Characteristics of *in-vitro* phenotypes of glutamic acid decarboxylase 65 autoantibodies in high-titre individuals

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Characteristics of *in vitro* phenotypes of GAD_{65} autoantibodies in high titer individuals

Running title: GADA phenotypes in high titer individuals

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Abbreviations: GAD65, glutamic acid decarboxylase; GADA, glutamic acid decarboxylase antibody; GABA, γ-aminobutyric acid; SPS, stiff person syndrome; T1D, type 1 diabetes.

Summary

Previous studies have indicated phenotypic differences in Glutamic acid decarboxylase 65 autoantibodies (GADA) found in Type 1 diabetes (T1D) patients, individuals at risk of developing T1D and Stiff-person syndrome (SPS) patients. In a phase II trial using aluminum formulated GAD_{65} (GAD-alum) as an immunomodulator in T1D, several patients responded with high GADA titers after treatment raising concerns whether GAD-alum could induce GADA with SPS-associated phenotypes. This study was aimed to analyze GADA levels, IgG1-4 subclass frequencies, b78- and b96.11-defined epitope distribution and GAD_{65} enzyme activity in sera from four cohorts with very high GADA titers: T1D patients (n=7), GAD-alum-treated T1D patients (n=9), T1D High-risk individuals (n=6), and SPS patients (n=12).

SPS patients showed significantly higher GADA levels and inhibited the *in vitro* GAD_{65} enzyme activity stronger compared to the other groups. A higher binding frequency to the b78-defined epitope was found in the SPS group compared to T1D and GAD-alum individuals, whereas no differences were detected for the b96.11-defined epitope. GADA IgG1-4 subclass levels did not differ between the groups, but SPS patients more frequently had higher IgG2 and lower IgG4 distribution.

In conclusion, the *in vitro* GADA phenotypes from SPS patients differed from the T1D- and High-risk groups, and GAD-alum treatment did not induce SPS associated phenotypes. However, occasional overlap between the groups exists, and caution is indicated when drawing conclusions to health or disease status.

Introduction

Glutamic acid decarboxylase (GAD) is a pyroxidal 5'-phosphate (PLP) dependent enzyme responsible for synthesis of the main inhibitory neurotransmitter γ-aminobutyric acid (GABA) from glutamate. The two GAD isoforms, GAD_{65} and GAD_{67}, have 65% identical amino acid sequences, with 74% homology in the C-terminal- and 25% homology in the N-terminal regions [1-2]. In humans, GAD_{65} is expressed both in pancreatic β-cells and in the synaptic vesicles of neurons, while GAD_{67} is restricted to the neural cytoplasm [3]. The function of GAD_{65} in β-cells still remains uncertain.

Stiff-person syndrome (SPS) is a rare autoimmune neurological disorder estimated to affect one per million in the general population [4-5], where clinical examination show progressive muscle stiffness and spasms [6]. Symptoms arise due to deficient GABA levels which have been attributed to the inhibition of GAD_{65} enzyme activity, as GADA-positive serum from SPS patients have shown to inhibit the GAD_{65} catalyzed decarboxylation of glutamate to GABA [7-8]. Approximately 60% of SPS patients have high GADA levels in sera [8], and autoantibodies are also present in the cerebro spinal fluid (CSF) [9-11].

Type 1 diabetes (T1D) results from a selective autoimmune destruction of the pancreatic insulin producing β-cells, where GAD_{65} acts as one of the major autoantigens [12]. Approximately 70-80% of newly diagnosed T1D patients have detectable GADA in serum [13], and the presence of persistent GADA together with other T1D-associated autoantibodies is a strong predictor for progression to disease in healthy individuals [14-16]. Both T1D and SPS are characterized by GAD_{65}-specific cellular and humoral immune responses [17]. Whereas the majority of GADA in T1D are directed to GAD_{65} [18], SPS patients show high levels of GADA specific for both isoforms [19-20]. The shared immunological etiology is reflected in the coexistence of both diseases in as many as 30% of SPS patients also develop T1D [1, 9], however, only one in ten thousand individuals diagnosed with T1D is affected by SPS [21].

There are differences in the GADA phenotypes present in these two diseases, as expressed in titers, recognized epitopes, IgG subclass distribution and ability to inhibit GAD_{65} enzyme activity. It has been reported that SPS patients display significantly higher GADA titers compared to T1D individuals [22]. GAD_{65}-specific monoclonal antibodies and their recombinant Fab (rFab) have previously been used to map GADA epitopes associated with T1D and SPS. The GADA epitope defined by monoclonal antibody b96.11 is located in the

middle region of GAD_{65}, and appears to be associated with progression to T1D [23-25]. In contrast, SPS patients recognize a GADA epitope defined by monoclonal antibody b78 which is located in the C-terminal region [7]. While sera from SPS patients characteristically inhibit GAD_{65} enzyme activity, an event associated to presence of b78-defined GADA [26], this phenomenon is only observed for a minority of GADA-positive T1D patients [8]. Furthermore, previous studies of GADA IgG subclass distribution have shown that IgG1 is the dominant subclass in newly diagnosed T1D patients and individuals at risk of developing T1D as well as in SPS patients [11, 17, 27-28]. However, SPS patients more frequently present a broader range of subclasses other than IgG1 [11, 17], whereas T1D patients show higher levels of IgG3 [17]. In contrast, individuals with a susceptibility to T1D, which display a higher frequency of GADA IgG2 [28] and/or IgG4 [27], stay non-diabetic longer than those with a broader subclass response lacking the emergence of IgG4.

