



# GAD Autoantibody Affinity in Adult Patients With Latent Autoimmune Diabetes, the Study Participants of a GAD65 Vaccination Trial

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## OBJECTIVE

Patients with latent autoimmune diabetes in adults (LADA) express autoantibodies against the 65-kDa isoform of GAD antibody (GADA). Intervention with recombinant human GAD65 formulated with aluminium hydroxide (GAD-alum) given twice subcutaneously to LADA patients at intervals of 4 weeks was safe and did not compromise  $\beta$ -cell function in a Phase II clinical trial. GADA affinity has been shown to predict progression to type 1 diabetes. Here, we asked whether GADA affinity was affected by the GAD65 antigen-specific vaccination and/or associated with  $\beta$ -cell function in participants of this trial.

## RESEARCH DESIGN AND METHODS

GADA affinity was measured in sera of 46 LADA patients obtained prior to the 1st week and 20 weeks after the second injection with GAD-alum or placebo using competitive binding experiments with [<sup>125</sup>I]-labeled and unlabeled human GAD65.

## RESULTS

At baseline, GADA affinities ranged from  $1.9 \times 10^7$  to  $5.0 \times 10^{12}$  L/mol (median  $2.8 \times 10^{10}$  L/mol) and were correlated with GADA titers ( $r = 0.47$ ;  $P = 0.0009$ ), fasting ( $r = -0.37$ ;  $P = 0.01$ ) and stimulated ( $r = -0.40$ ;  $P = 0.006$ ) C-peptide concentrations, and HbA<sub>1c</sub> ( $r = 0.39$ ;  $P = 0.007$ ). No significant changes in affinity were observed from baseline to week 24. Patients with GADA affinities in the lower first quartile ( $<4 \times 10^9$  L/mol) had better preserved fasting C-peptide concentrations at baseline than those with higher affinities (mean 1.02 vs. 0.66 nmol/L;  $P = 0.004$ ) and retained higher concentrations over 30 months of follow-up (mean 1.26 vs. 0.62 nmol/L;  $P = 0.01$ ).

## CONCLUSIONS

Intervention with GAD-alum in LADA patients had no effect on GADA affinity. Our data suggest that patients with low GADA affinity have a prolonged preservation of residual  $\beta$ -cell function.

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Islet autoantibodies are characteristic features of type 1 diabetes and valuable instruments to diagnose the early pre-clinical stage of this disease (1,2). Latent autoimmune diabetes in adults (LADA) is considered a subform of type 1 diabetes characterized by the presence of autoantibodies against the 65-kDa isoform of GAD (GADA) and a slow disease progression phenotype (3). Not all patients with LADA require insulin therapy, and a meaningful proportion of LADA patients have residual  $\beta$ -cell function at disease diagnosis.

In a Phase II clinical trial, subcutaneous vaccination with recombinant human GAD65 formulated with aluminum hydroxide (GAD-alum) was used to determine whether this intervention was safe and can improve  $\beta$ -cell function in GADA-positive LADA patients (4). The results of this study indicated that the intervention was well tolerated without any treatment-related adverse events after 5 years of follow-up and that a prime and boost injection of 20  $\mu$ g GAD-alum might preserve residual insulin secretion in LADA patients (5). However, subsequent Phase II/III clinical trials in recent-onset type 1 diabetic patients treated with GAD-alum have shown discordant results (6–8). While the first trial showed that treatment with two doses of 20  $\mu$ g GAD-alum induces tolerance to GAD65 resulting in preservation of  $\beta$ -cell insulin secretion in a subgroup of patients who were recruited within 6 months of diagnosis (6,9), these effects could not be

reproduced by two subsequent larger clinical trials that used the same drug for intervention (7,8). The reasons for these discrepancies are still unclear.

