthy blood donors. The cut-offs for pEx9 RBA were >39.0 U/ml and >59.0 U/ml representing the 97.5th and 99th percentile, respectively and the cut offs for pThGAD65 RBA were >31.0 U/ml and >59.0 U/ml representing the 97.5th and 99th percentiles, respectively.

The cut-offs provided by the manufacturer for the pGAD17 RBA were >1 U/ml and >1.5 U/ml, respectively [13]. The >1 U/ml given by the manufacturer corresponded to the 98.5th percentile when 200 of the 400 samples from healthy donors were analyzed. Thus, due to the good agreement, the cut-offs recommended by the manufacturer was accepted.

Statistics

The distribution of the GAD65Ab levels was positively skewed in all three assays therefore central tendency and variance are reported as median and interquartile range (IQR; distance between 25th and 75th centile), respectively. Log-transformed GAD65Ab levels were used in a scatter plot. Proportions (%) of GAD65Ab positive subjects are given with a 95% confidence interval (95% CI). The correlation between the assays was assessed using Spearman's correlation test (r_s). Concordance between two assays was calculated by summing up the proportions of subjects the assays jointly categorized as positive and negative. The agreement between methods was tested by Cohen's kappa with a 95% CI. In the analyses of concordance and agreement between assays two cut-off levels per assay were used, as described above. The data analysis was performed with SPSS version 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Plasma samples from the 112 individual patients were analyzed for GAD65Ab with the three different RBA methods. The median GAD65Ab levels were low according to all three RBAs (Table I). There was no significant correlation between GAD65Ab and sex, age or BMI in either method (data not shown).

In order to test the influence of unspecific binding in the assays we performed a displacement study by adding cold rhGAD65 to five positive samples with GAD65Ab levels in the lower range. As can be seen in Figure 1, all subjects became GAD65Ab negative after the displacement in all three assays indicating that there was no unspecific binding.

We performed cumulative plots to study the distribution of GAD65Ab in the three assays (Figure 2(a–c)). The distribution of GAD65Ab in the pEx9 RBA (Figure 2(a)) displayed a slow increase in GAD65Ab levels beginning from the level of 20% of the patients. This was in contrast to the pThGAD65 RBA (Figure 2(b)) and the pGAD17 RBA (Figure 2(c)) where the GAD65Ab levels increased after 75 and 84%, respectively. The correlation between the pEx9 RBA and the pThGAD65 RBA was high but their respective correlation to pGAD17 RBA was low (Table I).

We analyzed the proportion of GAD65Ab levels in the three RBAs applying the different cut-off values that were used in the laboratories (Table I). The pEx9 RBA had a very high proportion of subjects in the intermediate level of GAD65Ab in contrast to both the pThGAD65 RBA and the pGAD17 RBA.

We calculated the concordance between the RBAs, i.e. how many unique subjects were classified as GAD65Ab positive and negative in two RBAs applying the different cut-off values (Table I). As expected, the concordance improved when higher cut-off vales were used that also can be seen in the scatter plots shown in Figure 3(a–c). It can be noted that only seven samples were correlated at a high level in all three assays (Figure 3(a–c)).

Table I. Median values and IQR of GAD65Ab levels (U/ml), bivariate correlation, proportion (%, 95% CI) of GAD65Ab positivity at different cut-off levels, concordance, and agreement (Cohen's kappa, 95% CI) between the pEx9 RBA, pThGAD65 RBA, and pGAD17 RBA, respectively.

	pEx9 RBA	pThGAD65 RBA	pGAD17 RBA
Median value (IQR)	18.3 (27.0)	9.0 (6.5)	0.2 (0.2)
Bivariate correlation (r_s)			
pEx9 RBA	1	0.56***	0.12
pThGAD65 RBA	0.56***	1	0.32***
Proportion of GAD65Ab levels (%)			
Negative (95% CI)	75.0 (66.4–82.4)	82.1 (73.8–88.7)	87.5 (79.9–93.0)
Low levels (95% CI)	12.5 (7.3–19.6)	4.5 (1.6–9.6)	0.9 (0.0–4.9)
High levels (95% CI)	12.5 (7.3–19.6)	13.4 (8.0–20.7)	11.6 (6.6–18.6)
Concordance (low cut-off)			
pEx9 RBA (%)	100	89.3	77.6
PThGAD65 RBA (%)	89.3	100	84.8
Concordance (high cut-off)			
pEx9 RBA (%)	100	97.3	88.4
PThGAD65 RBA (%)	97.3	100	87.4
Cohen's kappa (low cut-off)			
pEx9 RBA (95% CI)	1	0.68 (0.52–0.85)***	0.30 (0.09–0.50)*
PThGAD65 RBA (95% CI)	0.68 (0.52–0.85)***	1	0.43 (0.20–0.65)***
Cohen's kappa (high cut-off)			
pEx9 RBA (95% CI)	1	0.88 (0.75–1.01)***	0.45 (0.20–0.70)***
pThGAD65 RBA (95% CI)	0.88 (0.75–1.01)***	1	0.43 (0.18–0.68)***