During a previous clinical phase II trial, using aluminum formulated GAD_{65} (GAD-alum) as an immunomodulator for T1D [29], treated patients displayed up to a 57-fold increase in GADA titers. These findings raised concerns whether induction of GADA titers by treatment could be accompanied by development of a GADA phenotype similar to that observed in SPS patients. Thus, the aim of the present study was to determine phenotypical differences in GADA titers, ability to inhibit GAD_{65} enzyme activity as well as GADA epitope and IgG subclass distribution in four groups of high GADA titers; T1D patients, T1D patients treated with GAD-alum, individuals at high risk for T1D, and SPS patients.

Material and Methods

Study populations

Four groups of high GADA positive individuals were included in the present study, for a detailed description of patient characteristics, see Table I.

Type 1 diabetes patients (T1D)

Samples from the T1D group (n=7) were obtained from patients participating in a Swedish nationwide prospective cohort study, Better Diabetes Diagnosis (BDD), involving newly diagnosed T1D patients ≤ 18 years recruited from 40 pediatric clinics [30]. For the current

study, samples with the highest GADA titers (>95th percentile of GADA positive patients) were selected from BDD patients recruited at the Linköping University Hospital pediatric clinic (n=198).

Type 1 diabetes High-risk individuals (High-risk)

The High-risk group (n=6) was selected from the ABIS (All Babies in Southeast of Sweden) cohort, where 17,055 children born 1997-1999 have been prospectively followed with regular biological sampling [31]. From this cohort, children testing positive for several T1D-associated autoantibodies at ≥ two time points (n=23), have been classified as having high risk for developing the disease [32]. Here we included 6 of the children with the highest GADA levels, of which three developed manifest T1D after sample collection.

Stiff-person syndrome patients (SPS)

Serum from the SPS group (n=12) were chosen exclusively based on sample availability, all SPS patients were GADA positive. Serum samples from ten patients were kindly donated by Mohammed Hawa and David Leslie at the Queen Mary University of London, UK, while two samples were collected from patients recruited from the Östergötland county council, Sweden. Eight out of twelve SPS individuals were also diagnosed with T1D.

Type 1 diabetes patients treated with GAD-alum (GAD-alum)

Samples from the GAD-alum group (n=9) were selected from a previous clinical phase II trial described elsewhere [29]. The treatment significantly increased GADA levels compared to patients receiving placebo, with the highest levels detected 3 months after initiation of treatment. At this time-point approximately 1/3 (n=11) of patients receiving GAD-alum displayed a GADA fold-change of 10-35 times, while the remaining 2/3 of the patients (n=24) displayed a GADA fold-change of less than 10 times compared to baseline. The maximum increase of GADA from baseline observed during the trial was a fold-change of 57 times, detected in one patient at 3 months. For the present study, serum samples from the 3 month visit were selected based on the highest quartile of GADA levels within the treated group.

Determination of GADA titers

Serum GADA titers were determined using a radio-binding assay employing ^{35}S-labeled recombinant human GAD_{65}, as previously described [33]. The assay is validated through the Diabetes Autoantibody Standardization Program (DASP) workshop, and in 2010 the assay had 100% specificity and 80% sensitivity.

GAD_{65} enzyme activity assay

Recombinant human GAD_{65} enzyme activity was measured in duplicates in the presence of patient serum by a $^{14}CO_2$-trapping method based on the enzymatic conversion of glutamate to GABA as previously described [33]. Mean results were expressed as a percentage of the maximum GAD_{65} enzyme activity.

Epitope-specific Radioligand Binding assay (ES-RBA)

Monoclonal antibodies b96.11 and b78 were derived from a patient with Autoimmune Polyendocrine Syndrome – type 2 [34], and recognize conformational epitopes formed by the 3D structure of amino acid residues 308–365 and 451–585, respectively. Both mAbs recognized GAD_{65} in its native conformation and do not bind GAD_{67}. The capacity of their recombinant Fab (rFab) to inhibit GAD_{65} binding by human serum GADA was tested in a competitive ES-RBA as previously described [25]. The two rFab were added to separate wells at a concentration sufficient to compete binding of the originating intact mAb to GAD_{65} by at least 80%. Non-competitive GAD_{65} binding was established by no addition of rFab. The cut-off for specific competition was determined as >15% by using a negative control rFab CG7C7 specific to insulin, at 2 μg/ml. Each sample was measured in triplicates, and the mean value was calculated. A control serum was included on each plate to correct for inter-plate variations. Binding of GADA to GAD_{65} in the presence of rFab was expressed as follows: Ratio = GADA cpm in the presence of rFab (competed) / GADA cpm in the absence of rFab (non-competed). A higher binding to GAD_{65} in the presence of an rFab indicates a lower proportion of GADA binding to the respective epitope. Cases where the rFab competed sample resulted in higher cpm than the non-competed sample, were regarded to have a 100% binding capacity (i.e. no GADA with the epitope specificity in question).

