Combined Etanercept, GAD-alum and vitamin D treatment: an open pilot trial to preserve beta cell function in recent onset type 1 diabetes

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Funding information
Barndiabetesfonden (Swedish Child Diabetes Foundation); Diabetesfonden (Swedish Diabetes Association); FORSS (the Research Council of Southeast Sweden); ALF (Region Östergötland); Diamyd Medical

Abstract
Aim: We aimed to study the feasibility and tolerability of a combination therapy consisting of glutamic acid decarboxylase (GAD-alum), Etanercept and vitamin D in children and adolescents with newly diagnosed with type 1 diabetes (T1D), and evaluate preservation of beta cell function.

Material and Methods: Etanercept Diamyd Combination Regimen is an open-labelled multi-centre study pilot trial which enrolled 20 GAD antibodies positive T1D patients (7 girls and 13 boys), aged (mean ±SD): 12.4 ± 2.3 (8.3–16.1) years, with a diabetes duration of 81.4 ± 22.1 days. Baseline fasting C-peptide was 0.24 ± 0.1 (0.10–0.35) nmol/l. The patients received Day 1-450 Vitamin D (Calciferol) 2000 U/d per os, Etanercept sc Day 1-90 0.8 mg/kg once a week and GAD-alum sc injections (20 μg, Diamyd™) Day 30 and 60. They were followed for 30 months.

Results: No treatment related serious adverse events were observed. After 6 months 90-min stimulated C-peptide had improved in 8/20 patients and C-peptide...
In spite of a modern devices and drugs, the treatment of type 1 diabetes is heavy and the disease cause complications and increased mortality. Residual insulin secretion facilitates treatment, improves metabolic control, decreases the risk of both acute and long-term complications. Some immune interventions have shown limited efficacy to preserve residual beta cell function, but the effect has been transient and some treatments have caused risks and adverse events. Autoantigen treatment could be a way to get a more specific and long-lasting effect with less adverse events. Glutamic acid decarboxylase (GAD-alum) treatment has been easy for both patients and staff, tolerable and without treatment-related adverse events. Efficacy was found in a Phase II trial using two GAD-alum sc injections, but the treatment failed in another Phase II trial with a different age range of patients and three sc injections. In a European Phase III trial, GAD-alum treatment failed to reach primary endpoint but efficacy was seen in pre-specified subgroups. Swine influenza epidemic and accompanying vaccination parallel to the GAD-alum treatment seemed to contribute to lack of efficacy, and patients in the European Phase III trial, who had already been followed for 30 months when the trial was interrupted, and got no swine flu vaccination in connection with the GAD-alum treatment, showed significant efficacy. A meta-analyses showed >97% probability that GAD-alum does preserve residual beta cell function.

We decided to conduct a series of pilot experiments to see if the GAD-alum efficacy could be improved. We have earlier reported a combination therapy using GAD-alum sc, combined with vitamin D and Ibuprofen. In that study, we found support for the use of vitamin D, while addition of a short period with Ibuprofen did not improve efficacy. Here we report results from the Etanercept Diamyd Combination Regimen (EDCR) study where we used the same GAD-alum treatment and oral vitamin D, but instead of Ibuprofen we tried combination with the tumour necrosis factor-α (TNF-α) inhibitor Etanercept. Vitamin D is said to cause Th2 deviation, improve dendritic cell function, increase T-regulatory cells, and perhaps also protect beta cells and improve insulin sensitivity. TNF-α-inhibition should be justified as TNF-α is involved in the autoimmune process leading to beta cell destruction.

Etanercept is a recombinant soluble TNF-α receptor fusion protein that binds to TNF-α. It acts by clearing TNF-α from the circulation, thereby blocking the biological activity of this inflammatory cytokine. Although Etanercept is used in the treatment of many autoimmune diseases including ankylosing spondylitis, juvenile rheumatoid arthritis, psoriasis, psoriatic arthritis and rheumatoid arthritis, it has to our knowledge only been tested once earlier in young people with type 1 diabetes (T1D). In that study, the beta cell function was preserved for 6 months and the percent change in C-peptide area under the curve from baseline to Week 24 showed a 39% increase in the Etanercept group compared to a 20% decrease in the placebo group ($p < 0.05$), while insulin dose decreased significantly more, as well as HbA1c, in the treatment group compared to placebo.