The following cut-off values were used; pEx9 RBA >39 U/ml (low cut-off), >59 U/ml (high cut-off); pThGAD65 RBA: >31 U/ml (low cut-off), >59 U/ml (high cut-off), pGAD17 RBA: ≥1.0 U/ml (low cut-off), ≥1.5 U/ml (high cut-off). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Finally, we tested the agreement between the RBAs applying the different cut-off levels (Table I). When the low cut-off levels were applied the agreement between pEx9 compared to the pGAD17 RBA was lower as compared to the agreement between pEx9 RBA compared with pThGAD65 RBA and pThGAD65 RBA with pGAD17 RBA, respectively. The agreement improved when applying high cut-off levels, especially between the pEx9 RBA and the pThGAD65 RBA, while it remained lower between pGAD17 RBA and the other two RBAs.

Discussion

In this study, we found an inconsistency to define which subjects were GAD65Ab positive when comparing the concordance between three RBA employing three different GAD65 preparations. The most remarkable discrepancy was observed when comparing pGAD17 RBA with pEx9 RBA, especially when the low (97.5th percentile) cut-off values were used. Applying the higher cut-offs resulted in a similar proportion (~12%) of subjects categorized as GAD65Ab positive in the three assays. However, the concordance between the pGAD17 RBA and the two other RBAs was low also when the higher (99th percentile) cut-off value were applied.

The different proportions of GAD65Ab positive subjects could be explained by unspecific binding especially in the pEx9 RBA as indicated in Figure 2(a). The pEx9 construct differs from the pThGAD65 by the non-coding region preceding the start site of transcription. The pEx9 plasmid has a transcription-enhancing Kozak consensus sequence different from the sequence used in pThGAD65. The pEx9 plasmid may generate an alternative start site, which is not present in the pThGAD65 plasmid. An alternative explanation to the unspecific binding could be a due to lower transcription/translation efficiency of the pEx9 plasmid. It could be due to the truncation of 45 amino acids at the NH_2-terminal end of the GAD65 used in the commercial kit. Several of the NH_2-terminal located amino acids are hydrophobic [14]. The deletion could lead to a change in the structure of the protein. Another result of the truncation could be the loss of GAD65Ab binding sites [15]. The NH_2 terminal is a prominent binding site of singular GAD65Ab which are often of low affinity and titer and therefore are not associated with T1D [16].

However, the difference in concordance between pGAD17 RBA and the two ^{35}S-labeled GAD65 RBAs could probably not be explained solely by unspecific binding based on the results of our displacement study (Figure 1). An alternative explanation to the low concordance could be differences in epitope exposure. In LADA patients initially classified with T2D, GAD65Ab predominantly occurs as single autoantibody and often in low titer, of which a significant amount is restricted to the NH_2-terminus of the molecule [17]. In contrast, in T1D the predominant NH_2-terminal

