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GAD65 Autoantibody Immune Responses in Newly Diagnosed Type 1 Diabetes Children and in GADA Positive Children from General Population

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Type 1 Diabetes is an autoimmune disease caused by the destruction of insulin secreting beta cells in pancreas. There are at least 50 potentially causal genes, but environmental factors act as the trigger. Before and after Type 1 Diabetes has developed, autoantibodies start appearing in the bloodstream, which could potentially predict the risk of developing the disease. The four major autoantibodies are: IAA, GADA, IA-2A and ZnT8A.

The focus of this study was GADA and its epitopes: many healthy people with no risk of developing Type 1 Diabetes are GADA positive. Serum samples were analyzed for autoantibodies with N-truncated GADA (tGADA). The method used in quantitative analysis of antibodies was radiobinding assay (RBA). Samples used in this study were from newly-diagnosed Type 1 Diabetes subjects in Finnish Pediatric Diabetes Register and from the DIABIMMUNE study 3–5-year-old children from the general population who were GADA positive.

The aim of this study was to determine whether specificity of the assay can be improved without losing sensitivity when using tGADA. Secondary goals were to determine which epitopes are the focus of immune response in multipositive, T1D positive and only-GADA positive subjects.

Correlation between GADA and tGADA assays was good (0.63–0.96). In all subjects, the immune response was directed towards M- and C-epitopes in those who were multipositive or T1D positive. Immune response against N-epitope was mostly found on subjects who were only-GADA positive and who were not multipositive or T1D positive.

With RBA, tGADA (96–585) recognizes T1D positives as well as GADA (1–585), but tGADA is better at recognizing those children who are at risk of developing type 1 diabetes or multipositivity in the future, thus tGADA is more specific, since it leaves out those who are only N-epitope positive. N-epitope positivity does not seem to have any effect on the risk of developing multiple autoantibodies or T1D.

Keywords	T1D, GADA, tGADA, RBA, Autoantibodies, Epitopes

Tekijä Otsikko Sivumäärä Aika	Aleksi-Mikael Kivelä GAD65 autovasta-aineiden immuunivasteet tyypin 1 diabetekseen vastasairastuneilla lapsilla ja GADA positiivisilla lapsilla valtaväestöstä 31 sivua + 1 liite 14.3.2018
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Tyypin 1 Diabetes on haiman insuliinia tuottavien beetasolujen tuhoutumisesta johtuva autoimmuunisairaus. Ihmisestä on löydetty ainakin 50 potentiaalisesti kausaalista geeniä, mutta ympäristölliset syyt toimivat autoimmuunivasteen laukaisijana. Ennen tyypin 1 diabeteksen puhkeamista ja sen jälkeen, vereen erittyy autovasta-aineita, jotka voivat mahdollisesti ennustaa sairastumisriskiä; näistä suurimmat ovat IAA, GADA, IA-2A ja ZnT8A. Tutkimuksen kohteena oli erityisesti GADA ja sen epitoopit: monilta terveiltä ihmisiltä on löydetty GAD vasta-aineita verestä ilman, että heillä on riskiä sairastua tyypin 1 diabetekseen.

Tutkimuksen tarkoituksena oli selvittää, pystytäänkö menetelmän spesifisyyttä parantamaan käyttämällä N-leikattua GADA:a (tGADA) menettämättä sensitiivisyyttä. Toissijaisena tarkoituksena oli tutkia mitkä epitoopit ovat immuunivasteen kohteena multipositiivisilla, diabetekseen sairastuneilla ja vain GADA positiivisilla. Näyteseerumeista analysoitiin autovasta-aineiden vasteet käyttämällä N-leikattua GADA:a. Autovasta-aineiden kvantitatiivisessa analyysissä käytettiin radiobinding menetelmää. Tutkimusaineistona käytettiin näyteseerumeita vastasairastuneilta lapsilta Suomen Lasten Diabetesrekisteristä ja DIABIMMUNE-tutkimuksessa 3–5 vuotiailta lapsilta kerättyjä GADA-positiivisia näytteitä, jotka olivat yleisestä väestöstä.