With this background, we found it reasonable to try a combination therapy with GAD-alum sc, vitamin D per os and Etanercept given sc. with the aim to see if such a therapy was feasible, safe and tolerable, and efficacious enough to justify possible risks.
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(Continues)
international guidelines. Patients with active or inactive (latent) tuberculosis should be excluded. The evaluation included a detailed medical history regarding previous potential exposure for tuberculosis, recent travel to countries with possible high exposure to tuberculosis and previous and/or current immunosuppressive treatments. Pulmonary X-ray as well as QuantiFeron® test was performed. All patients were also tested for hepatitis B and C. Patients with prior or active hepatitis B and C were excluded from the study.

Out of 47 patients screened at eight Swedish paediatric clinics 20 were eligible (Figure S1). Clinical characteristics at baseline are shown in Table 1. The patients got from Day 1 to 450 Vitamin D (Calciferol) 2000 U/d per os; Day 1-90 Etanercept (TNF-α inhibitor) 0.8 mg/kg body weight (max 50 mg) given sc once a week; and 20 μg Diamyd™ sc at Day 30 and 60. During the Etanercept treatment the patients were carefully observed.

All patients and their parent(s)/legal guardian(s) were informed by the investigator and study team that they should seek medical help immediately in case the patient treated with Etanercept developed signs and/or symptoms of blood dyscrasia or infections (long-lasting low-grade fever, sore throat, bruises, bleedings, weight loss, unexpected productive cough lasting 2-3 weeks or more and/or paleness (Table S1).

The patients were followed for a total of 30 months. The primary endpoint was to evaluate the tolerability of this combination therapy with Diamyd, Vitamin D and Etanercept. Secondary endpoints were to evaluate how the above-mentioned treatment influences the immune system and endogenous insulin secretion: C-peptide (90 min value and AUC_{mean 0-120 min}) during an Mixed Meal Tolerance Test (MMTT), proportion of patients with a stimulated maximum cytokine secretion level was calculated by subtracting the spontaneous secretion (i.e., secretion from PBMC cultured in medium alone) from the one following stimulation with GAD65 or CD3/CD28 beads.

Next, lymphocyte proliferation, and cytokines and chemokines secretion analysis were performed in samples from baseline and 1, 2, 3, 6, 9, 15 and 30 months.

Cytokines were quantified in PBMC supernatants, after cell culture for 7 days in the presence of 5 μg/ml recombinant human GAD65 (Diamyd Medical), CD3/CD28 beads (Gibco; Life Technologies AS) or in medium (AIM-V) alone at 37°C in 5% CO2. The cytokines interleukin IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-17, IL-12(P70), TNF-α and interferon (IFN)-γ were using Bio-Plex Pro Cytokine Panel (Bio-Rad) according to the manufacturer’s instructions. The chemokines G-CSF, GM-CSF, MCP-1, MIP-1 b and IL-8 were quantified PBMC supernatants. Data were collected using the LumineX 200 ™ (LumineX xMAP™ Corporation). The antigen-induced cytokine secretion level was calculated by subtracting the spontaneous secretion (i.e., secretion from PBMC cultured in medium alone) from the one following stimulation with GAD65 or CD3/CD28 beads.

PBMC proliferative response was analysed in the presence of 5 μg/ml rhGAD65 (Diamyd Medical), CD3/CD28 beads (Gibco; Life Technologies AS) and in medium alone. Data were expressed as stimulation index, calculated as the mean of triplicates in presence of stimulus divided by the mean of triplicates with medium alone.

### TABLE 1 (Continued)

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<tr>
<td></td>
<td>Max</td>
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<tr>
<td>GADA (U/ml)</td>
<td>Mean (SD)</td>
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<td>Median</td>
<td>157.6</td>
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</table>

Abbreviations: GADA, glutamic acid decarboxylase antibodies; T1D, type 1 diabetes.
4 | STATISTICS

Demographics and baseline characteristics are presented using descriptive statistics. Efficacy data regarding C-peptide and immune system as well as serious(S) adverse events (AE) and other safety data are also summarized descriptively. The AE/SAE are presented using a standardized tabulation of the frequency and incidence rate of all observed AEs/SAEs. The frequencies and incidence rates are calculated on a per patient basis. p-values <0.05 were considered statistically significant.