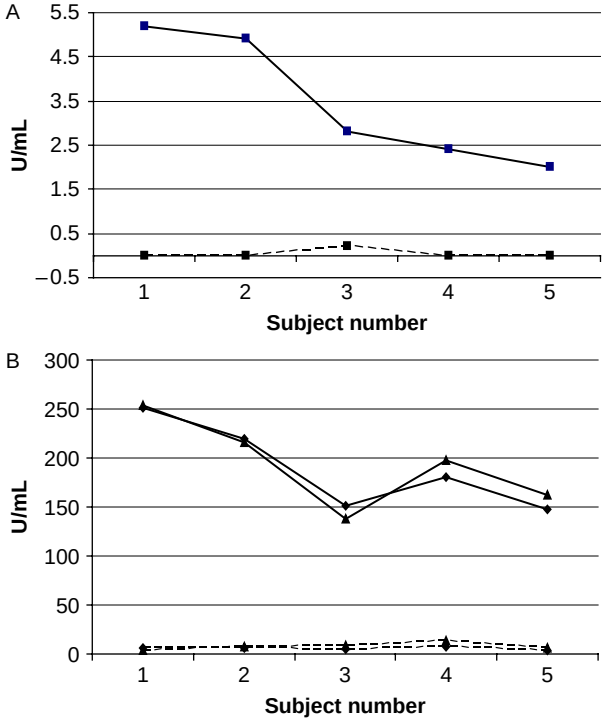


Figure 1. Displacement with rhGAD65 in five GAD65Ab positive subjects with low levels of autoantibodies applying the pGAD17 RBA, pEx9 RBA and pThGAD65 RBA assays. (A) Displacement with rhGAD65 in five GAD65Ab positive subjects with low levels of autoantibodies applying the pGAD17 RBA. Full line denotes GAD65Ab levels (U/ml) before displacement with rhGAD65. Broken line denotes GAD65Ab levels (U/ml) after displacement with rhGAD65. (B) Displacement with rhGAD65 in five GAD65Ab positive subjects with low levels of autoantibodies applying the pEx9 RBA and pThGAD65 RBA assays. Full line (triangles and diamonds) denotes GAD65Ab levels (U/ml) measured with the pEx9 RBA and pThGAD65 RBA, respectively, before displacement with rhGAD65.Broken line (triangles and diamonds) denotes GAD65Ab levels (U/ml) measured with the pEx9 RBA and pThGAD65 RBA, respectively, after displacement with rhGAD65.