Korrelaatio GADA ja tGADA menetelmien välillä oli hyvä (0.63–0.96). Kummassakin aineistossa immuunivaste kohdistui M- ja C-epitooppeja vastaan diabetekseen sairastuneilla ja multipositiivisilla. Vasteet N-epitooppia kohtaan esiintyivät lähinnä GADA-positiivisilla potilailla, jotka eivät olleet multipositiivisia, eivätkä diabeetikoita.

RBA menetelmällä tGADA (96–585) tunnistaa diabetekseen sairastuneet yhtä hyvin kuin GADA (1–585), mutta tGADA on parempi tunnistamaan riskilapset eli ne lapset, joilla on vaarana tulevaisuudessa muodostaa useita autovasta-aineita tai sairastua tyypin 1 diabetekseen. tGADA on siis spesifisempi, sillä se jättää pois vain N-epitooppi positiiviset. N-epitooppi positiivisuudella ei näyttäisi olevan vaikutusta sairastumisvaaraan tai useiden autovasta-aineiden kehittymiseen.

Avainsanat	T1D, tGADA, GADA, RBA, Autovasta-aineet, Epitoopit

Contents

Abbreviations

ECL	Electrochemiluminescence
ELISA	Enzyme-linked Immunosorbent Assay
GAD	Glutamic Acid Decarboxylase
GADA	Glutamic Acid Decarboxylase Autoantibody
HLA	Human Leukocyte Antigen
IAA	Insulin Autoantibody
IA-2	Islet Antigen 2
IA-2A	Islet Antigen 2 Autoantibody
ICA	Islet Cell Autoantibody
LADA	Latent Autoimmune Diabetes of the Adult
RBA	Radiobinding Assay
T1D	Type 1 Diabetes
tGADA	(N-)Truncated Glutamic Acid Decarboxylase Autoantibody
ZnT8	Zinc Transporter Protein 8
ZnT8A	Zinc Transporter Protein 8 Autoantibody

1 Introduction

Type 1 Diabetes, an autoimmune disease, is characterized by immune-mediated response against islets of Langerhans, eventually leading to insulin deficiency. During the pre-diabetic period, autoantibodies against pancreatic beta cell antigens appear in circulation [1]. Type 1 Diabetes is usually diagnosed in childhood and adolescence and remains as one of the most common autoimmune diseases in that age group. The HLA antigen II confers most of the genetic susceptibility, but there are at least 49 other genes that act as potential triggers [2]. The true cause of type 1 diabetes is unknown, but both viral and chemical, as well as environmental causes have been suggested and it could be triggered by a combination of two or more [1].

Pancreatic beta-cell antigens are the targets of immune response in type 1 diabetes. The most important autoantibodies that act as biomarkers for the disease are islet cell antibodies (ICA), insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibody (GADA), islet antigen-2 autoantibodies (IA-2A), zinc transporter 8 autoantibodies (ZnT8A). Appearance of GADA often means that the subject eventually develops type 1 diabetes. However, depending on which GAD epitope the autoantibodies are directed against, the risk may be non-existent [3.]

In Finland, over 60 cases of type 1 diabetes per 100 000 people are diagnosed annually. The study of type 1 diabetes is important because the disease increases risk for cardiovascular events, such as myocardial infarction, and the outcome is less-favorable when compared to non-diabetic patients. The risk for microvascular and other macrovascular complications is also higher in individuals with type 1 diabetes [1.]

The main goal of the study was to determine, whether specificity of GADA assays can be improved without losing sensitivity when using N-terminally truncated GAD65 (tGAD). The secondary goals were to see which epitopes are focus of the immune response in type 1 progressors, multiantibody positive and single GADA positive subjects, and whether the results correlate with previous studies done on this subject.

2 Type 1 Diabetes

Type 1 Diabetes (T1D) is an autoimmune disease where loss of insulin-producing pancreatic beta cells leads to hyperglycemia. It is usually diagnosed in childhood and adolescence, but also increasingly in adults [1; 2].

The steps from initiation to progression of beta cell destruction are still unclear, but it is believed that beta-cell autoantigens are processed by antigen presenting cells (B cells, macrophages, dendritic cells) and presented to naive T cells in pancreatic lymph nodes, followed by generation of autoreactive T cells (both CD4+ and CD8+) [1; 25].

