LUND UNIVERSITY
Faculty of Medicine

Version 3.1.4

Short title: GADA IgG subclasses three years after clinical onset

The GAD_{65}Ab IgG subclass profile differs between adult onset type 1 diabetes and latent autoimmune diabetes in adults (LADA) up to three years after clinical onset

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Words in abstract: 238

Words in fulltext: 2259

Abstract

Background

Autoantibodies against glutamic acid decarboxylase 65 (GADA) are frequently found in patients with autoimmune diabetes. IgG_1 is the most frequent subclass among the GADA IgG subclasses. IgG_4 is a more common subclass in latent autoimmune diabetes in adults (LADA) at clinical onset compared to in type 1 diabetes. The aim was to study the different GADA-IgG subclass profiles during a three year follow-up in these groups of autoimmune diabetes.

Material and Methods

Adult onset subjects, classified as either type 1 (n=40) or LADA (n=43) were included in the study. New samples were collected every year from these patients. In addition to conventional GADA analyses, GADA-IgG subclasses, were also analyzed with a radioimmunoprecipitation assay using biotin conjugated antibodies (directed against human IgG subclasses and IgM) and streptavidin Sepharose.

Results

During three years follow-up, all the IgG subclass levels decreased in type 1 diabetes, IgG_1; $p<0.001$, IgG_2; $p<0.001$, IgG_3; $p<0.001$, IgG_4; $p<0.05$, (Friedman's´ test), while levels remained stable for all four subclasses in LADA. GADA IgM, however, decreased in both groups, ($p<0.001$).

Conclusions

Patients with LADA have higher GADA IgG_3 and IgG_4 at clinical onset and seem to maintain levels and profile of their IgG subclasses up to three years after clinical onset, while all the GADA IgG subclass levels decreases in type 1 diabetic patients. This indicates a persistent different immune response in LADA compared to in type 1 diabetes and further points out the difference in pathogenesis.

Keywords

IgG subclasses, autoimmune diabetes, type 1 diabetes, LADA, isotype class switch

Introduction

The term “latent autoimmune diabetes in adults” (LADA) [1-3] have been used since the early 1990s to describe adult onset subjects who develop a phenotypic type 2 diabetes (T2DM) but with the presence of beta cell specific autoantibodies (GADA, IA-2A or ICA) and with a slower progression to beta cell failure compared to classical type 1 diabetes.
Due to the slower progression, it is considered that subjects with LADA have no immediate need for insulin during the first six months and sometimes for up to several years after clinical onset. However, it is worth noticing that the individual requirement for insulin is based upon the treating physicians’ subjective judgment. Usually, the features of LADA include an onset above 30 years of age and normal or above normal C-peptide level. The BMI in LADA subjects is often similar to that in type 2 diabetic subjects [4-6] or sometimes less but they are not phenotypically different from T2DM. LADA was recommended to be included as a separate subgroup of diabetes in the World Health Organization (WHO) criterion in1998 [7]. Nevertheless, later on it has been questioned whether LADA is a distinct etiological entity or just adult onset type 1 diabetes in subjects with high insulin resistance [8, 9]. LADA has been suggested to differ in islet cell antigenicity compared to type 1 diabetes. Antibodies against GAD_{65} are important markers for both type 1 diabetes and LADA while IA-2A is more frequent in type 1 diabetes [10]. Also, there seem to be differences in T cell reactivity to epitopes between the groups [11]. Even though both adult onset subjects with type 1 diabetes and LADA have an increased frequency of HLA susceptibility genes [12, 13] there also seems to be other genetic differences between these two groups in MHC class I chain-related gene A (MICA) 5.0/5.1 allele [14] as well as in the tumor necrosis factor (TNF) allele [15, 16].

While patients with type 1 diabetes commonly have a combination of diabetes specific autoantibodies at clinical onset, LADA patients usually only have one of the beta cell specific antibodies and most common is the antibody directed against glutamic acid decarboxylase 65 (GADA). A majority of these LADA patients require insulin treatment within three years after clinical onset [17]. From this point of view, it appears like LADA by time become more similar to type 1 diabetes since they develop beta cell failure and insulin dependence.