GADA IgG subclass assay

The GADA IgG1, 2, 3 and 4 subclasses were measured using a modification of the conventional GADA assay, as previously described [33]. The cut-off value for each subclass was determined using a GADA-negative control, which was run in duplicates in each assay. Results were expressed as cpm, and positivity of each sample was calculated by subtraction of the mean cpm value plus three times the standard deviation (SD) obtained for the negative control. Due to sample limitations in the SPS group, GADA subclass distribution analysis were performed for 10/12 patients.

Statistics

As data sets were determined to be significantly different from a Gaussian distribution using Shapiro-Wilk test, non-parametric tests corrected for ties were used. Unpaired analysis was performed using Kruskal-Wallis followed by the Mann-Whitney *U*-test and correlations were calculated using Spearman's rank correlation coefficient test. Differences within the groups were analyzed by Friedman's test followed by Wilcoxon signed rank test; p-values <0.05 were considered statistically significant. The statistical analyses were performed using IBM SPSS Statistics version 19 (SPSS inc).

Ethics

Informed consent from the participants, or their guardians, was obtained as part of previous clinical- and epidemiological studies according to the Helsinki Declaration.

Results

Higher GADA levels in SPS patients

The SPS group displayed higher GADA levels (median: 424,300 U/ml, range: 2,019-4,992,000) compared to the T1D- (median: 21,140 U/ml, range: 12,040-48,000; p=0.003), GAD-alum- (median: 14,770 U/ml, range: 9,145-158,300; p=0.002) and High-risk (median: 13,678 U/ml, range: 1,020-70,350; p=0.003) groups (Fig. 1). In average, SPS patients had a

20-fold higher GADA titer compared to the T1D group. While the coexistence of T1D in SPS patients did not affect GADA levels, and GADA levels of these individuals were evenly distributed within the SPS group, the four SPS patients without T1D showed GADA titers above the median level for the whole group (Fig. 1).

T1D, GAD-alum and High-risk individuals inhibit GAD_{65} enzyme activity to a lower extent compared to SPS patients

The *in vitro* GAD_{65} enzyme activity was significantly lower in SPS patients (median: 45%; range: 34-67%) compared to the T1D- (median: 66%; range: 42-81%; p=0.010), GAD-alum- (median: 93%; range: 54-100 %; p<0.001), and High-risk (median: 75%; range: 38-88%; p=0.032) groups (Fig. 2). Sera from GAD-alum-treated patients inhibited the activity to a lesser extent than sera from the T1D group (p=0.042). Coexistence of T1D and SPS did not seem to affect the enzymatic inhibition differently. In addition, one T1D patient and one High-risk individual inhibited GAD_{65} enzyme activity to the same extent as the median inhibition observed for the SPS group.

Correlation analysis revealed a relationship between high GADA titers and low GAD_{65} enzyme activity in the GAD-alum (r=-0.883; p=0.002) and High-risk groups (r=-0.812; p=0.050), and a trend in T1D patients (r=-0.721; p=0.068) (Fig. 3A-D). However, this association was not observed for the SPS patients. No other association between GAD_{65} enzyme inhibition, GADA titers, IgG subclass distribution or epitope pattern was observed.

Higher frequency of GADA with the b78 phenotype in SPS patients

For the majority of SPS patient sera (11/12, 92%), binding to GAD_{65} was significantly reduced in the presence of rFab b78, while only 2/7 T1D- (28%), 3/9 GAD-alum- (33%) and 2/6 High-risk individuals (33%) were affected (Fig. 4A). The majority of individuals in all groups showed significant reduction in binding to GAD_{65} in the presence of rFab b96 with no significant differences between the groups (Fig. 4B).

No significant differences in GADA IgG1-4 subclass distribution

Analysis of the GADA IgG 1-4 subclass distribution (in cpm) revealed no significant differences between the groups (Fig. 5). We further assessed the relative contribution (%) of each subclass to the entire GADA titer for each individual; still no differences were found between the groups (data not shown). However, while the levels of each subclass did not differ between the groups, a difference in subclass hierarchy within the groups was found. The most frequent subclass in all groups was IgG1. While IgG2 was the lowest prevalent in T1D-, GAD-alum- and High-risk individuals, similar distribution of the other classes was found in the T1D- and GAD-alum groups (IgG3>IgG4>IgG2), but not in the High-risk group (IgG4>IgG3>IgG2). In contrast, SPS patients more frequently showed lower proportions of IgG4 (IgG3> IgG2>IgG4).

