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IgG4 subclass Glutamic acid decarboxylase antibodies (GADA) are associated with a reduced risk of developing type 1 diabetes as well as increased C-peptide levels in GADA positive gestational diabetes

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## Abstract

Some women with gestational diabetes (GDM) present with autoantibodies associated with type 1 diabetes. These are usually directed against glutamic acid decarboxylase (GADA) and suggested to predict development of type 1 diabetes. The primary aim of this study was to investigate if GADA IgG subclasses at onset of GDM could assist in predicting postpartum development. Of 1225 women diagnosed with first-time GDM only 51 were GADA-positive. Total GADA was determined using ELISA. GADA subclasses were determined with radioimmunoassay. Approximately 25% of GADA-positive women developed type 1 diabetes postpartum. Titers of total GADA were higher in women that developed type 1 diabetes (142.1 vs 74.2 u/mL;  $p=0.04$ ) and they also had lower titers of GADA IgG<sub>4</sub> (index = 0.01 vs 0.04;  $p=0.03$ ). In conclusion we found that that women with high titers of total GADA but low titers of GADA IgG<sub>4</sub> were more prone to develop type 1 diabetes postpartum.

## Introduction

Gestational diabetes mellitus (GDM) is a heterogeneous disorder affecting approximately 2.5% of pregnant women in southern Sweden. A few per cent of these women in turn present with antibodies targeting islet specific antigens, glutamic acid decarboxylase antibodies (GADA) in particular [1-3]. The presence of GADA at onset of GDM [4] or at delivery [1] has been suggested to predict future development of type 1 diabetes.

Type 1 diabetes is considered to be a cellular Th1/Th17 mediated disease. The exact mechanisms are debated but the topic concerning T cell subsets, tolerance interference and post-translational modifications of antigens is comprehensively reviewed by Haskins and Cook [5]. The adaptive immune system during pregnancy, however, is polarized towards a humoral Th2 response [6]. In setting dominated by Th1/Th17 cells, the local cytokine profile will induce an isotype switch towards the human IgG<sub>1</sub> subclass [7, 8], while Th2 cells will induce an isotype switch towards the human IgG<sub>4</sub> subclass [9]. Whether this actually may prevent type 1 diabetes or not will need to be further investigated in future studies, but promising results have been shown in NOD mice [10, 11].

We have previously shown that patients with latent autoimmune diabetes in adults (LADA) have a higher frequency of GADA IgG<sub>4</sub> antibodies compared to patients with adult onset type 1 diabetes [12]. We have suggested that the Th1 and Th2 balance within pancreatic beta cells may be different between patients with type 1 diabetes and LADA since the IgG<sub>4</sub> isotype switch is mediated mainly by Th2 cytokines. Thus it seems reasonable to hypothesize that also GADA positive women with GDM could have higher titers of GADA IgG<sub>4</sub> antibodies. Only one study has previously reported GADA reactivity, including GADA IgG<sub>1</sub> and IgG<sub>4</sub>, as well as epitope binding during GDM [13]. The GADA IgG<sub>4</sub> positivity was reported to be 44%, while GADA IgG<sub>1</sub> positivity was as high as 76% in the 34 women with GDM. The authors could not find a difference in circulating GADA IgG subclasses between patients with type 1 diabetes and GDM, although a reduced and more restricted antibody response was suggested due to lesser epitope spreading in patients with GDM.

The association between the HLA-region at chromosome 6 and autoimmune diabetes has been known for more than three decades [14] and while certain variants of the DQ beta chain encodes susceptibility motifs (DQB1\*0201 and DQB1\*0302 and also known as DQ2 and DQ8 respectively), others are protective (DQB1\*0602) [15]. Both DQB1\*0201 and DQB1\*0302 are reported in one study to be slightly more common in Scandinavian women with GDM compared to in pregnant controls [16]. The presence of GADA was especially associated with DQB1 risk genotypes in this study. Another study suggested the protective genotype to be less frequent in GDM patients but without being able to confirm an increased frequency of high risk genotypes [17]. A third study investigated HLA risk alleles in autoantibody positive women with GDM and concluded that there was an association with autoantibodies and the development of type 1 diabetes postpartum [18].