Previously, we suggested an increased frequency of the GADA IgG_4 subclass at clinical onset in some subjects with LADA compared to in subjects with type 1 diabetes [18]. This could be a reflection of a different cytokine profile in and around the islets of Langerhans in LADA with a higher participation of T_h2 regulation at clinical onset compared to in type 1 diabetes.

The aim of this study was to follow the profiles of IgG subclasses and IgM in patients with type 1 diabetes and LADA up to three years after clinical onset to see if this profile remains or changes in any way during this period.

Material and Methods

Subjects

Subjects were recruited from a study in a defined area in southern Sweden as well as from a population based study in Sweden [17, 19] including patients with newly diagnosed diabetes who were followed annually for three years. The first blood sample was collected within 24 hours after clinical diagnosis. All patients fulfilling the diagnostic criteria for LADA including; age above 30 years, phenotypically classified as type 2 diabetes, positivity for GADA and without insulin treatment for at least six months after clinical onset were included (n=43). Adult onset patients (>18 years), initiated on insulin treatment at diagnosis and clinically classified as type 1 diabetes (n=40) were selected for comparison. Clinical data is presented in table 1. Although the subjects with LADA did not receive insulin for the first six months after diagnosis, more than 80% (35/43) had insulin therapy three years after clinical onset. This study was approved by the Ethical Committee at Lund University and informed consent of all subjects was obtained.

Methods

Total GADA was analyzed with a ^{35}S-based radioimmunoprecipitation assay (IPA) [20, 21] as the samples arrived, meaning that three years passed between the analysis of the first and last sample. The assay was based on a precipitation of IgG in protein A Sepharose which basically means that it captures IgG_1, IgG_2 and IgG_4. The IgG subclasses were also analyzed with an IPA, based on the same principles as for total GADA as previously described [18]. However, in this assay the biotin conjugated antibodies directed against the human IgG subclasses were incubated together with the plasma sample and ^{35}S labeled antigen in a liquid phase over night at 4°C. This was followed by precipitation on streptavidin Sepharose for 60 min at room temperature. The follow-up samples in the IgG subclass assays were analyzed together with the first sample, so the subclass assays did not have any inter assay variation. The GADA IgM was analyzed with the same technique as for the IgG subclasses with the use of biotin conjugated antibodies (555781, PharMingen, SD, USA) directed against human IgM. Antibody concentration for optimal binding capacity, IgG_1 (15 µg x ml^{-1}), IgG_2 (22 µg x ml^{-1}), IgG_3 (10 µg x ml^{-1}), IgG_4 (20 µg x ml^{-1}) and for IgM (5 µg x ml^{-1}), was determined by titration with high titer positive (n=4) and low titer positive (n=4) in-house controls for each subclass. The intra assay variations for the subclass assays (n=18) were as follows; IgG_1 (12%), IgG_2 (17%), IgG_3 (17%), IgG_4 (11%) and IgM (9%). The inter- and intra assay variation for the total GADA assay was 33% (n=52) and 16% (n=40) respectively [22].

C-peptide

Non-fasting C-peptide levels were analyzed with a commercial ^{125}I-based radioimmunoassay kit (Euro-Diagnostica, Malmö, Sweden) at the department of Clinical Chemistry, Lund University Hospital, Sweden. The reference interval was 0.25-1.0 nmol x L^{-1}and the detection limit was 0.13 nmol x L^{-1}. The intra assay variation was 5% in the measurement interval 0.5-3.5 nmol x L^{-1} and total variation (sum of intra- and inter variation) was 7% in the same measurement interval.

Statistics

The Kolmogorov-Smirnov test was used to test if the material was normally distributed. If $p<0.05$, normality was rejected [23]. The Mann-Whitney U-test was used to analyze differences in IgG subclass- or IgM levels between type 1 diabetes and LADA for each year. Wilcoxon signed rank test was used to test paired differences in C-peptide levels from clinical onset and three years after clinical onset. Correlation between total GADA and IgG subclasses or IgM (n=83) was studied with the Spearman Rank test (r_s). Friedman analysis was used to test for changes in antibody- or subclass levels for repeated measurement of paired samples, from clinical onset until three years after. All of the statistical data was analyzed with the software SPSS for Windows® version 12.0 (SPSS Inc, Chicago, IL) with the exception of the Wilcoxon signed rank test that was analyzed with the software MedCalc for Windows® version 7.4 (Mariakerke, Belgium).