In pre-diabetic phase, the disease is characterized by the appearance of autoantibodies produced by B cells in the bloodstream of the patient. These are directed against one or more of the following: ICA, IAA, GADA, IA-2A. The autoantibodies act as a reproducible biomarker during the development of the disease; the destruction of the β-cells is caused by autoreactive CD8+ T cells [1].

Measurement of autoantibodies can also be used to distinguish T1D from other forms of diabetes. There are many hypotheses of how beta-cell autoimmunity is initiated, but none of them alone are responsible for the development of the disease. Both genetic predisposition and environmental factors are thought to affect both the initiation and the rate of disease progression. One promising avenue of study has been the impact of viral infections in pathogenesis, specifically Enteroviruses [1; 2.]

It has been proposed that the development of type 1 diabetes happens in three stages. In the first stage, beta-cell autoimmunity is initiated, and the autoantibodies appear in circulation. At this point the beta-cell mass remains high and none of the usual symptoms of the disease have manifested. In stage two, there is a change in blood sugar regulation and the beta-cell mass starts to decrease, even though the disease itself remains asymptomatic. In the third stage, the beta-cell mass has dropped so low that it leads to insulin deficiency, and the usual symptoms of type 1 diabetes start appearing (Figure 1.) [4.]

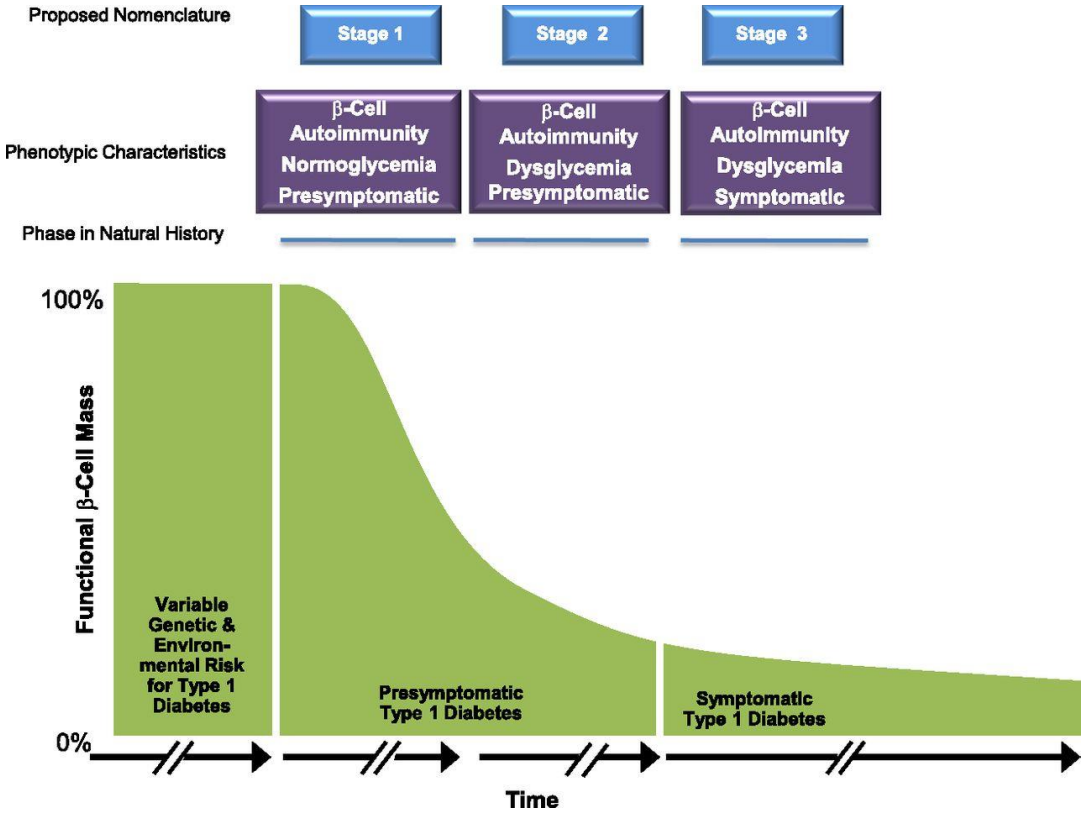


Figure 1. Staging of type 1 diabetes [4]

2.1 Genetics

There are at least 50 potentially causal disease genes associated with type 1 diabetes and 58 genomic regions show genome-wide evidence for association. The human leukocyte antigen (HLA) gene complex, or a group of genes, confers the largest contribution (about 50%) to the total genetic risk of developing type 1 diabetes.