Type 1 diabetes–related islet autoantibodies in children are typically restricted to high-affinity responses (10–12). In populations at increased risk of type 1 diabetes, islet autoantibody affinity varies and ranges from high- to low-affinity responses, but only high-affinity antibodies predict who progresses to diabetes (10,11). The measurement of autoantibody affinity is therefore a useful strategy to improve disease specificity and distinguish between disease-related and unrelated antibodies. Little is known about the affinity of GADAs and its predictive value in LADA patients. We therefore examined GADA affinity in LADA patients participating in the GAD65 vaccination trial to ask whether antibody affinity was similarly restricted to high-affinity responses or heterogeneous and was affected by vaccination with GAD-alum. We also questioned whether the maturity of the antibody response against GAD65 is critical in determining the disease pathogenesis and clinical phenotype in LADA patients and hypothesized that only mature high-affinity responses are associated with a more rapid decline of  $\beta$ -cell function.

## RESEARCH DESIGN AND METHODS

### Patients and Trial Design

The GAD65 vaccination trial was conducted as a randomized, double-blind, placebo-controlled, group comparison,

dose-escalation Phase II clinical study in GADA-positive LADA patients, as previously described in detail (4). Briefly, 47 patients between 30 and 70 years of age received two subcutaneous injections of 4  $\mu$ g ( $n = 9$ ), 20  $\mu$ g ( $n = 8$ ), 100  $\mu$ g ( $n = 9$ ), or 500  $\mu$ g ( $n = 8$ ) GAD-alum or placebo ( $n = 13$ ) at intervals of 4 weeks and were followed for 5 years (4,5). All patients had been diagnosed with diabetes within the previous 5 years. When the patients entered the trial, their diabetes was treated only by diet and/or oral hypoglycemic agents. Fasting and 2-h Sustacal-stimulated C-peptide concentrations were measured prior to the first injection (baseline) and then at 2, 6, 9, and 12 months and thereafter every half year up to year 5. HbA<sub>1c</sub> was similarly measured. Stimulated C-peptide and HbA<sub>1c</sub> were no longer assessed in patients starting insulin treatment, while fasting C-peptide continued to be measured after patients started insulin treatment. The criteria for the introduction of insulin treatment were left to the discretion of each attending physician. GADA and autoantibodies to insulinoma-associated antigen-2 (IA-2A) and insulin (IAA) were determined as previously reported (5). For this study, autoantibodies to zinc transporter-8 (ZnT8A) were determined at the Institute of Diabetes Research, Helmholtz Zentrum München, as previously described (13). This assay showed a sensitivity of 72% and specificity of 99% in the Diabetes Antibody Standardization Program 2009 workshop (14). Table 1 summarizes the demographic

**Table 1—Demographic data and markers of diabetes in placebo and treatment dose groups at baseline**

|                               | Placebo          | 4 $\mu$ g<br>GAD-alum | 20 $\mu$ g<br>GAD-alum | 100 $\mu$ g<br>GAD-alum | 500 $\mu$ g<br>GAD-alum |
|-------------------------------|------------------|-----------------------|------------------------|-------------------------|-------------------------|
| <i>n</i>                      | 13               | 9                     | 8                      | 9                       | 8                       |
| Age                           | 56 (37–66)       | 58 (39–69)            | 57 (48–67)             | 57 (30–69)              | 53 (39–62)              |
| Male                          | 12 (92)          | 7 (78)                | 6 (75)                 | 6 (67)                  | 8 (100)                 |
| <i>HLA DQB1*0302</i>          | 5 (38)           | 3 (33)                | 4 (50)                 | 5 (56)                  | 4 (50)                  |
| GADA positive                 | 13 (100)         | 9 (100)               | 8 (100)                | 9 (100)                 | 8 (100)                 |
| IA-2A positive                | 1 (8)            | 0                     | 2 (25)                 | 1 (11)                  | 1 (13)                  |
| IAA positive                  | 1 (8)            | 0                     | 0                      | 1 (11)                  | 0                       |
| ZnT8A positive                | 2 (15)           | 2 (22)                | 2 (25)                 | 3 (33)                  | 3 (38)                  |
| BMI (kg/m <sup>2</sup> )      | 26 (23–32)       | 27 (20–35)            | 28 (23–33)             | 27 (20–39)              | 26 (21–33)              |
| HbA <sub>1c</sub> (%)         | 5.9 (4.7–7.4)    | 6.7 (5.5–10.9)        | 5.9 (5.1–9.9)          | 6.0 (4.6–7.1)           | 5.9 (5.4–8.1)           |
| HbA <sub>1c</sub> (mmol/mol)  | 41.0 (27.9–57.4) | 49.7 (36.6–95.6)      | 41.0 (32.2–84.7)       | 42.1 (26.8–54.1)        | 42.1 (35.5–65.0)        |
| Fasting C-peptide (nmol/L)    | 0.7 (0.3–1.7)    | 0.6 (0.3–1.5)         | 0.7 (0.5–1.4)          | 0.7 (0.3–1.5)           | 0.6 (0.3–1.8)           |
| Stimulated C-peptide (nmol/L) | 1.6 (0.5–3.7)    | 1.3 (0.7–2.9)         | 1.5 (1.0–2.0)          | 2.0 (0.6–3.9)           | 1.3 (0.8–5.1)           |