Our aim with this study was to investigate if GADA IgG<sub>1</sub> and IgG<sub>4</sub> subclass distribution in women with GDM is associated with the endogenous insulin secretion and development of type 1 diabetes postpartum. We were also interested in whether DQB1 risk types (DQ2 and DQ8) could be associated with the GADA subclass distribution or could predict the development of manifest type 1 diabetes postpartum in our material.

## Material and methods

### Participants

Women diagnosed with new-onset GDM at Skåne University Hospital in Lund, Sweden, 1996-2013 (n=1225) were asked to participate in these studies. All women consented and were screened for autoantibodies. Criteria for GDM diagnosis were blood glucose values of  $\geq 10$  mmol/L after a 2 hour 75g oral glucose tolerance test (OGTT). Patients were followed up postpartum to make sure that the pathological blood glucose value was normalized or a classification of manifest diabetes was used instead of GDM. All GDM women are offered follow-up at 3-month to 1-year intervals postpartum.

The Study was conducted in accordance with the Helsinki declaration and approved by the Regional Ethical Review Board at Lund University (LU526/00; 849/2005; 244/2007, 2009/307 and 2014/78).

### Blood tests

Blood was drawn in serum and EDTA plasma vials and sent to the laboratory by hospital post. Plasma and serum was separated by centrifugation at 2000 x g and stored at -70°C until use.

### Detection of GADA positivity

GADA was identified using commercially available enzyme linked immunosorbent assay's (ELISA) from RSR Ltd with a reported specificity and sensitivity of 100% and 90% respectively [19]. Absorbance was measured in a FLOUstar Optima (BMG Labtech). The cut-off level for positivity was set at 10 u/ml. A total of 53 women were positive for GADA (4.3%) and were included in the study. Serum or EDTA plasma was available from 51 of the GADA positive patients

### GADA IgG<sub>1</sub> and IgG<sub>4</sub> subclasses

A liquid phase binding radioimmuno-precipitation assay (LPBA) was used to detect GADA IgG<sub>1</sub> and IgG<sub>4</sub> subclasses in GADA positive women as described in detail elsewhere [20]. Briefly, serum or plasma was incubated in duplicates with recombinant <sup>35</sup>S (Perkin-Elmer)-labeled GAD65, followed by incubation with anti-human IgG<sub>1</sub> antibodies (15µg/ml, BD Pharmingen) or anti-human IgG<sub>4</sub> antibodies (25µg/ml, BD Pharmingen) overnight at 4°C, forming immune complexes between GADA and GAD65, as well as between human IgG-subclasses and mouse anti-human subclass antibodies. The immune complexes were precipitated onto streptavidin sepharose (40% dilution, GE healthcare) in 96-well filter plates (Millipore, France) pre-coated with 1% BSA to avoid unspecific binding and punched out into vials with 4 ml scintillation fluid (Ultima Gold, Perkin-Elmer). Counts per minute (cpm) were measured in a beta-counter (Packard Tri-carb 2100 TR) for 2 minutes per sample. Quadruplicates of in-house standards were used and indexes were calculated to estimate IgG-subclass titers. An index of 0.04 was used as cut-off level for GADA IgG<sub>1</sub> positivity and 0.03 for GADA IgG<sub>4</sub> positivity [20].

### C-peptide

C-peptide levels were measured with commercially available ready-to-use ELISA kits (Merckodia) according to manufacturers' instructions. Internal controls were also purchased from the same manufacturer. The reported detection limit was 15 pmol/l and absorbance was measured at 450 nm in a FLOUstar Optima (BMG Labtech).