The appearances of islet autoantibodies are known to be associated with *HLA-DR-DQ* haplotypes. The patients with *HLA-DR4-DQ8* haplotype had usually insulin as the first autoantibody, whereas those homozygous with *HLA-DR3-DQ2* had GADA as the first autoantibody. Other HLA class II molecules, *HLA-DRB3; HLA-DRB4* and *HLA-DRB5*, could be contributing to the initial triggering of beta-cell autoimmunity and type 1 diabetes. In children younger than 12 years, HLA class II haplotypes *DRB1*1501* and *DQA1*0102-DQB1*0602* confer disease resistance against type 1 diabetes [4.] Genetic polymorphisms in *INS* and *PTPN22* genes respectively contribute to the genetic cause of diabetes and to the risk of developing insulin autoantibodies as the first islet autoantibody. Genetics are an important factor with the development and predilection of type 1 diabetes, but environmental factors are the triggers for the disease [2].

2.2 Environmental Factors

Type 1 diabetes is thought to have many environmental triggers, from certain viruses and diet to hygiene and route of neonatal delivery, antibiotics and host microbiome [4; 5]. Enteroviruses have been suggested as potential triggers in type 1 diabetes and latent autoimmune diabetes of adults (LADA), specifically the *Coxsackie* -family of viruses [6]. Some epidemiological and immunological studies indicate that the early introduction of complex proteins early in infancy may increase beta-cell autoimmunity and diabetes risk [7; 8]. Researchers have studied the effect of removing bovine insulin from cow's milk formula or the use of hydrolysed infant formula in beta-cell autoimmunity. Pilot studies indicated that the use of bovine insulin free milk or hydrolysed milk formula reduced the incidence of autoantibodies in children with genetic risk [7; 8]. However, an international clinical trial with 2159 newborn infants showed that neither the beta-cell autoimmunity nor the incidence of type 1 diabetes was reduced among children who were weaned to hydrolysed formula when compared to conventional formula [9; 10].

The DIABIMMUNE study aims at assessing the role of environmental factors, especially the role of hygiene in the pathogenesis of type 1 diabetes, celiac disease and allergies. According to the hygiene hypothesis, early exposure to specific micro-organisms and allergens in infancy is beneficial to developing immune system and accordingly confers protection against many allergic and autoimmune diseases. Indeed, there is a regional difference in the prevalence of type 1 diabetes in Finland, Estonia and Russian Karelia, even though the genetic background between these regions is relatively similar [11].

The difference is significant: type 1 diabetes is most prevalent in Finland, where >60 cases per 100 000 people are diagnosed annually, compared to Estonia where about 10 cases per 100 000 people and in Russian Karelia less than 10 cases per 100 000 people are diagnosed annually [12]. The hypothesized reason for such major differences in numbers is thought to be the difference in childhood hygiene, where children exposed to a larger number of bacteria and allergens in childhood have a smaller risk of developing type 1 diabetes and other autoimmune diseases [13].

The DIABIMMUNE studies indicate that diversity of gut microbiome decreases in T1D progressors after autoantibody seroconversion [5]. The studies also indicated the predominance of *Bacteroides* species, low immune stimulators, in Finnish and Estonian children, while they are low in abundance in Russian Karelian children [13].

2.3 Autoantibodies

The appearance of autoantibodies against islet cell antigens is the first easily detectable sign of a possible beta cell autoimmunity. In young children the insulin autoantibodies are often the first ones to appear, in contrast to adult diabetics who usually display GAD65 or IA-2 autoantibody positivity when diagnosed [27, p. 383, Figure 2]. Testing positive for multiple autoantibodies is often indicative of an increased risk of progression to clinical type 1 diabetes. A study by Knip M. *et al.* [14] found that the highest positive predictive value (PPV) of 91.7% was connected to recurring positivity with IAA and ICA, with PPV decreasing as positivity for antibodies increased: testing positive for all four antibodies yielded a PPV of 55.7%, however it also yielded the highest disease sensitivity (54.4%) [14].