Results

There was a statistical significant correlation between total GADA and IgM (r_s=0.24; p=0.03, n=83) and a strong correlation was also found with the IgG_1 subclass (r_s=0.63; p<0.001). No significant correlation was found between total GADA and the other IgG subclasses. Further analyses showed a significant correlation between age at onset and IgG_3 (r_s=0.28; p=0.01) as well as IgG_4 (r_s=0.29; p<0.01) when including all subjects (n=83). There was also correlation between BMI and IgG_3 (r_s=0.30; p<0.01) and IgG_4 (r_s=0.32; p<0.01).

IgM and IgG subclasses in type 1 diabetes

Friedman test indicated a highly significant decrease of the mean rank in GADA levels of IgG_1, IgG_2 and IgG_3 (Fig 1, panel A-C, p<0.001) and also a significant decrease in IgG_4 (Fig 1, panel D, p=0.02) for subjects with type 1 diabetes. Also the IgM levels (Fig 1, panel E) showed a significant decrease in patients with type 1 diabetes (p<0.001). The decreasing trend was not significant in total GADA (Fig 1, panel F, p=0.07) even though the pattern was similar to the IgG_1 subclass levels (Fig 1, panel A).

IgM and IgG subclasses in LADA

The Friedman test indicated a significant decrease in GADA IgM levels three years after clinical onset (Fig 1, panel E, p=0.01) but there was no decrease in the mean rank in any of the GADA IgG subclasses (Fig 1, panel A-D) or total GADA (Fig 1, panel F, p=0.11).

Comparison of levels between the groups

The group of LADA had significantly higher levels of IgG_3 (Fig 1, panel C, p<0.01) and IgG_4 (Fig 1, panel D, p<0.05) at clinical onset compared to the group of type 1 diabetes. The difference between the groups further increased with longer duration for the IgG_3-subclass (Fig 1, panel C, p<0.001) while the IgG_4-subclass more or less maintained the same difference between the groups (Fig 1, panel D). In addition, even though the IgG_2-subclass did not appear to be significantly different between type 1 diabetes and LADA at clinical onset, a significant difference in levels of IgG_2 (Fig 1, panel B, p<0.001) was observed after a year and sustained up to three years after diagnosis. The Mann-Whitney U-test did not indicate a significant difference in levels of total GADA (Fig 1, panel F) or IgM (Fig 1, panel E) between LADA and T1DM at clinical onset or annual follow up.

C-peptide levels in type 1 diabetes and LADA

The C-peptide levels were significantly lower in the subjects with type 1 diabetes at clinical onset as well as after three years (table 1). However, only patients with LADA showed a significant decrease in C-peptide over time (Fig 2).

Discussion

All the GADA IgG subclass levels decreased in the group of type 1 diabetic subjects while they were more sustained in the LADA group. The GADA IgM levels decreased over the years similarly in both groups.

The number of subjects included in the study was adequate and the methods were quite well established. However, it would have been desirable to achieve a similar age distribution in both groups. GADA IgG_3 and IgG_4 correlated well with age at onset and it seems reasonable to consider if the subclass response might be the result of the aging immune system rather than the subtype of diabetes. It has been suggested that elevated serum levels of immunoglobulin isotypes could be associated with aging [24]. A more recently published study indicated that IgM and IgG were actually reduced with increased age and no age dependent increase in IgG subclasses was reported [25]. Increased levels of GADA IgG_3 and IgG_4 also correlated with increased BMI. Since numerous lines of evidence supports a link between adipose tissue and activity of immunocompetent cells [26-29] it might be questioned if the subclass response is the result of adipose tissue stimulated immunity rather than the subtype of diabetes. However, there is no evidence that link the increase in subclasses to high BMI. Instead, over weight seems to be related to a reduced antibody production [30, 31]. Therefore, it is reasonable to believe that the presence of other subclasses beside IgG_1 is directly due to LADA rather than age and BMI.