5 | RESULTS

The treatment was feasible, well tolerated, with no serious adverse events related to the treatment. AEs are shown in Table S2. No patient dropped out from the study.

Compared to baseline 90-min stimulated C-peptide improved in 8/20 patients after 6 months (Figure 1A). Of these patients, seven were ≥12 years old. One female patient aged ≥12 years had a mild disease with only 6% C-peptide decrease from Baseline to Month 30. Otherwise the decreases were ≥43%.

C-peptide AUC after MMTT increased up to 6 months in five patients, 4/5 > 12 years old (Figure 1B), but thereafter an age-independent consistent decline in C-peptide values was seen. The female patient aged ≥12 years with a mild disease had only 2% C-peptide decrease from baseline to Month 30; otherwise the decreases were ≥46%. The proportion of patients with a MMTT stimulated C-peptide maximum level >0.2 nmol/L decreased consistently during the study: 100.0% at Baseline, 94.4% at Month 6, 70.0% at Month 15 and 42.1% at Month 30. The mean fasting C-peptide values peaked at Month 1, declined thereafter and were above the Baseline level up to Month 6. The mean decreases from baseline were 0.02 nmol/L at Month 6, 0.10 nmol/L at Month 15 and 0.15 nmol/L at Month 30.

HbA1c reached lowest levels at Month 1, increased thereafter. The mean increases from baseline were 0.80 mmol/mol at Month 6, 6.15 mmol/mol at Month 15 and 7.55 mmol/mol at Month 30. The external insulin requirements (per kg body weight and 24 h) were lowest at Month 1, increased thereafter. The mean increases in insulin requirements were 0.01 IU at Month 6, 0.25 IU at Month 15 and 0.42 IU at Month 30. The insulin dose-adjusted Hba1c was lowest Month 1, increased thereafter. The mean increases from baseline were 0.09 units at Month 6, 1.56 units at Month 15 and 2.37 units at Month 30. (Table S3). Vitamin D in serum is shown in Table S4.
5.1 Autoantibodies in serum

GADA autoantibody levels started to rise after the first injection of GAD-alum, and were significantly higher at 3 months, after the second GAD-alum dose. The levels started to wane thereafter, with a statistically significant reduction over the study (Figure 2). Levels of IA-2 were not affected by the treatment (data not shown).

![Graph showing GADA levels over time](image)

**Figure 2** Levels of glutamic acid decarboxylase (GAD65) from baseline to 30 months. GAD antibodies (GADA) were measured in serum samples (n = 20) using 35S-labelled recombinant human GAD65, and levels were expressed as U/ml. The significance differences were indicated as p values.

5.2 Cytokines and chemokines secretion

Levels of cytokine and chemokine secretion in PBMC supernatants cultured in medium alone did not change along the study, with the exception of IL-17 that was significantly increased between baseline and 3 months (Figure 3A).

Following 1 month administration of Etanercept in vitro stimulation with GAD65 induced higher levels of IL-4 (p = 0.03; Figure 3B). IL-4 further increased at 2 months, after the first GAD-alum dose, but dropped following the second injection (Figure 3B). Higher IL-13 levels were also detected at 2 months (p = 0.03), but decreased after the second GAD-alum dose (p = 0.03; Figure 3C). Levels of GAD-induced IFN-gamma and also TNF-alpha were enhanced following administration of Etanercept and were higher at 1 month compared to baseline samples (p = 0.002 and p = 0.02, respectively) (Figure 3D, E).

Subcutaneous injections of GAD-alum did not produce any further effect, and both cytokines decreased at 6 months compared to 1 month levels (p = 0.005 and p = 0.003, respectively; Figure 3D, E).

GAD65-induced levels of the chemokines GM-CSF and MIP were enhanced after 1 month administration of Etanercept (p = 0.02; Figure 3F, G), but the first injection of GAD-alum did not lead to a further increase. MCP-1 started to increase at 1 month, and became higher than baseline at 3 months (p = 0.02; Figure 3H). The chemokines started to wane after the second injection of GAD-alum and were significantly lower at 6 months compared to 2 months levels (Figure 3F–H).