Data are median (range) or count (percentage) unless otherwise indicated.

data and markers of diabetes in the placebo and treatment dose groups at baseline (Table 1). The clinical trial was approved by a national regulatory agency and local ethics committee, and written informed consent was obtained from participating individuals in accordance with the Declaration of Helsinki. Because the trial was initiated before July 2005, the protocol was not registered in a registry.

In this study, we used sera from 46 trial participants obtained at baseline and 24 weeks after administration of the prime dose for GADA affinity measurements. Sera of sufficient volume for affinity measurements were not available from one patient in the placebo group.

#### GADA Affinity Measurements

GADA affinity was measured by competitive binding experiments using a similar protocol as previously described (11). Briefly, serum (2  $\mu$ L) was incubated in duplicate for 48 h at 4°C in Tris-buffered saline with Tween (TBST) buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Tween 20, pH 7.4) in the presence of  $9.4 \times 10^{-16}$  mol [ $^{125}$ I]-recombinant human GAD65<sub>aa1-585</sub> (Medipan, Berlin, Germany) and five increasing quantities of unlabeled human GAD65<sub>aa1-585</sub> ( $1.5 \times 10^{-15}$ ,  $1.5 \times 10^{-14}$ ,  $1.5 \times 10^{-13}$ ,  $1.5 \times 10^{-12}$ , and  $1.5 \times 10^{-11}$  mol, respectively [RSR Ltd.]) or TBST buffer only in a final volume of 47  $\mu$ L. Immune complexes were precipitated for 60 min with 1.5 mg protein A sepharose (GE Healthcare, Chalfont St Giles, U.K.) preswelled in 50  $\mu$ L TBST, subsequently washed five times with 1.8 mL ice-cold TBST buffer,

and measured using a  $\gamma$  counter (Packard Instruments Co., Meriden, CT). Results were expressed as counts per minute. Half-maximal inhibitory concentration ( $IC_{50}$ ) and  $K_d$  values were calculated by nonlinear regression analysis using the GraphPad Prism 3 program (GraphPad Software, Inc., San Diego, CA), and GADA affinity was expressed as reciprocal  $K_d$  value (liters per mol). The calculation of  $K_d$  values was limited to samples with  $IC_{50}$  values greater than the concentration of labeled GAD65 (0.02 nmol/L). For samples with an  $IC_{50} < 0.02$  nmol/L, the affinity was set at  $5.0 \times 10^{12}$  L/mol.

#### Statistical Analysis

The Mann-Whitney  $U$  test was used to compare GADA affinities between groups. Fischer exact test was used to compare frequencies between groups. Spearman correlation was used to determine the correlation between variables (i.e., GADA affinity vs. GADA titer, fasting C-peptide, stimulated C-peptide, and HbA<sub>1c</sub>, respectively). The Wilcoxon matched pair test was used to analyze changes in GADA affinity from baseline to week 24. The unpaired  $t$  test was used to compare fasting and stimulated  $\log_{10}$  C-peptide concentrations and  $\log_{10}$  HbA<sub>1c</sub> levels between groups at baseline and after 2, 6, 12, 18, 24, and 30 months, respectively. For all analyses, a two-tailed  $P$  value of  $<0.05$  was considered significant. The statistical analyses were performed using the GraphPad Prism 3 program (GraphPad Software, Inc.) and SPSS (SPSS 20.0; SPSS, Chicago, IL, USA).