2.3.1 Islet Cell Autoantibodies

Islet cell autoantibodies (ICA) against pancreatic islets were first detected by Botazzo *et al.* in 1974. This discovery led to metabolic studies of islet function and to the modelling of progressive disease stages in type 1 diabetes by George Eisenbarth in 1986 [2]. These autoantibodies are detected with immunofluorescence using pancreatic tissue from organ donors in blood group 0 as substrate. Islet cell autoantibodies consist from a heterogenous group of autoantibodies including IA-2A, GADA and ZnT8A. Circulating IA-2A levels, followed by ZnT8A correlate strongest with ICA titers in the blood, whereas the correlation was weaker between GADA and ICA titers. In general, ICA stain all islet cells but restricted or beta cell specific autoantibodies have also been found. Restricted autoantibodies have not been found to predict type 1 diabetes, unlike non-restricted autoantibodies [14.]

2.3.2 Insulin

Insulin is a protein expressed almost exclusively in the pancreas, specifically in pancreatic beta cells. It is used to regulate glucose levels in human blood and lack of insulin and insulin production is what leads to type 1 diabetes. Some extra-pancreatic expression of insulin is in thymic medullary epithelial cells at both mRNA and protein levels.

The insulin autoantibody (IAA) epitopes have been identified in both the A chain and the B chain of insulin, within residues 8–13 and 28–30 respectively [3]. IAA are the least frequent autoantibody in newly diagnosed type 1 diabetes children, with only 44.8% showing positivity. However, IAA often appears as the first autoantibody in genetically susceptible infants [14.]

2.3.3 Glutamic Acid Decarboxylase

Glutamic acid decarboxylase (GAD65) is an enzyme in the biosynthesis of gamma-aminobutyric acid (GABA), which is an important neurotransmitter in the central nervous system. Some diabetic patients can also develop antibodies against GAD67, the homologue of GAD65. GAD65 consists of the amino (N), middle (M) and carboxy (C)-terminal regions with a length of 65 kDa (Figure 2). GAD65 is not solely expressed in the beta cells of pancreas but are present in several tissues where GABAergic systems exist, such as neurons of the nervous system [3.]