Discussion

In this study we assessed whether GADA phenotype characteristics observed in different groups of individuals with very high GADA titers correlated with disease status. Only high GADA titer groups were included when comparing GADA phenotypes to SPS patients, in contrast to most previous studies which have selected individuals based solely on GADA positivity. This evaluation is also of clinical relevance, as GAD-alum immunization triggers a significant increase in GADA titers, raising concerns about the possible induction of SPS-like GADA phenotypes. While our data support previous findings of disease-specific GADA phenotypes on a group basis, we found phenotypic overlaps among individuals from the different groups. A previous report including high-titer patient groups suggested that GADA phenotypic patterns may be associated to the high GADA titers usually found in SPS patients, rather than the disease *per se* [35]. Even though sera from GAD-alum, T1D and High-risk groups for this study were selected based on their very high GADA titers, levels in SPS patients were still significantly higher than the other groups. It has previously been reported that GADA titers in SPS patients are 50-500 fold greater than those found in general T1D populations [21-22]. The 20-fold difference in GADA levels between the SPS and T1D group in our study is considerably lower, as our T1D cohort was selected on basis of extremely high GADA titers. Even though GADA titers in the SPS group overall exceeded that of the other

groups, some SPS patients had levels similar to those found in the other cohorts. Further, two individuals in the GAD-alum group had titers similar to the SPS group median level.

A similar pattern was observed when analyzing the inhibition of GAD_{65} enzyme activity. Thus, while sera from SPS patients inhibited the *in vitro* GAD_{65} enzyme activity significantly more compared to the other groups, the inhibition in three SPS patients was close to the median inhibition observed for T1D patients, and an overlap for certain individuals within each group was observed. The lower inhibition of enzyme activity observed by sera from the GAD-alum treated group compared to that of T1D individuals further supports the safety of GAD-alum treatment in T1D patients. An inverse correlation was found between GADA titers and GAD_{65} enzyme activity in the GAD-alum and High-risk group, but not for SPS individuals. Previous studies have not been able to establish a correlation between GADA titers in serum or CSF with disease severity in SPS patients [7, 36], which might explain the lack of correlation between GADA titers and enzyme inhibition in our SPS group. Due to ethical and practical reasons it was unfortunately not possible to include CSF sampling as a part of this study.

The analysis of GADA epitopes showed that the b78-defined epitope, previously described as a marker for SPS [7], was indeed recognized significantly better by SPS patients. Our results, including selected high-titer GAD-alum treated patients, are in line with our previous report including the whole study cohort, where we found no change in recognition of the b78-defined epitope, and only a transient increase in binding to the b96.11-defined epitope [37]. Here we add new data showing that even a 57-fold increase in GADA titers did not induce an SPS-associated phenotype. Noteworthy, all patients participating in the GAD-alum phase II trial have been followed at 4 [38], 5 and 6 years after the trial was initiated (unpublished data) and no neurological or other clinical adverse events have been reported. No induction of SPS associated GADA phenotypes were detected during the phase II GAD-alum trial [33, 39] and none of the participants in the trial has developed neurological complication after several years. To be able to assess the persistence of the GADA-phenotypes observed during the study, additional future sampling is needed.

Previous studies have shown that the b96.11-defined epitope is commonly recognized by GADA in T1D individuals [7] and in individuals progressing to T1D [40]. The majority of samples from all groups recognized the b96.11-defined epitope with no significant differences

between the groups. This may be due to the fact that the majority of SPS patients were also diagnosed with T1D, and half of the High-risk individuals developed T1D after sampling.

Analysis of GADA IgG1-4 subclass distribution in absolute values (cpm) or relative contribution (%) revealed no differences between the groups. As previously described [11, 17, 27] IgG1 was the most frequent subclass in all groups, and the similar IgG subclass hierarchy observed for the T1D- and GAD-alum groups is in line with a previous study showing higher IgG3 frequencies in T1D patients [17]. The subclass hierarchy observed for the High-risk individuals is also in agreement with previous findings showing relatively higher IgG4 frequencies [27], and the two individuals with highest IgG4 levels have not developed T1D yet. In contrast, SPS patients more frequently displayed higher prevalence of the IgG3 and IgG2 subclasses and low IgG4. Indeed it has been reported that SPS patients more frequently show a broader subclass distribution [17], including a higher frequency of the IgG4 subclass. However, another study could only detect IgG1 and IgG2, but no IgG3 and IgG4 in sera and CSF from SPS patients [11]. It has also been proposed that increased frequency of IgG2, IgG3 and IgG4 may reflect the high antibody titers normally found in SPS individuals [35], highlighting the difficulty to establish a consistent subclass hierarchy for these patients. Due to the lack of samples at the time of T1D diagnosis for the SPS patients with co-existing diseases, it was impossible to asses GADA-phenotypes during this period.

In conclusion, here we show that *in vitro* phenotypes of GADA from SPS patients differed from high GADA titer positive T1D patients and T1D High-risk individuals, and that GAD_{65} injections did not induce SPS associated phenotypes in T1D patients responding with very high GADA titers to GAD-alum treatment. However, despite the low number of patients in each group, overlaps between these groups exist, suggesting caution when drawing conclusions from *in vitro* analyses regarding association of GADA phenotypes to health or disease status.

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MC, RC, CH, JL designed the research study; MC, CH performed the research; MC, RC analyzed the data; MC wrote the paper; RC, JL, CH critically revised the paper.