### HLA-genotyping

DNA was extracted from leukocytes with a salt-out precipitation method [21]. Concentration and purity was determined by measuring the ratio of absorbance at 260/280 nm. HLA-DQβ1 risk alleles (DQ2/DQ8) were determined using a commercially available MutaGEL<sup>®</sup> HLA-DQ2+8 kit

(immundiagnostik AG) with specific primers for HLA DQ2 (allele combination DQ $\alpha$ 1\*05/DQ $\beta$ 1\*0201) and HLA-DQ8 (allele combination DQ $\alpha$ 1\*03/DQ $\beta$ 1\*0302). All genotyping was performed according to the manufacturers' instructions.

### Statistical analyses

Distribution of data was estimated using D'Agostino-Pearson test [22]. Results are reported as mean  $\pm$  standard deviation for data that followed a normal distribution and median followed by interquartile range (IQR) in brackets when normal distribution was rejected. The 2-tailed Mann-Whitney U-test was used to analyze differences in IgG-subclass titers between the two groups and the Wilcoxon signed rank test to compare IgG-subclass titers between the first and second pregnancy with GDM. The Spearman rank correlation was used to detect associations between antibodies and/or C-peptide levels. Fischer's exact test was used to test for differences in frequency of GADA IgG-subclass positivity in the groups.  $P < 0.05$  was considered to be statistically significant. The statistical software MedCalc for Windows<sup>®</sup> v.14.12.0 was used to analyze the data.

### Results

Clinical and laboratory data are presented in table 1. A total of 53 of the 1225 GDM women were positive for GADA (4.3%) and serum or EDTA plasma was available from 51 of the GADA positive GDM women for IgG subclass analyses. The two women without available serum or EDTA plasma samples were excluded from the study. Neither one of these two women were reported to have developed manifest diabetes during follow-up. All GADA positive GDM women participated in follow-up at regular 3-month to 1-year intervals. Twelve of the 51 GDM women with GADA positivity included in the study developed type 1 diabetes within 0-5 years after onset of GDM ( $1.4 \pm 1.1$  years). Some of the GADA positive women ( $n=15$ ) returned, during their second pregnancy with GDM, 1-9 years after postpartum ( $4.5 \pm 2.8$  years). Two of them also had a third pregnancy with GDM within two years after the second.

Total GADA titers were significantly higher in patients that developed type 1 diabetes; 142.1 (61.6-821.5) u/ml, compared to in patients that did not; 74.2 (30.1-227.8) u/ml ( $p=0.04$ ).

The GADA IgG<sub>4</sub> titers, to the contrary, were significantly lower in women that developed type 1 diabetes ( $p=0.03$ ) and the frequency of GADA IgG<sub>4</sub> positivity was significantly decreased in this group (Fig 1,  $p=0.04$ ). No significant differences of the GADA IgG<sub>1</sub> levels were found between the groups.

All patients positive for GADA IgG<sub>4</sub> were also positive for GADA IgG<sub>1</sub>. However, positivity for GADA IgG<sub>1</sub> was twice as frequent as GADA IgG<sub>4</sub> in our material with frequencies of 82% (42/51) and 41% (21/51) respectively. Total GADA correlated significantly with GADA IgG<sub>1</sub> ( $r_s=0.65$ ;  $p < 0.0001$ ) but not with GADA IgG<sub>4</sub> ( $r_s=0.11$ ;  $p=0.47$ ).

C-peptide levels were not significantly different between women who developed type 1 diabetes; 1.09 (0.42-1.40) nmol/L and women who did not; 1.35 (0.64-1.90) nmol/L. However, a positive correlation was found between C-peptide levels and GADA IgG<sub>4</sub> (Fig 2;  $r_s=0.32$ ;  $p=0.04$ ). There was no such correlation between C-peptide and GADA IgG<sub>1</sub> ( $r_s=-0.03$ ;  $p=0.86$ ).

Women with GDM during their second pregnancy ( $n=15$ ), and in a few cases also their third pregnancy ( $n=2$ ), retained their GADA levels as well as their IgG subclass profile. Only one of the women, with very low levels of total GADA, IgG<sub>1</sub> and IgG<sub>4</sub> at clinical onset, in her first pregnancy, turned out to be GADA negative during her second pregnancy with GDM.