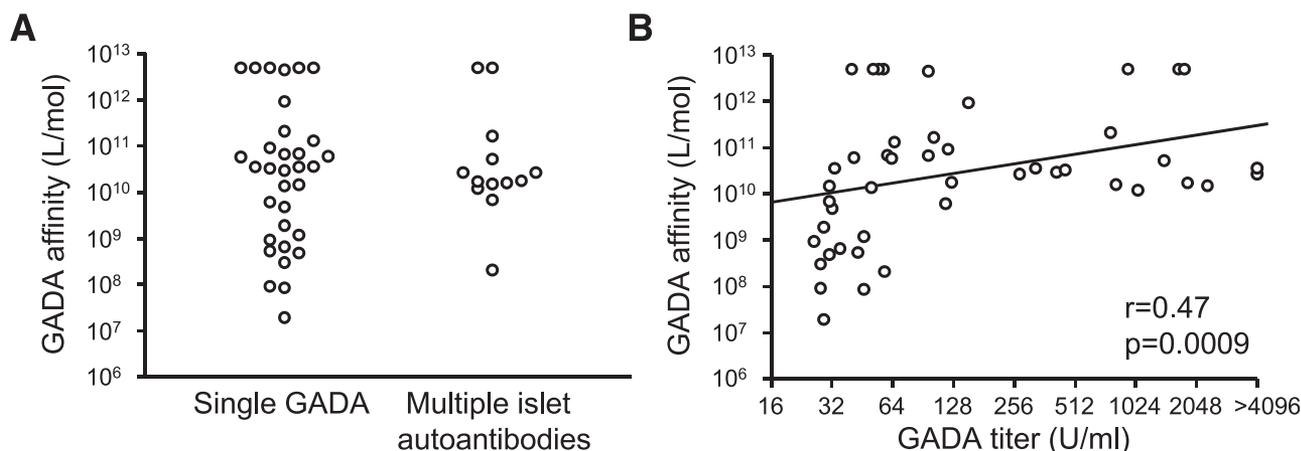
## RESULTS

### GADA Affinity Varies and Is Unaffected by GAD-Alum Vaccination

At baseline, GADA affinity ranged from  $1.9 \times 10^7$  to  $5.0 \times 10^{12}$  L/mol among the 46 LADA patients (median affinity  $2.8 \times 10^{10}$  L/mol [interquartile range (IQR)  $4.1 \times 10^9$ – $1.4 \times 10^{11}$ ]). Affinities in the lower first quartile ( $<4 \times 10^9$  L/mol) were found in 10 (30.3%) of 33 single GADA-positive patients and 1 (7.7%) of 13 patients with multiple islet autoantibodies (i.e., patients who were positive for GADA and at least one additional autoantibody of IA-2A, IAA, or ZnT8A;  $P = 0.14$ ) (Fig. 1A). GADA affinity and titer were positively correlated ( $r = 0.47$ ;  $P = 0.0009$ ) (Fig. 1B). On follow-up, GADA affinity remained relatively constant. No significant changes in affinity could be observed from baseline to week 24 in any of the intervention groups (Supplementary Fig. 1). Affinity did not increase substantially in the 11 patients who had GADA affinity in the lowest quartile, regardless of GAD-alum dose received. GADA titer also remained relatively constant after treatment in these 11 patients. In contrast, the titer increased substantially in 8 of 11 patients with higher-affinity GADAs who received either 100 or 500  $\mu$ g GAD-alum (Supplementary Fig. 2).

### GADA Affinity Is Associated With $\beta$ -Cell Function

At baseline, GADA affinity was inversely correlated with fasting C-peptide ( $r = -0.37$ ;  $P = 0.01$ ) and stimulated C-peptide concentrations ( $r = -0.40$ ;  $P = 0.006$ ) and correlated with HbA<sub>1c</sub> levels



**Figure 1**—GADA affinities in serum samples of 46 LADA patients at baseline are plotted (○) with respect to islet autoantibody status (A) and against GADA titers (B) at baseline.