Stimulation of samples with CD3/CD28 beads did not induce any change in cytokines and chemokines secretion along the study.

![Graphs showing cytokine and chemokine secretion](image)

**Figure 3** Cytokine and chemokine secretion in peripheral blood mononuclear cells (PBMCs). PBMC samples from the patients (n = 20) collected at baseline (Day 1) and after 1, 2, 3, 6, 15 and 30 months were cultured for 7 days in in medium (AIM-V) alone (spontaneous secretion) or in the presence of 5 μg/ml rhGAD65 (Diamyd Medical) at 37°C in 5% CO2. Cytokines and chemokines were measured using BioPlex Pro Cytokine Panel (Bio-Rad). GAD65-induced levels were calculated by subtracting the spontaneous secretion, and levels were expressed as pg/ml. The significance differences are indicated as p values.
controls, even sc and inhibitor, did trial, seen a had though The combination we‐no to to plus‐including substantial C of with GAD65 and c‐peptide preservation. GADA levels from baseline to 30 months were compared in patients stratified patients according to the preservation of c‐peptide at 15 months: good responders: patient with loss of c‐peptide AUC ≤ 30% (n = 5, blue colour) and poor responders: patient with loss in c‐peptide AUC > 30% (n = 15, red colour). Significant differences are indicated as p‐values.

5.3 Proliferative response to GAD65

GAD65‐induced proliferation was not affected by GAD‐alum injections. The low proliferation observed at baseline decreased along the study, and it was lower at 30 months than baseline (Figure 4A). No changes were observed in CD3/CD28‐induced proliferation (data not shown).

6 GADA IN RELATION TO C‐PEPTIDE PRESERVATION

We stratified patients according to the preservation of c‐peptide at 15 months: (i) good responders (GR): patient with loss of C‐peptide AUC ≤ 30% (n = 5, Figure 4B, blue colour) (GR) and (ii) poor responders (PR): patient with loss in c‐peptide AUC > 30% (n = 15, Figure 4B, red colour) GADA titres increased after GAD‐alum treatment both in GR and PR, but GADA was significantly higher in the GR individuals at 3 months. No other association between C‐peptide and immune responses was observed.

7 DISCUSSION

The combination treatment including Etanercept, an effective TNF‐α inhibitor, was well tolerated and gave no treatment‐related SAEs. However, even though we have no controls, we dare to conclude that a 3‐month treatment period with adequate doses of Etanercept, a TNF‐α inhibitor, given parallel to sc GAD‐alum plus vitamin D did not lead to any substantial preservation of C‐peptide, as had been seen in a previous 6‐month trial, and this type of combination therapy did not improve GAD‐alum therapy. Somewhat surprisingly we got no decrease of TNF‐α concentrations and instead of the usual Th2‐deviation we are used to see in connection with GAD‐alum therapy, the increase of IL4 and IL‐3 was very transient, but instead we noticed a stable increase of IL‐17. Thus, we observed that spontaneous secretion of IL‐17α started to increase after the administration of Etanercept, and it remained higher throughout the study. It has been shown that treatment with Etanercept inhibited Th‐17 cells and IL‐17α levels in psoriasis patients. In contrast, anti‐TNF therapy (adalimumab) of patients with rheumatoid arthritis induced both Th17‐cells and IL‐17 secretion, irrespectively of disease activity, suggesting a different disease‐related outcome. The pathogenic contribution of Th17 cells with T1D progression of T1D has been suggested by several studies, and circulation of Th17 lymphocytes was observed in peripheral blood of long‐standing T1D patients. Enhanced IL‐17 expression in the pancreas of newly diagnosed T1D patients and increased expression of IL‐17 genes on circulating lymphocytes of T1D patients have been also reported. It was interesting that administration of Etanercept in our study did not reduce TNF‐α spontaneous secretion from PBMC in T1D, but on the contrary GAD65‐induced TNF‐α increased 1 month after Etanercept treatment. Interleukin‐17, which increased, initiates inflammation by enhancing production of pro‐inflammatory cytokines such as TNF‐α, IL‐1β and IL‐6. It has been shown that circulating CD4+ T cells from T1D patients produced IL‐17 when activated by β‐cell autoantigens including proinsulin, insulinoma‐associated protein and GAD65 peptides.