In our material 15 women were positive for the investigated HLA risk alleles DQ2 ( $n=7$ ), DQ8 ( $n=6$ ) or both ( $n=2$ ). No statistically significant increase of the analyzed HLA risk alleles was found in women

who developed type 1 diabetes. However, GADA IgG<sub>4</sub> positive women had a significantly lower frequency of DQ2 or 8 (2/21; 11%, both being DQ8) compared to GADA IgG<sub>4</sub> negative patients (13/30; 43%; p=0.012). Also, women with the DQ2 genotype (n=9) had higher levels of GADA; 240.6 (132.1-816.7) u/mL compared to in women without DQ2 (n=42); 74.3 (48.7-140.5) u/mL, although this was not statistically significant (p=0.07).

## Discussion

Our main finding was that not only GADA positivity in itself, but also GADA subclass distribution could influence the risk of developing type 1 diabetes postpartum. GADA positive women with GDM and higher titers of GADA IgG<sub>4</sub> were less prone to develop type 1 diabetes, regardless of GADA titers. We also found a positive correlation between GADA IgG<sub>4</sub> and endogenous insulin secretion, as reflected by the C-peptide levels, which seemed to be higher in IgG<sub>4</sub> positive women.

One strength of this study is that it was conducted in a region of Sweden, where a screening program for GDM includes all pregnant women. An OGTT is offered to all pregnant women at 28 weeks of gestation and also as early as the 12<sup>th</sup> week of gestation in the case of known risk factors. These include a previous GDM, 1<sup>st</sup> degree relative with diabetes or BMI>30.

Virtually all women in the region accept the offer of OGTT. Therefore practically all pregnancies with GDM are detected. A limitation of the study is the relatively small number of participants due to the low amount of GADA positive women with GDM. The association between C-peptide and GADA IgG<sub>4</sub> antibodies must be interpreted with caution due to the small number of patients.

In our material GADA IgG<sub>1</sub> positivity was twice as frequent as GADA IgG<sub>4</sub> positivity with frequencies of 82% and 41% respectively. These figures are well in accordance with another GDM study reporting frequencies of 76% and 44% respectively [13]. However, the authors also reported presence of GADA IgG<sub>4</sub> in patients with type 1 diabetes, which we have not yet ourselves observed in two previous GADA studies in adult patients [12, 23]. This discrepancy could be due to differences in methodology and determination of the cut-off levels for positivity in each IgG-subclass.

There was no difference in GADA IgG<sub>1</sub> levels between patients that developed type 1 diabetes postpartum and those who did not. This suggests that the cytotoxic T cell response in Th1/Th17 cells are similar in both groups of women, but might be better regulated with Th2 cells present in the patients without development of type 1 diabetes. Most of the women who developed type 1 diabetes presented within the first two years postpartum. One GADA IgG<sub>4</sub> positive woman with GDM did actually develop type 1 diabetes (Fig 2). Although, not until five years postpartum, which suggests that the presence of Th2 cell cytokines - as reflected by the IgG<sub>4</sub> subclass - in pancreatic lymphoid tissues might slow down the beta cell destruction.

Autoantibody positivity, especially GADA positivity, has been implied as a risk factor for the development of type 1 diabetes postpartum in several studies [1-4] GADA titers in women with GDM have not yet assembled such interest. However, in LADA patients GADA titers have been reported to be of prognostic value for beta cell function [24-26], which makes it reasonable to assume that this would be the case also in GADA positive women with GDM. In most cases there was a good correlation between total GADA and GADA IgG<sub>1</sub> levels, suggesting that IgG<sub>1</sub> is the most abundant heavy chain in the circulation in GADA positive women. In some women with high total GADA both GADA IgG<sub>1</sub> and IgG<sub>4</sub> were lower than expected. This could be due to higher GADA IgM levels in these subjects or that the epitopes recognized by the assays are different.