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Table I. Patient characteristics of SPS and T1D patients, GAD-alum treated T1D patients and healthy High-risk T1D individuals.

Patient	Age at sampling	Sex	Age T1D	Age SPS
SPS 1	53	M	15	51
SPS 2	48	F	25	N/A
SPS 3	45	M	24	42
SPS 4	48	F	32	47
SPS 5	65	F	60	63
SPS 6	71	F	no	69
SPS 7	37	F	no	34
SPS 8	33	F	28	31
SPS 9	61	F	no	60
SPS 10	71	F	31	68
SPS 11	64	M	29	49
SPS 12	56	F	no	47
T1D 1	10	F	10	no
T1D 2	17	M	17	no
T1D 3	10	F	10	no
T1D 4	5	M	5	no
T1D 5	12	F	12	no
T1D 6	4	F	4	no
T1D 7	17	M	13	no
High-risk 1	5	F	no	no
High-risk 2	8	M	13	no
High-risk 3	8	F	no	no
High-risk 4	8	F	11	no
High-risk 5	8	M	no	no
High-risk 6	8	M	13	no
GAD-alum 1	18	F	17	no
GAD-alum 2	15	F	15	no
GAD-alum 3	17	F	16	no
GAD-alum 4	12	M	11	no
GAD-alum 5	15	F	14	no
GAD-alum 6	18	M	18	no
GAD-alum 7	17	F	17	no
GAD-alum 8	11	F	10	no
GAD-alum 9	15	F	15	no

SPS, Stiff-person syndrome; T1D, Type 1 diabetes; GAD-alum, aluminum-formulated glutamic acid decarboxylase

Figure legends

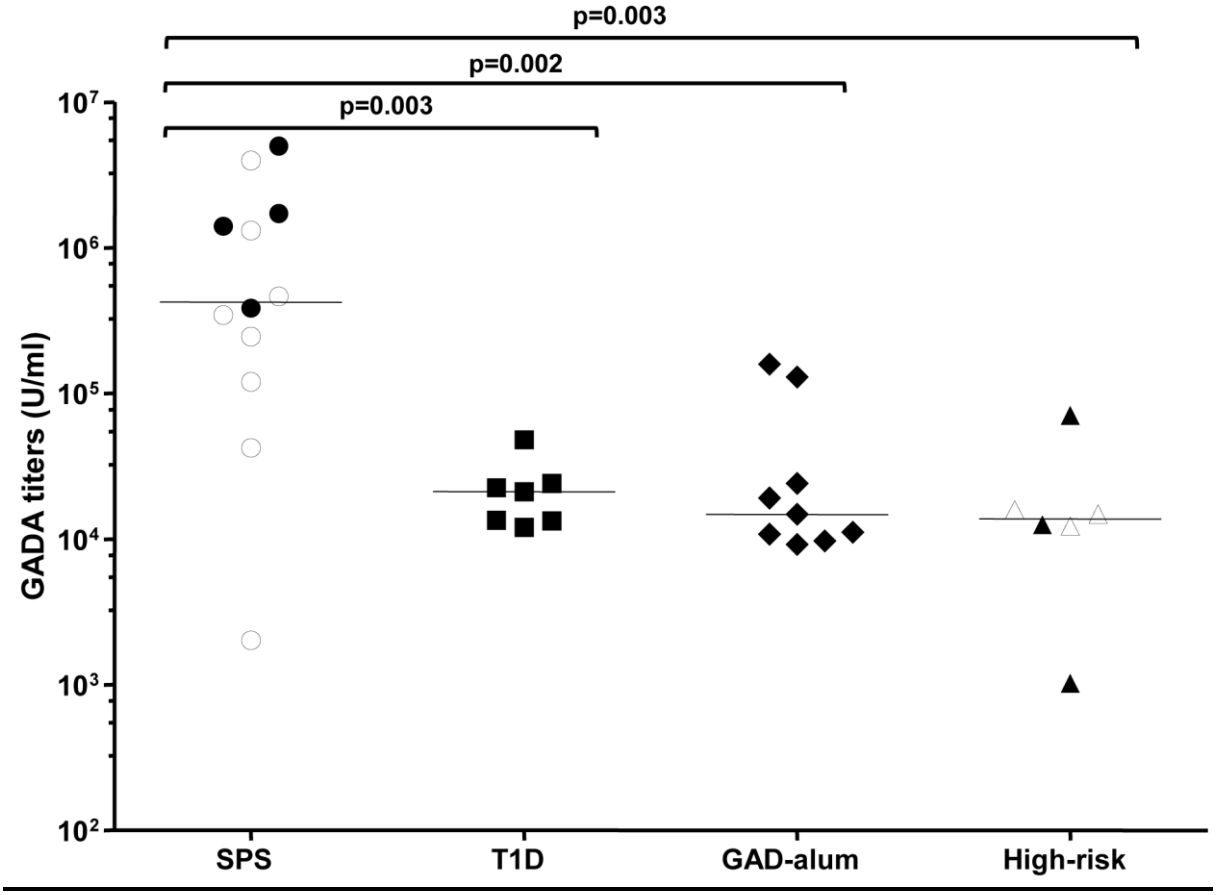

Figure 1. Serum GADA titers (U/ml) in SPS (circles, n=12), T1D (squares, n=7), GAD-alum (rhombuses, n=9) and High-risk (triangles, n=6) groups. Empty circles in the SPS group (n=8) represent individuals with coexistent T1D whereas empty triangles in the High-risk group (n=3) represent individuals that developed T1D after sampling. Significant differences are indicated as p-values and horizontal lines represent the median.