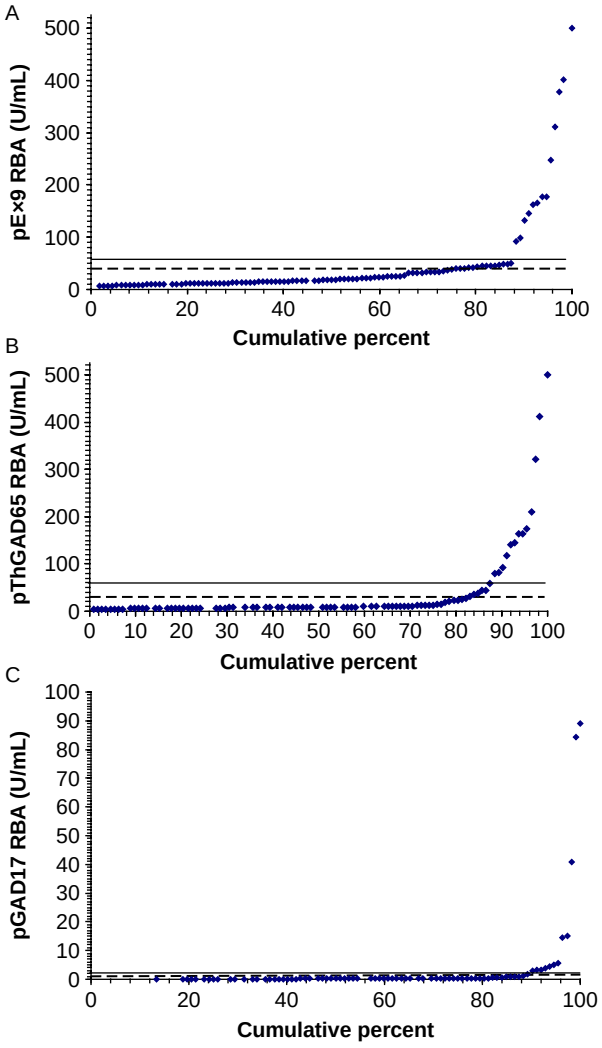
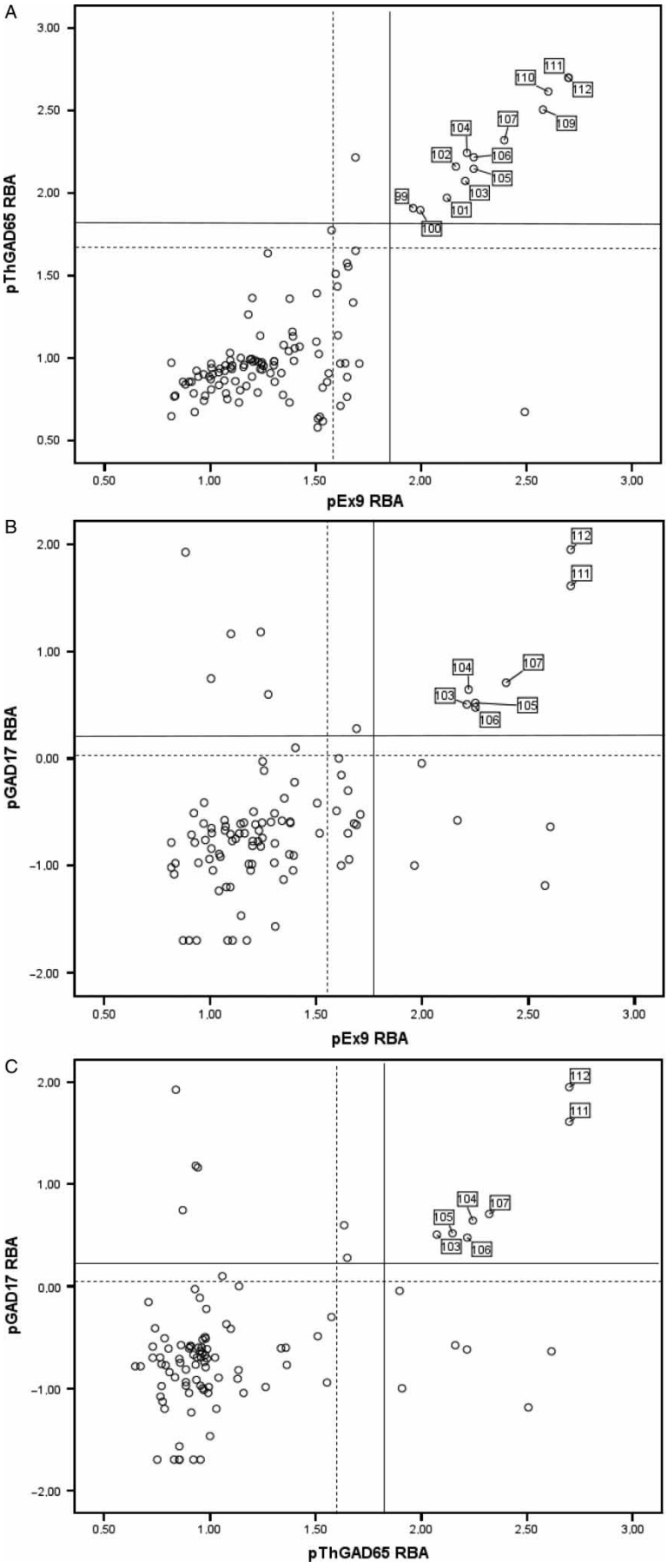


Figure 2. Cumulative plots of pEx9 RBA, pThGAD65 RBA, and pGAD17 RBA. (A) Cumulative plot of pEx9 RBA (U/ml). Broken line and full line on the *y*-axis denotes low cut-off (> 39 U/ml) and high cut-off (> 59 U/ml), respectively. (B) Cumulative plot of pThGAD65 RBA. Broken line and full line on the *y*-axis denotes low cut-off (> 31 U/ml) and high cut-off (> 59 U/ml), respectively. (C) Cumulative plot of pGAD17 RBA. Broken line and full line on the *y*-axis denotes low cut-off (> 1.0 U/ml) and high cut-off (> 1.5 U/ml), respectively.

binding is rare. In these patients GAD65Ab occur together with other autoantibodies, and they are restricted to the C-terminus and to conformational epitopes of the molecule and are of higher affinity.

Our result of low agreement between the pGAD17 RBA and the two other RBAs is in contrast to previous studies [8,13,18]. In the study by Powell et al. [13], the agreement between the pEx9 RBA and pGAD17 RBA was compared with sera from T1D subjects. The Pearson correlation coefficient between the pEx9 RBA and pGAD17 RBA was $r = 0.91$ and 66/94 and 67/94 T1D patients, respectively, were classified as GAD65Ab positive in the two RBAs. Borg et al. [18] reported similar results when they compared the performance of the pEx9 RBA and the pGAD17 RBA with a Spearman correlation coefficient of $r_s = 0.93$. They also compared the frequency of GAD65Ab in ICA positive children as estimated by the pEx9 RBA and the pGAD17 RBA. The pEx9 RBA and the pGAD17 RBA identified 59/81 and 51/81, respectively, as GAD65Ab positive. Finally, the performance of the pEx9 RBA and the pGAD17 RBA were evaluated in a proficiency study organized by the DASP [8] using sera from T1D patients only. The assay performances were compared by evaluating the area under the curve (AUC) in a receiver operator characteristic (ROC) and it was reported that the AUC did not differ significantly between the pEx9 RBA (AUC 0.97, 95% CI 0.93–1.0) and the pGAD17 RBA (AUC 0.94, 95% CI 0.90–0.99).