It has been suggested, that the IgG subclass response to GADA could be titer dependent rather than reflecting the ongoing immune response based on high titer positive patients with Stiffman Syndrome (SMS) in comparison to type 1 diabetes [32]. Thus, high titer GADA should correlate with high titers of the IgG_2, IgG_3 or IgG_4 subclass. A significant correlation was found with the IgG_1 subclass which basically means that the major subclass in total GADA is IgG_1. However, in accordance with another study [33]we were not able to find any statistical significant correlation for the other subclasses. During the first and second year after clinical onset, the box plot (Fig 1, panel F) gives the impression that levels of total GADA were higher in the group of LADA, even though lack of statistical significance (p=0.11). That observation would not exclude the fact that it could be a disease dependent response. Maybe, it could be the difference in underlying mechanism of autoimmunity that causes the high antibody titers in some subjects, perhaps due to polyclonal B lymphocyte activation including both types of $CD4^+$ T cells with a broader IgG subclass response as a result. The GADA IgM levels significantly decreased in both type 1 diabetes and LADA as groups and no correlation was found with age, C-peptide or BMI. Since IgM is the first immunoglobulin subclass produced during a primary response to an antigen, alternating levels over the years could indicate epitope spreading. This was observed in some of the individuals but not significantly different between the two groups.

Since the IgG_1 subclass is the most prevalent subclass of GADA, we expected the GADA IgG_1 subclass to follow the same pattern as for total GADA. The pattern was quite similar although the total GADA did not show a statistically significant decrease in levels over the years for type 1 diabetes.

In conclusion, it appears to be immunological differences between the two groups of autoimmune diabetes as reflected by the distinction in GADA IgG subclass levels three years after clinical onset.

The differences are seen even though several of the LADA patients decreased their C-peptide levels and started insulin treatment within the three years. No correlation between total GADA and IgG subclasses besides IgG_1 was found and since the levels of total GADA did not differ between type 1 diabetes and LADA we suggest that the differences are disease dependent rather than titer dependent.

Acknowledgements

The members of the DISS study group are as follows: Hans Arnqvist, Göran Blohmé, Jan Bolinder, Per-Ola Carlsson, Soffia Gudbjörnsdottir, Lennarth Nyström, Olof Rolandsson and Jan Östman

The study was financed by the Swedish Medical Research Council and funds from Region Skåne. Maggie Stephens' Foundation and the Royal Physiograhical Foundation in Lund are thanked for travel funds.

Mrs Birgitta Persson, Berit Persson, Birgitte Ekholm, Eine Valterson and Miss Ulrika Olsson are thanked for their expert technical assistance.

Dr Anders Isaksson, Dept of Clinical Chemistry, Lund University Hospital is thanked for providing the C-peptide analyses.

The authors have no conflicts of interest.