( $r = 0.39$ ;  $P = 0.007$ ) (Fig. 2). The correlations with stimulated C-peptide ( $r = -0.42$ ;  $P = 0.006$ ) and HbA<sub>1c</sub> ( $r = 0.5$ ;  $P = 0.0006$ ) remained stable after 24 weeks. The mean  $\pm$  SEM concentrations of fasting C-peptide were  $1.02 \pm 0.13$  nmol/L (range 0.57–1.80) in patients with GADA affinities  $<4 \times 10^9$  L/mol (lower first quartile) compared with  $0.66 \pm 0.05$  nmol/L (0.27–1.55;  $P = 0.004$ ) in patients with higher GADA affinities at baseline. Throughout 30 months of follow-up, the fasting C-peptide concentrations remained elevated in patients with low GADA affinities (Fig. 3A) and were  $1.26 \pm 0.29$  nmol/L (0.50–2.80) at month 30, as compared with  $0.62 \pm 0.10$  nmol/L (0.10–2.20 nmol/L;  $P = 0.01$ ) in those with high GADA affinities. A similar association was seen for stimulated C-peptide concentrations (Fig. 3B), whereas no significant difference in HbA<sub>1c</sub> levels was seen after 12 months of follow-up (Fig. 3C). Affinity was  $>1 \times 10^{10}$  L/mol in all 15 patients who started insulin treatment during the 30 months of follow-up (Fig. 2). Overall, 32 (69.6%) patients had GADA affinities  $>1 \times 10^{10}$  L/mol. In comparison, fasting and stimulated C-peptide and HbA<sub>1c</sub> were not different between patients who had only GADA and patients who had GADA and other islet autoantibodies (Supplementary Fig. 3).

## CONCLUSIONS

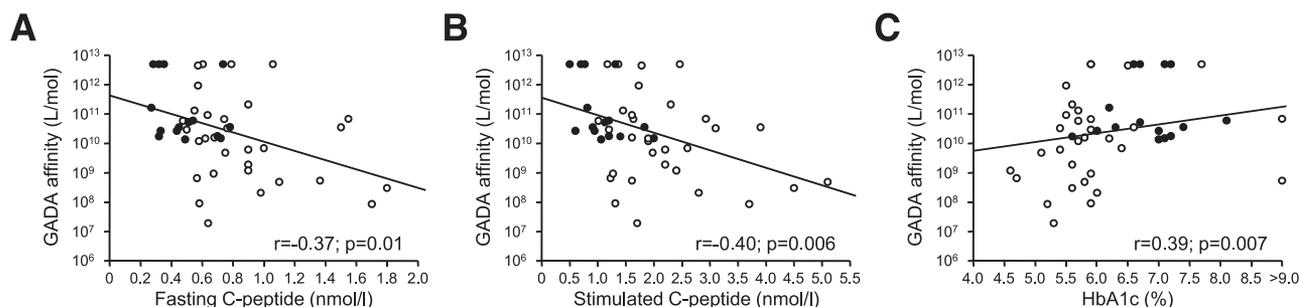
In the current study, we show that subcutaneous injections of different doses of GAD-alum had no effect on GADA affinity in LADA patients participating in a GAD65 vaccination trial. We also show that GADA affinity can range widely (up to 10,000-fold) in GADA-positive LADA

patients and that this variation at baseline correlated with  $\beta$ -cell function and subsequent need of insulin treatment in the trial participants. Patients with low-affinity GADAs had increased fasting and stimulated C-peptide concentrations and lower HbA<sub>1c</sub> levels at baseline and retained relatively high fasting C-peptide concentrations (mean  $>1$  nmol/L) over a time course of 30 months. Concordantly, all patients who started insulin treatment during this time course had high-affinity GADAs. Although high affinity did not predict further decompensation in  $\beta$ -cell function in our cohort, it identified lower function at baseline that persisted over 30 months of follow-up. Based on these findings, we suggest that high GADA affinity could be a marker for reduced  $\beta$ -cell function in LADA patients and may improve our ability to identify single GADA-positive patients who are at highest risk of requiring insulin therapy.