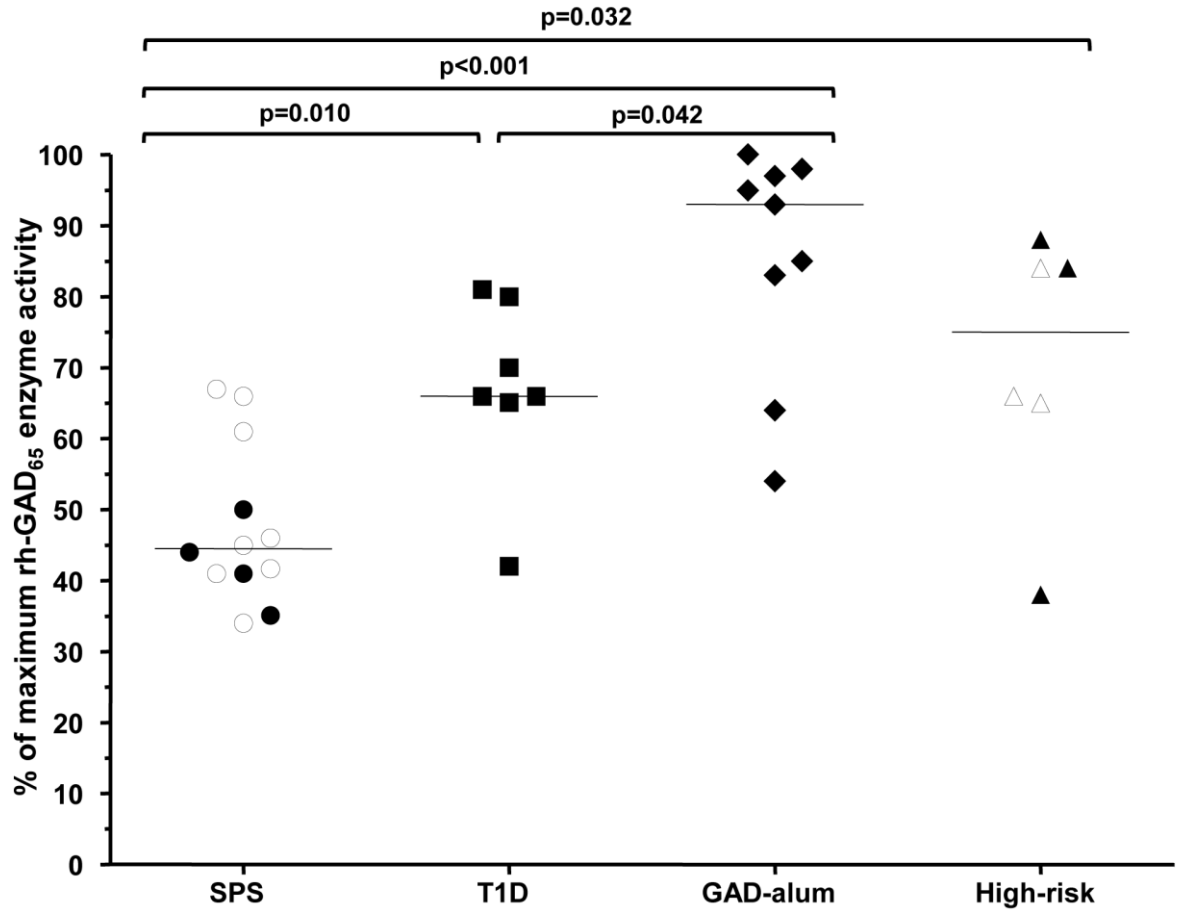

Figure 2. Recombinant human GAD_{65} *in vitro* enzyme activity in the presence of sera from SPS (circles, n=12), T1D (squares, n=7), GAD-alum (rhombuses, n=9) and High-risk (triangles, n=6) groups. Empty circles in the SPS group (n=8) represent individuals with coexistent T1D whereas empty triangles in the High-risk group (n=3) represent individuals that developed T1D after sampling. Results are expressed as a percentage of maximum GAD_{65} enzyme activity. Significant differences are indicated as p-values and horizontal lines represent the median.