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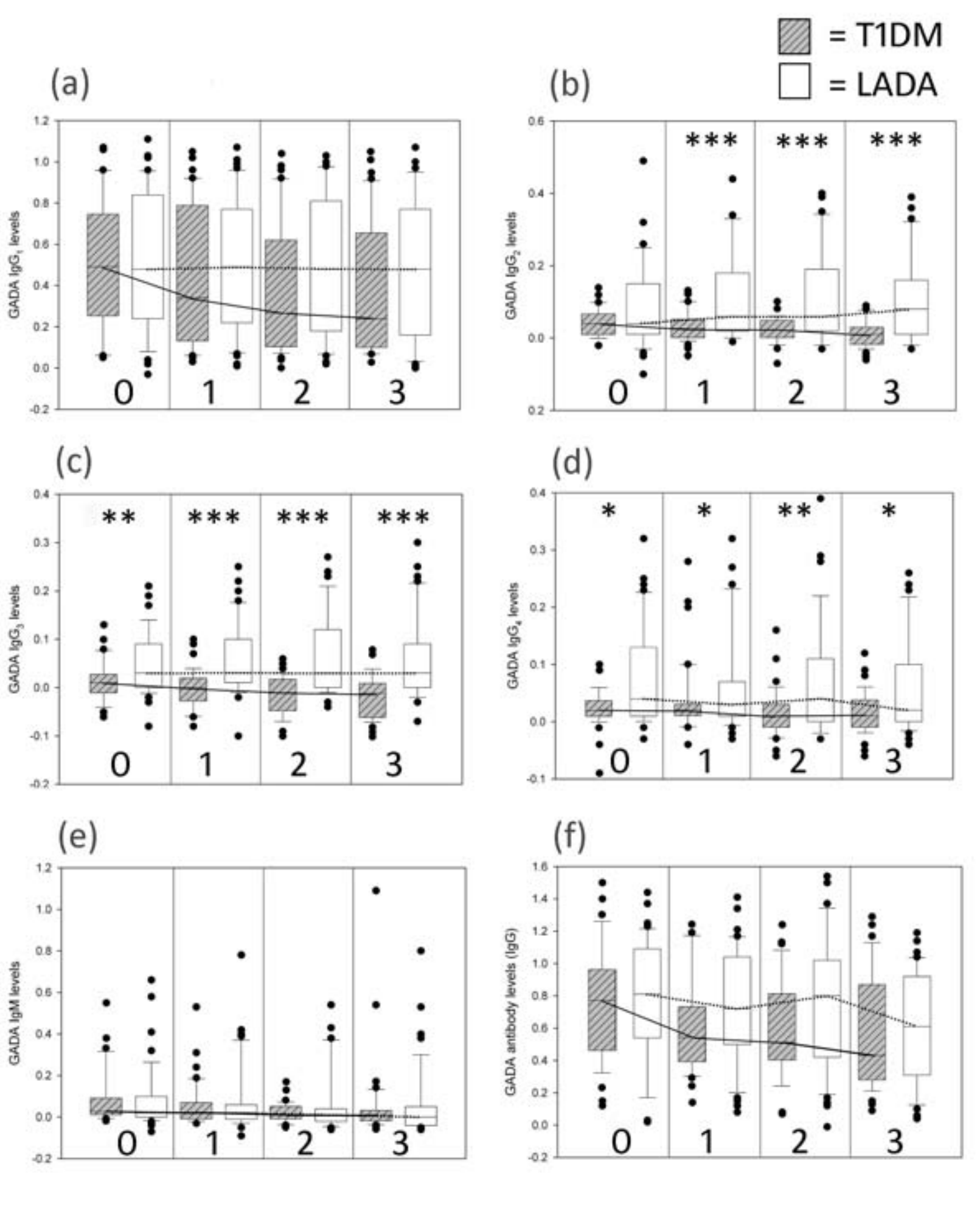
Table 1. Clinical data of the subjects at onset and the C-peptide levels three years after clinical onset.

	T1DM (n=40) Median (min-max)	LADA (n=43) Median (min-max)	p-value
Age at clinical onset (years)	28 (18-65)	36 (30-79)	<0.001[†]
BMI at clinical onset (kg/m^2)	20.9 (15.2-25.4)	25.6 (18.7-46.6)	<0.001[†]
Gender M/F	26/14	23/20	NS[‡]
C-peptide at clinical onset (nmol/L)	0.22 (0.10-0.45)	0.58 (0.38-2.80)	<0.001[†]
C-peptide after 3 yrs (nmol/L)	0.12 (0.10-1.10)	0.44 (0.10-2.90)	<0.001[†]

[†] Mann-Whitney U-test
[‡] χ^2-test

Fig 1. GADA antibody levels observed in subjects with type 1 diabetes (T1DM; n=40) and LADA (n=43). The numbering below the boxes starts with 0 (time of diagnosis) and indicates the number of years after clinical onset. The stars above the boxes indicates the significance of differences in subclass levels between type 1 diabetes and LADA as given by the Mann-Whitney U-test, * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. There seemed to be a decrease in IgG subclass levels for all subclasses in the group with type 1 diabetes (as indicated by the unbroken lines between the medians) that was not observed in LADA (dotted lines; panel A to D). The Friedman test indicated a decrease in mean rank for subclass levels in type 1 diabetes: IgG_1 (panel A, $p<0.001$), IgG_2 (panel B, $p<0.001$), IgG_3 (panel C, $p<0.001$) and IgG_4 (panel D, $p=0.02$) but no significant change was observed in LADA. The GADA IgM levels (panel E) decreased over the years in both type 1 diabetes ($p<0.001$) and LADA ($p=0.001$). The total GADA was expected to have similar pattern as for IgG_1. However, the Friedman test did not indicate a statistically significant decrease in total GADA levels for type 1 diabetes ($p=0.07$) nor LADA ($p=0.11$).