When comparing titers of total GADA between women with and without DQ2 or DQ8 no differences were found. However, we did find a trend towards higher levels of GADA in women with the DQ2

allele alone, which also is in accordance with the NIRAD study group's report on LADA patients. DQ2 was reported to be more prevalent in the high-titer GADA group while DQ8 was equally distributed between high- and low-titer GADA patients with LADA [25]. In our material only one woman with DQ2 and only two women with DQ8 were positive for GADA IgG<sub>4</sub>. Similarly, only 11% (2/21) of GADA IgG<sub>4</sub> positive women had risk alleles compared to 43% (13/30) of the GADA IgG<sub>4</sub> negative women.

Previous studies on HLA risk alleles in GDM have reported the presence of GADA to be associated with DQ risk genotypes in Scandinavian women [16]. A further study found no statistically significant association between DQ risk types and GDM but instead that the protective allele DQB1\*0602 (DQ6) was less frequent in Swedish women with GDM [17]. An explanation to these different findings could be the varying inclusion criteria in the studies. In our study only GADA positive GDM women were included, which would explain why DQ risk types were more common in the material.

GADA positive women with more than one GDM pregnancy retained their GADA positive and IgG subclass profile during their second and third pregnancy, suggesting a remaining risk for development of type 1 diabetes postpartum, often within two years after clinical onset of their first GDM.

Future studies should be conducted in larger populations of GADA positive women with GDM to further investigate the association between C-peptide and IgG subclasses. More investigation is required before this information may be used for prognostic evaluation of residual beta-cell function.

In conclusion we found that that women with GDM that had high titers of GADA were more prone to develop type 1 diabetes postpartum. However, presence of GADA IgG<sub>4</sub> was positively correlated with C-peptide levels in our study, associated with a lower frequency of DQβ1 risk alleles and reduced risk of type 1 diabetes.

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#### Conflict of interest

The authors declare no conflict of interest.

#### Author contributions

JD: Laboratory analyses, compilation of data, statistical analyses and writing parts of the manuscript.

CN: Review of medical records for follow up of manifest diabetes mellitus postpartum, ethical application, critically review of the manuscript.

HS: Critical review of the manuscript, proofreading, language and collection of blood samples.

MLO: Parts of the study design, ethical application and collection of blood samples.

MH: Study design, ethical application, method development, compilation and interpretation of data, statistical analyses and writing parts of the manuscript.



Table 1. Serum or plasma was available for 51 GADA positive women at clinical onset of GDM. Twelve women developed type 1 diabetes within 5 years postpartum. Age is reported as mean  $\pm$  standard deviation. Antibody titers and C-peptide levels are reported as median followed by interquartile range. Presence of HLA-DQ2 (DQB1\*0201) and/or HLA-DQ8 (DQB1\*0302) is given in total.

|                               | No postpartum<br>type 1 diabetes<br>(n=38) | Type 1 diabetes<br>postpartum<br>(n=13) | P-value |
|-------------------------------|--|---|---------|
| Age (years)                   | 31.8 $\pm$ 5.3                             | 31.4 $\pm$ 5.0                          | 0.80    |
| Total GADA (u/ml)             | 74.2 (30.1-227.8)                          | 142.1 (61.6-821.5)                      | 0.04    |
| GADA IgG <sub>1</sub> (index) | 0.16 (0.04-0.41)                           | 0.41 (0.12-0.54)                        | 0.19    |
| GADA IgG <sub>4</sub> (index) | 0.04 (0.02-0.06)                           | 0.01 (0.00-0.03)                        | 0.03    |
| C-peptide (nmol/L)            | 1.35 (0.64-1.90)                           | 1.09 (0.42-1.40)                        | 0.23    |
| HLA DQ2/8 (yes/no)            | 10/28 (26.3%)                              | 5/8 (38.5%)                             | 0.48    |

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Figure 1. GADA IgG<sub>4</sub> titers in women that developed type 1 diabetes (n=13) compared to in women without manifest type 1 diabetes (n=38; p=0.03).

Figure 2. A significant correlation was found between endogenous insulin secretion and GADA IgG<sub>4</sub> subclass of antibodies ( $r_s=0.32$ ,  $p=0.04$ ) which suggests a protective role of GADA IgG<sub>4</sub>.