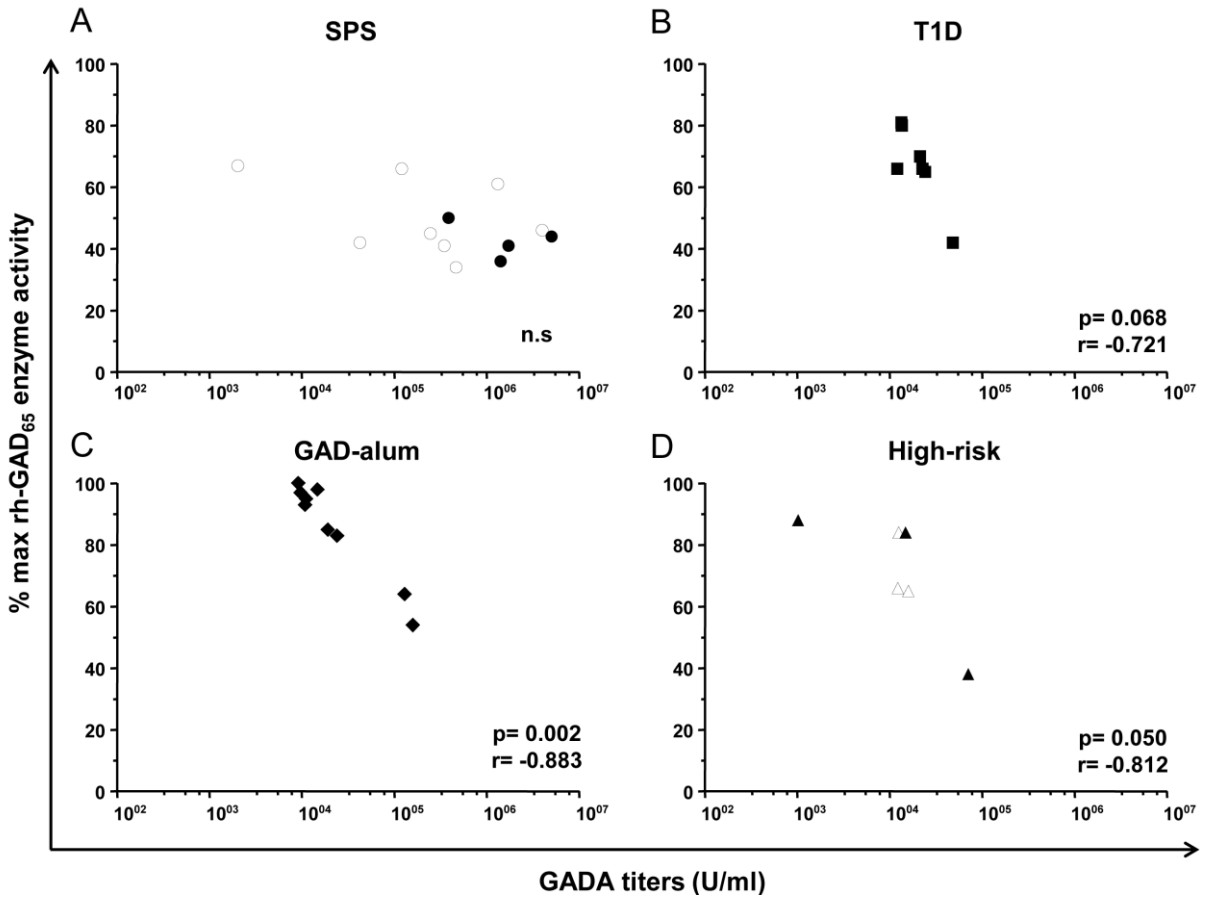

Figure 3. Correlation between GADA titers and GAD_{65} enzyme activity in SPS **(A)** and T1D patients **(B)**, GAD-alum treated T1D patients **(C)** and High-risk T1D individuals **(D)**. Empty circles in the SPS group (n=8) represent individuals with coexistent T1D whereas empty triangles in the High-risk group (n=3) represent individuals that developed T1D after sampling. Significant differences or trends are indicated as p-values, and the correlation coefficient as r.