However, even though the correlation, evaluated by correlations test or ROC curves, was good between the pEx9 RBA and the pGAD17 RBA in T1D patients, it was not reported whether the methods identified the same subjects as GAD65Ab positive.



The explanation to the discrepancy between our study and previous study could be the different study populations. The three previous studies included only younger T1D subjects and by using young T1D patients or older T2D /LADA patients different levels of GAD65Ab are found. The level of GAD65Ab was reported to be higher in patients with T1D compared to LADA patients [19]. Thus, it is important to compare agreement and concordance at different cut-offs in different populations.

The strength of our study was that the study had a clinically relevant question with population-based design. Moreover, the blood samples were blinded to the assay laboratories. However, there are some flaws in our study. It is acknowledged that our study included a low number of positive cases. The major problem was the lack of a gold standard assay to which new assays could be compared, such an assay is yet to be identified. In addition, the proportion of subjects classified as GAD65Ab positive was low leading to wide 95% confidence intervals. A general methodological problem in our and previous studies, is the uncertainty to what extent the assays detect specific GAD65Ab and their concentration. Competition assays using unlabeled GAD65 at a known concentration in displacement analyses are warranted. Finally, there are some differences between the in-house assays and the commercial assay with a shorter incubation time with 2 h in the latter compared to the former using over night incubation. However, the antibody-antigen equilibrium is probably attained after 2 h in both methods and the discrepancy between in-house and commercial assays is not explained by differences in end-point titers.

In conclusion, the comparison of three assays for GAD65Ab applied in patients diagnosed with T2D showed discrepancies mainly in determining GAD65Ab positivity between the three assays. The measurement of GAD65Ab in patients with T2D is important in terms of prognosis and therapy [20]. As discrepancies in determining the GAD65 positivity constitutes a problem when used for clinical purposes, further methodological GAD65Ab assays studies are needed.

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Declaration of interest. The authors declare that there is no duality of interest to declare associated with this manuscript. The authors are responsible for the content and writing of this paper.

References

[1] Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 1998;15:539–553.

[2] Jahromi MM, Eisenbarth GS. Cellular and molecular pathogenesis of type 1A diabetes. Cell Mol Life Sci 2007;64:865–872.

[3] Zimmet P, Thomas CR. Genotype, obesity and cardiovascular disease–has technical and social advancement outstripped evolution? J Intern Med 2003;254:114–125.

[4] Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. Diabetes [0012-1797] 1993;42:359–362.

[5] Turner R, Stratton I, Horton V, Manley S, Zimmet P, Mackay IR, Shattock M, Bottazzo GF, Holman R. UKPDS 25: Autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. UK Prospective Diabetes Study Group. Lancet [0140-6736] 1997;350:1288–1293.

[6] Zimmet P, Turner R, McCarty D, Rowley M, Mackay I. Crucial points at diagnosis. Type 2 diabetes or slow type 1 diabetes. Diabetes Care 1999;2:B59–B64.

[7] Stenstrom G, Gottsater A, Bakhtadze E, Berger B, Sundkvist G. Latent autoimmune diabetes in adults: Definition, prevalence, beta-cell function, and treatment. Diabetes 2005;54(Suppl 2): S68–S72.

[8] Bingley PJ, Bonifacio E, Mueller PW. Diabetes Antibody Standardization Program: First assay proficiency evaluation. Diabetes 2003;52:1128–1136.

[9] Weinehall L, Hallgren CG, Westman G, Janlert U, Wall S. Reduction of selection bias in primary prevention of cardiovascular disease through involvement of primary health care. Scand J Prim Health Care 1998;16:171–176.

[10] Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, Karlsen AE, Boel E, Michelsen B, Lernmark A. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. Diabetologia 1994;37:344–350.