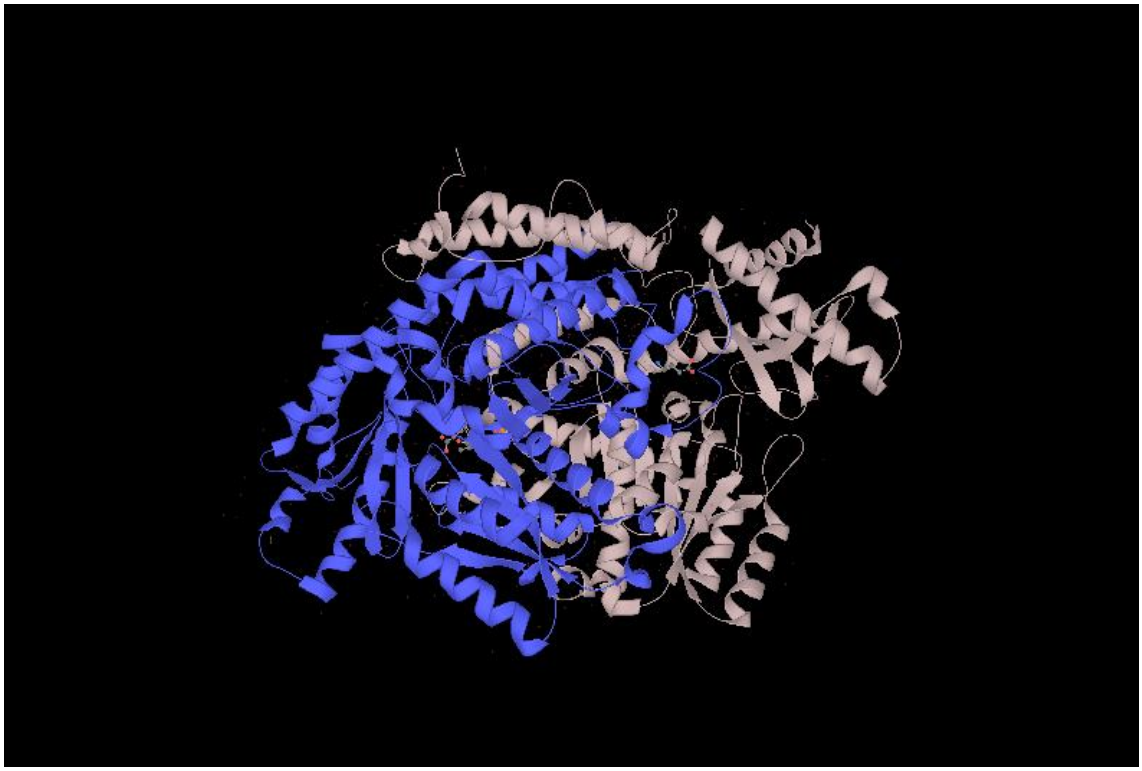


Figure 2. Structure of GAD65 [15]

Glutamic acid decarboxylase autoantibodies (GADA) have been implicated in several other autoimmune diseases of the central nervous system such as stiff person syndrome, Batten disease and cerebral ataxia. GADA are also common in patients with autoimmune polyendocrinopathy and immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome. While these patients frequently develop type 1 diabetes, the autoantibodies against GAD can be present even in the absence of T1D. Immune response against GAD is most common in adult-onset of type 1 diabetes, but it is also common in children, appearing often between ages 2 and 3 years. Accordingly, GAD is thought to be one of the primary targets in autoimmune process of T1D [3; 14.]

2.3.4 Islet Antigen 2

Islet Antigen 2 (IA-2) is a protein tyrosine phosphatase (PTP) like protein inherent to the membrane of secretory granules. It is not expressed exclusively in the pancreatic beta cells; it is also present in several neuroendocrine cells. The immune response is directed against epitopes in the cytoplasmic JM domain and epitopes in the PTP-like domains [3]. The biological role of IA-2 is not entirely known: it can influence insulin secretion, secretory granules biogenesis and homeostasis as well as beta cell expansion, but no ligand that would bind it to ectodomain has been identified. Neither has the PTP-like domain any phosphatase activity [3].

IA-2 autoantibodies are common autoantibodies in type 1 diabetes diagnosed children, with a prevalence of about 76 % [3; 14]. It is usually thought that the autoimmune process has already progressed far when IA-2 autoantibodies appear, but IA-2A can also be the first autoantibody to appear

2.3.5 Zinc Transporter Protein 8

Zinc Transporter Protein 8 (ZnT8) is a transmembrane protein that regulates cellular zinc content. It has six transmembrane domains and a histidine-rich loop between domains IV and V. ZnT8 is critical for the maintenance of stored insulin, because it accumulates zinc into the secretory granules. The protein is expressed almost exclusively in pancreatic islets, particularly in β cells, with low levels in α cells and possibly in the cells of the retina [3.]

The epitopes of ZnT8 are present in both the NH2 and COOH terminus. The immune response is almost completely directed against the COOH epitopes, with less than 10% of patients showing antibodies to the NH2 terminus. Some antibodies have been identified that are specific towards polymorphic variants of arginine or tryptophan at amino acid residue 325 in COOH terminus.

While ZnT8 autoantibodies are not as frequent as the other autoantibodies, still over 60% of type 1 diabetes diagnosed children are ZnT8A positive [3; 14.]

3 Analysis of Type 1 Diabetes-Associated Autoantibodies

3.1 Radiobinding Assay

Radiobinding assay (RBA) is a method used in detection of antibodies against a specific antigen. The principle of autoantibody radiobinding assay is shown in Figure 3. The serum samples are incubated with a labelled antigen, either 125-Iodine for IAA, or 35S-methionine for GADA, ZnT8A and IA-2. The immune complexes that form are precipitated with a protein A Sepharose and the unbound antigen is washed with a buffer, using either centrifugation or filtration technique. RBA is relatively cheap, low volume method that can be used to analyse a large number of samples. The downsides are the use of radioactive material and the differences in sensitivity and specificity between laboratories.