We have previously shown that GAD65‐recall response of PBMC from T1D patients who received subcutaneous administration of GAD‐alum was characterized by the secretion of several cytokines, including IL‐5, IL‐10, IL‐13, IL‐17, IFN‐γ and TNF‐α. In the present study, increase of the levels of GAD65‐induced
cytokines and chemokines was observed already after 1-month treatment with Etanercept, before the first injection of GAD-alum. Interestingly, PBMC proliferation was not enhanced after treatment, but instead it waned over time, and was lower after 30 months compared to baseline. This is in strong contrast with previous treatment with Etanercept, before the first injection of GAD-alum. Although studies in experimental animals can give valuable information, neither can studies in adults replace studies in children and adolescents as the T1D disease differ depend on age.40 However, when using new combination therapies, with possible risks the number of included children has to be kept as low as possible. Treatment with Etanercept in children means addition of more injections and could increase the risk for serious infections. Thus, for ethical reasons, we chose to do a pilot trial in a small group of patients, with deliberately no power to show clear efficacy unless this would be pronounced. For safety reasons, we chose not to do a double-blind placebo-controlled trial, but an open pilot trial without controls, which may be justified in early phase pilot trials.41,42 Our small pilot trial did give results clear enough regarding the lack of efficacy of a short treatment period with Etanercept given parallel to a GAD-alum/autoantigen treatment. Thus, we are able to add a piece of the puzzle regarding the design of future trials. The regimen we used can be avoided. However, whether another design, with Etanercept and GAD-alum/autoantigen given at different time-points, separated from each other, would give another result cannot be answered from our trial. As said in another paper, ‘progress in medicine is, at best, two steps forward and one step back, and progress towards halting or preventing T1D has been no different’. We agree. Our results are just one small piece of the puzzle.

In summary, the combination of GAD-alum, vitamin D and Etanercept was safe and tolerable. However, patients in the study did not go into pronounced or long remission and the beta cell function improved just in a minority of patients during the first 6 months but thereafter declined. The immune response did not suggest mitigation of the immune process. We can conclude that 3 months addition of sc Etanercept parallel to sc treatment with GAD-alum and oral vitamin D can be ruled out as a promising therapy.

ACKNOWLEDGEMENTS
We are grateful for the important work done by diabetes research nurses at the participating clinics, to paediatricians who have helped with seeing some patients at certain visits and laboratory staff such as Ingela Johansson and Gosia Smolinska. This trial was generously funded by Barn diabetesfonden (Swedish Child Diabetes Foundation), Diabetesfonden (Swedish Diabetes Association), FORSS (the Research Council of Southeast Sweden), ALF (Region Östergötland) and unrestricted grants from Diamyd Medical.

CONFLICT OF INTEREST
None of the authors has anything to disclose or any financial interest in the products studied.

ETHICAL APPROVAL
The study (EudraCT: 2014-001323-76; Clin Gov NCT 02464033) was approved by Medical Product Agency in Sweden and by the Research Ethics Committee, Linköping (Dnr 2014/148-31). All patients and their parents/caregivers gave their consent after oral and written information.

AUTHOR CONTRIBUTIONS
Johnny Ludvigsson had the idea, designed the EDCR study, was coordinating investigator and sponsor, conceived the study, and wrote the first draft of the manuscript. Indusmita Routray and Rosaura Casas performed experiments and analysed data. Rosaura Casas conceived the study, designed data set and data analysis. Tore Vigård, Ragnar Hanås, Björn Rathsman, Annelie Carlsson, Stefan Särnblad, Anna-Karin Albin, Carl-Göran Arvidsson, Ulf Samuelsson and Johnny Ludvigsson recruited and followed patients. All the authors read and approved the final version of the manuscript. Johnny Ludvigsson and Rosaura Casas are guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data, and the accuracy of the data analysis.

DATA AVAILABILITY STATEMENT
The data sets are available from the corresponding author on reasonable request.

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REFERENCES


SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Ludvigsson J, Routray I, Vigård T, et al. Combined Etanercept, GAD-alum and vitamin D treatment: An open pilot trial to preserve beta cell function in recent onset type 1 diabetes. *Diabetes Metab Res Rev.* 2021; e3440. https://doi.org/10.1002/dmrr.3440