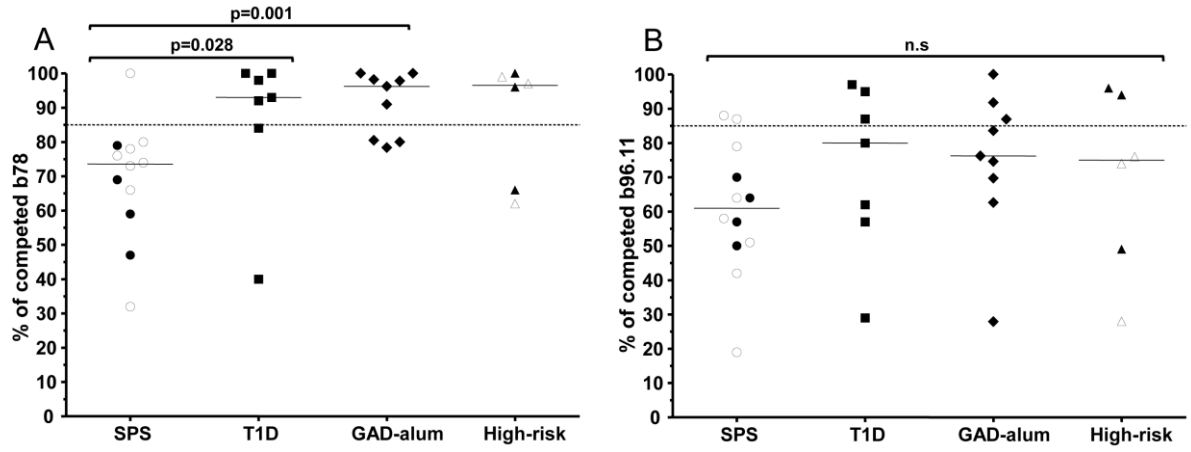

Figure 4 A-B. Binding to GAD_{65} in the presence of rFab b78 **(A)** and rFab b96.11 **(B)** presented as a ratio of competed / non-competed in SPS (circles, n=12), T1D (squares, n=7), GAD-alum (rhombuses, n=9) and High-risk (triangles, n=6) groups. Empty circles in the SPS group (n=8) represent individuals with coexistent T1D whereas empty triangles in the High-risk group (n=3) represent individuals that developed T1D after sampling. A higher binding to GAD_{65} in the presence of rFab indicates a lower proportion of GADA binding to the respective epitope. Samples with a calculated value below the 85% cut-off limit, represented as a dotted line, were regarded as positive for binding to the respective epitope. Significant differences are indicated as p-values and horizontal lines represent the median.

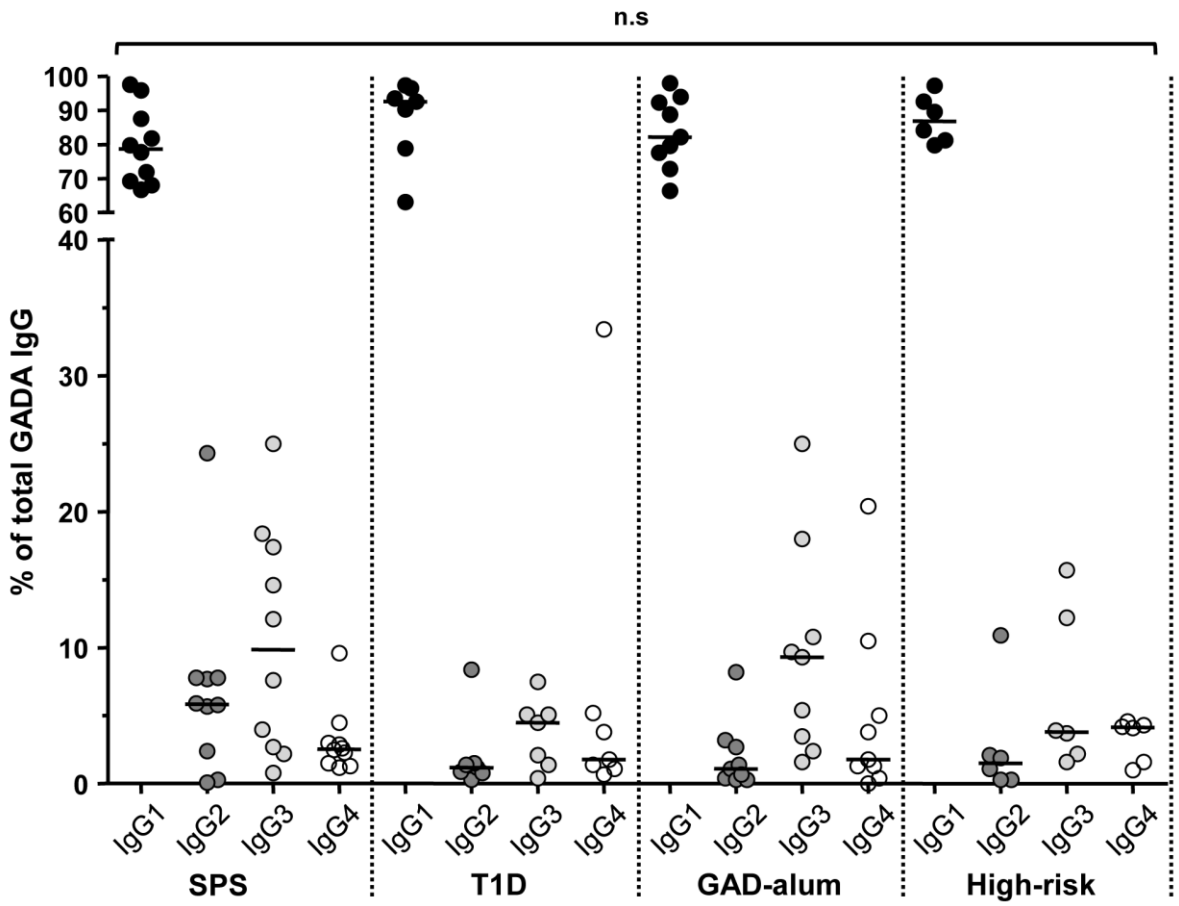

Figure 5. Serum GADA IgG 1-4 subclass distribution in SPS (n=10), T1D (n=7), GAD-alum (n=9) and High-risk (n=6) groups. Results are expressed as the relative contribution of each subclass (% of total GADA) and positivity of each sample was calculated by subtraction of the mean cpm value plus three times the standard deviation (SD) obtained for the negative control. Horizontal lines represent the median.