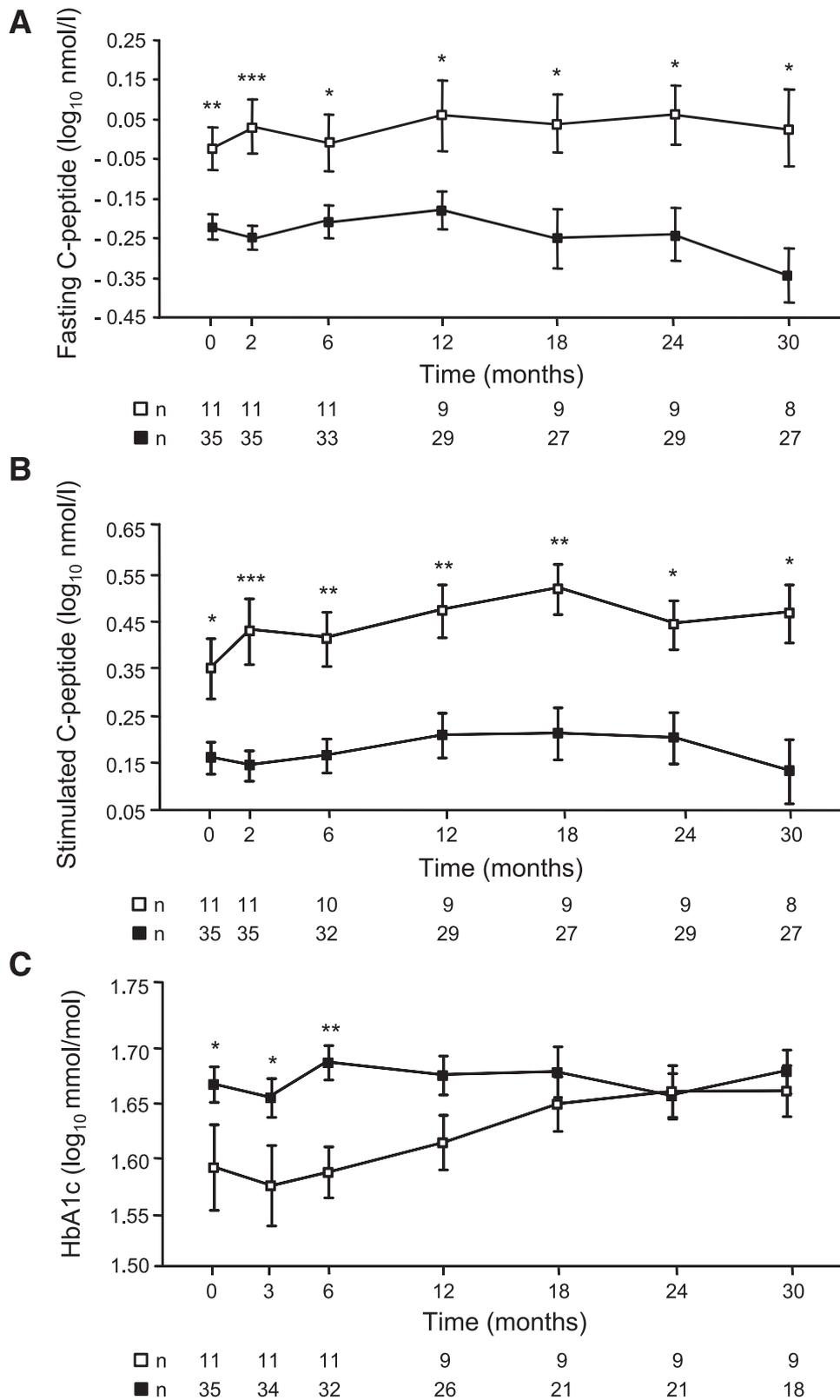
A limitation of our study is the relatively small sample size resulting in low statistical power to detect significant small differences and changes in GADA affinity. Our findings should therefore be validated in independent larger LADA cohorts. Moreover, the majority of the patients already had high affinity. A substantial increase in GADA titers was seen in patients with high-affinity GADAs who received the highest doses of GAD-alum vaccination. As expected, there were no further increases in affinity. There were 11 patients with affinities in the lowest quartile whose levels may thus be expected to increase upon vaccination if the primary target antigen of their GADAs was GAD65 and given in an effective dose; 6 of these received 100- or 500- $\mu$ g vaccine doses. Affinity

and titer did not increase substantially in these patients.