Fig 2. A box plot of the C-peptide levels in patients with type 1 diabetes (T1DM) and LADA. Levels of C-peptide were significantly lower in patients with T1DM compared to in LADA at clinical onset as well as three years after diagnosis. There was also a significant decrease in the LADA group over time indicated by the Wilcoxon signed rank test ($p=0.03$), however this was not evident in the group of type 1 diabetes ($p=0.17$).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GADA IgG1 levels
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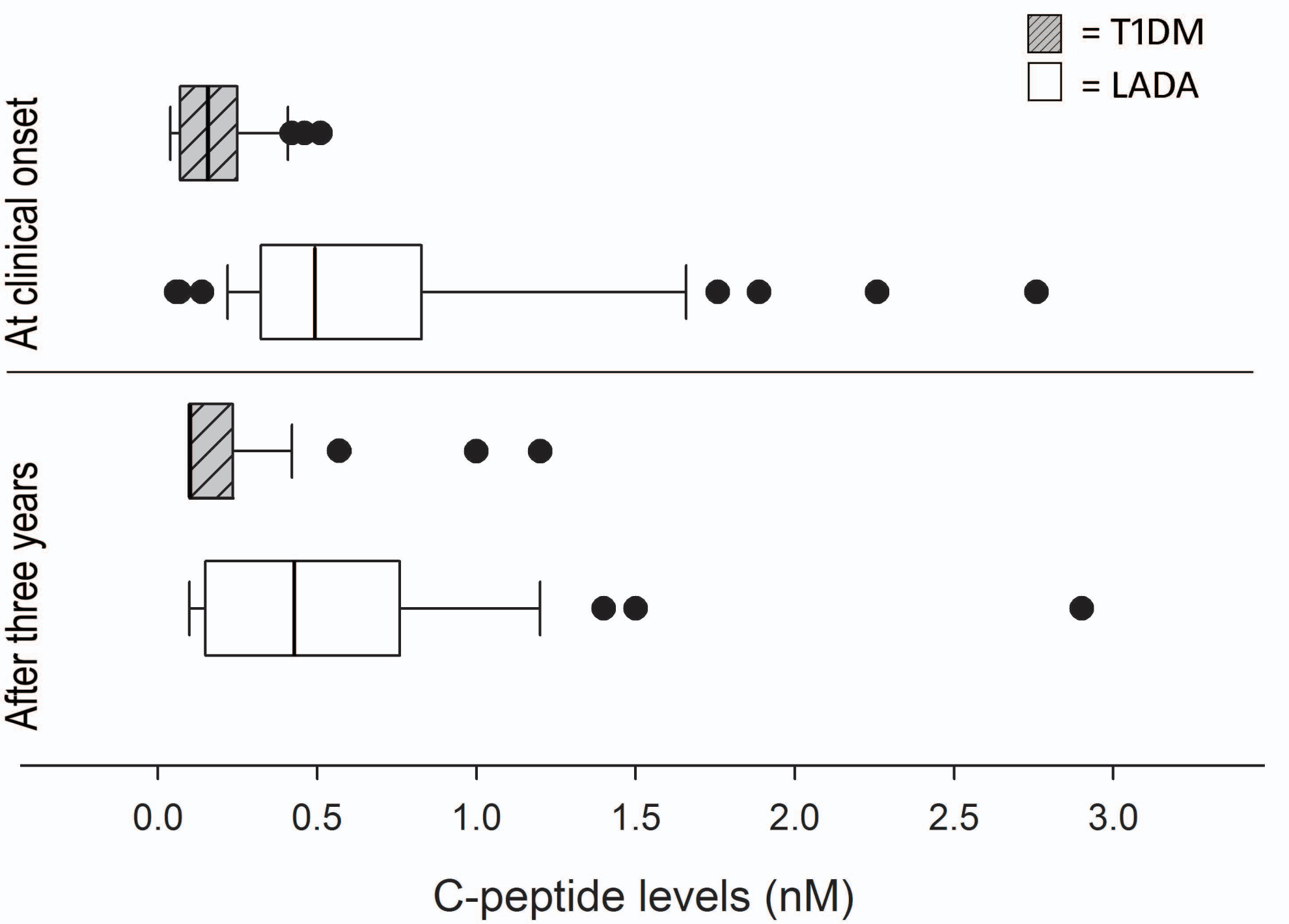
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At clinical onset
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C-peptide levels (nM)