Figure 3. Scatter plots between pEx9 RBA, pThGAD65 RBA, and pGAD17 RBA. (A) Scatter plot of pEx9 RBA (log-transformed U/ml) on the *x*-axis where broken line and full line denotes low cut-off (> 39 U/ml) and high cut-off (> 59 U/ml), respectively. On the *y*-axis pThGAD65 RBA (log-transformed U/ml) where broken line and full line denotes low cut-off (> 31 U/ml) and high cut-off (> 59 U/ml), respectively. Data labels are given for samples exceeding the higher cut-offs for both methods. (B) Scatter plot of pEx9 RBA (log-transformed U/ml) on the *x*-axis where broken line and full line denotes low cut-off (> 39 U/ml) and high cut-off (> 59 U/ml), respectively. On the *y*-axis pGAD17 RBA (log-transformed U/ml) where broken line and full line denotes low cut-off (> 1.0 U/ml) and high cut-off (> 1.5 U/ml), respectively. Data labels are given for samples exceeding the higher cut-offs for both methods. (C) Scatter plot of pThGAD65 RBA (log-transformed U/ml) on the *x*-axis where broken line and full line denotes low cut-off (> 31 U/ml) and high cut-off (> 59 U/ml), respectively. On the *y*-axis pGAD17 RBA (log-transformed U/ml) where broken line and full line denotes low cut-off (> 1.0 U/ml) and high cut-off (> 1.5 U/ml), respectively. Data labels are given for samples exceeding the higher cut-offs for both methods.

[11] Falorni A, Ortqvist E, Persson B, Lernmark A. Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using 35S or 3H recombinant human ligands. J Immunol Methods 1995;186:89–99.

[12] Torn C, Mueller PW, Schlosser M, Bonifacio E, Bingley PJ. Diabetes Antibody Standardization Program: Evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2. Diabetologia 2008;51:846–852.

[13] Powell M, Prentice L, Asawa T, Kato R, Sawicka J, Tanaka H, Petersen V, Munkley A, Morgan S, Rees Smith B, et al. Glutamic acid decarboxylase autoantibody assay using 125I-labelled recombinant GAD65 produced in yeast. Clin Chim Acta 1996;256:175–188.

[14] Karlsen AE, Hagopian WA, Grubin CE, Dube S, Disteche CM, Adler DA, Barmeier H, Mathewes S, Grant FJ, Foster D, et al. Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. Proc Natl Acad Sci USA 1991;88:8337–8341.

[15] Hampe CS, Hammerle LP, Bekris L, Ortqvist E, Kockum I, Rolandsson O, Landin-Olsson M, Torn C, Persson B, Lernmark A. Recognition of glutamic acid decarboxylase (GAD) by autoantibodies from different GAD antibody-positive phenotypes. J Clin Endocrinol Metab 2000;85:4671–4679.

[16] Mayr A, Schlosser M, Grober N, Kenk H, Ziegler AG, Bonifacio E, Achenbach P. GAD autoantibody affinity and epitope specificity identify distinct immunization profiles in children at risk for type 1 diabetes. Diabetes 2007;56:1527–1533.

[17] Hampe CS, Kockum I, Landin-Olsson M, Torn C, Ortqvist E, Persson B, Rolandsson O, Palmer J, Lernmark A. GAD65 antibody epitope patterns of type 1.5 diabetic patients are consistent with slow-onset autoimmune diabetes. Diabetes Care 2002;25:1481–1482.

[18] Borg H, Fernlund P, Sundkvist G. Measurement of antibodies against glutamic acid decarboxylase 65 (GADA): Two new 125I assays compared with [35S]GAD 65-ligand binding assay. Clin Chem 1997;43:779–785.

[19] Lohmann T, Kellner K, Verlohren HJ, Krug J, Steindorf J, Scherbaum WA, Seissler J. Titre and combination of ICA and autoantibodies to glutamic acid decarboxylase discriminate two clinically distinct types of latent autoimmune diabetes in adults (LADA). Diabetologia 2001;44:1005–1010.

[20] Davis TM, Wright AD, Mehta ZM, Cull CA, Stratton IM, Bottazzo GF, Bosi E, Mackay IR, Holman RR. Islet autoantibodies in clinically diagnosed type 2 diabetes: Prevalence and relationship with metabolic control (UKPDS 70). Diabetologia 2005;48:695–702.

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