GAD65 is one of the major autoantigens in human type 1 diabetes, and circulating GADAs are present in sera from the majority of new-onset patients as well as prior to the clinical onset of disease (1). Whereas children and adolescents usually develop type 1 diabetes accompanied by multiple islet autoantibodies (2), single GADA-positive individuals make up a large number of LADA cases at diagnosis (1). However, only a portion of these patients progresses to insulin treatment within subsequent years (15). It is therefore important to identify disease-specific antibody characteristics or other markers that will help distinguish which single GADA-positive LADA patient will progress to insulin treatment. We have previously shown that GADA responses in nondiabetic children with first-degree family history of type 1 diabetes can be heterogeneous with respect to affinity and epitope specificity and that diabetes development in these children is associated with high-affinity GADAs (11), suggesting that only mature autoantibody responses have disease relevance. Concordantly, high-affinity GADAs have been found in patients with type 1 diabetes (16). We also described a type of autoantibody response with a stunted maturation and limited progression to other islet antigens, which was unrelated to type 1 diabetes development (11). It is therefore possible that low-affinity GADA responses in LADA patients are not associated with destructive autoimmunity at the site of the pancreatic islets and decline of  $\beta$ -cell function. Since repeated GAD65 vaccination did not lead to maturation of low-affinity antibodies,



**Figure 2**—GADA affinities in serum samples of 46 LADA patients at baseline are plotted (○) against fasting (A) and stimulated (B) C-peptide concentrations and HbA<sub>1c</sub> levels (C) at baseline. Patients who started insulin treatment during 30 months of follow-up from baseline are indicated (●).



**Figure 3**—Fasting (A) and stimulated (B) C-peptide concentrations and HbA<sub>1c</sub> levels (C) at baseline and after 2, 6, 12, 18, 24, and 30 months of follow-up are shown for LADA patients with GADA affinities  $<4 \times 10^9$  L/mol (□) or higher GADA affinities at baseline (■). Values are shown as mean  $\pm$  SEM and compared between both groups at each time point (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Numbers below the x-axes indicate the number of patients per group at each time point for whom data were available.

it is also possible that GAD65 is not the primary target antigen for low-affinity GADs, which may arise from immunization events that are unrelated to the pathogenesis of type 1 diabetes, e.g., nonislet antigen, as previously shown for low-affinity IAAs (17). Alternatively, low-affinity GADs could result from similar immunization events as are associated with the corresponding high-affinity GADs, but with weaker intensity or reduced expansion, and may be non- or less responsive to antigen challenge.

Immunomodulatory effects of GAD-alum vaccination have been reported. In particular, an increase in GADA titers was observed in response to 500  $\mu$ g GAD-alum in the LADA patients (4), whereas GAD-alum vaccination did not induce changes in the GADA epitope pattern (18). Similar findings were reported in a subsequent Phase II clinical trial in recent-onset type 1 diabetic patients treated with 20  $\mu$ g GAD-alum (6) and in a recent GAD-alum vaccination study in mice (19). Changes in the T-cell responses to GAD65 were also observed (19,20). None of these studies has investigated changes in GADA affinity in response to GAD-alum vaccination.

In summary, we have found a remarkable association between GADA affinity and  $\beta$ -cell function in LADA patients. Our results have practical implications with respect to diagnosis and classification of LADA and prediction of  $\beta$ -cell function in the clinical course of LADA patients and suggest that GADA affinity should be considered for recruitment and randomization of LADA patients in clinical trials.

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**Duality of Interest.** M.P., J.F., and B.R.-S. are employees of RSR Ltd., U.K. RSR Ltd. is a developer of medical diagnostics including kits for measuring diabetes autoantibodies. No other potential conflicts of interest relevant to this article were reported.

**Author Contributions.** S.K. researched data; contributed to the discussion; drafted, reviewed, and edited the manuscript; and performed GADA affinity measurements. U.L. performed GADA affinity measurements, researched data, contributed to the discussion, and reviewed and edited the manuscript. C.-D.A., S.H., K.L., J.H., K.F.L., M.P., J.F., B.R.-S., E.B., A.G.Z., and A.L. researched data, contributed to the discussion, and reviewed and edited the manuscript. P.A. researched data; contributed to the discussion; and wrote, reviewed, and edited the manuscript. P.A. 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.

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