According to the 2002–2016 Islet Autoantibody Standardization Program (IASP) the sensitivities and specificities of the assays were: 42–62% and 92–99% for IAA, 64–90% and 90–98% for GADA, 62–72% and 93–100% for IA-2A, 48–70% and 97–100% for ZnT8A. The IASP and DASP Workshop results and diagrams were taken from a summary table that was sent to the participating laboratories, these tables are not available for public viewing. The workshop results vary widely between laboratories, autoantibodies and assays. The purpose of the DASP and IASP Workshop diagrams and results is to show the sensitivities and specificities of the assays in a comparable form, when analysing different autoantibodies.

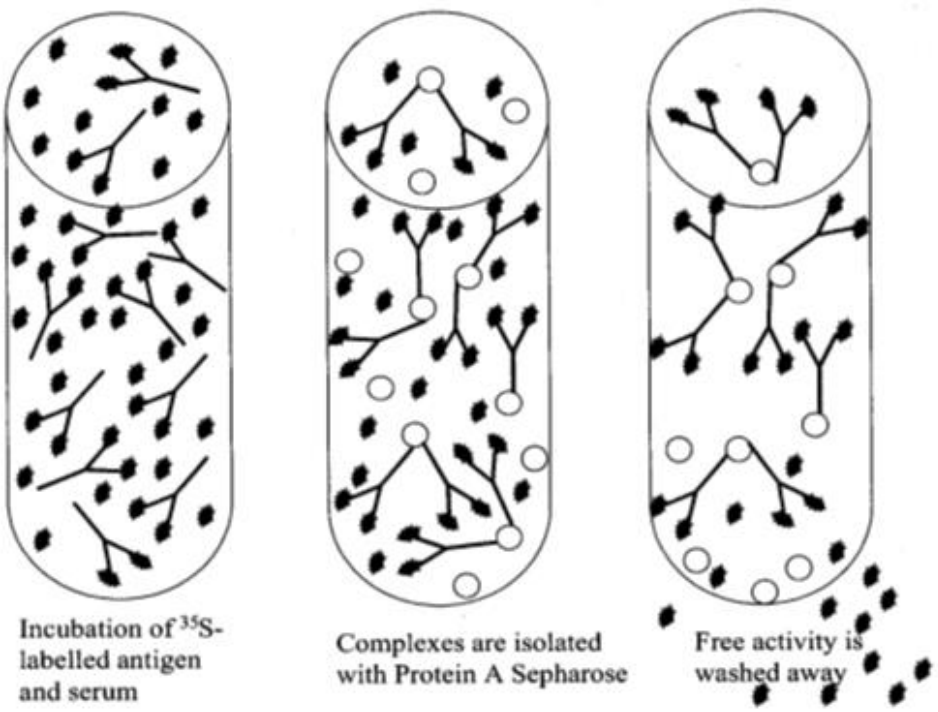


Figure 3. The radiobinding assay principle [26, p. 48]

3.2 Enzyme-linked Immunosorbent Assays

Enzyme-linked immunosorbent assays (ELISA) are a common immunogenicity method used in the analysis, identification and quantification of different antibodies and antigens. There are three methods used in immunogenicity assessment: Direct/Indirect ELISA, Bridging or “Sandwich” ELISA and Competitive ELISA. In direct ELISA assay an enzyme-labelled anti-immunoglobulin conjugated to an enzyme or a small molecule, such as biotin, is used to detect bound antibodies when plasma or serum samples are incubated with the immobilized antigen. The indirect method uses an antigen that is anchored using an immobilized capturing agent, such as streptavidin. The bridging ELISA uses the antigen for both capturing and detection of the antibody: an antigen is bound to the bottom of the assay plates and after the sample is added, the conjugated antigen is added to produce a colour change. The competitive ELISA uses competitive binding between the antibodies: the sample is first incubated with the antigen and then washed so that unbound antibodies are removed (Figure 4.)

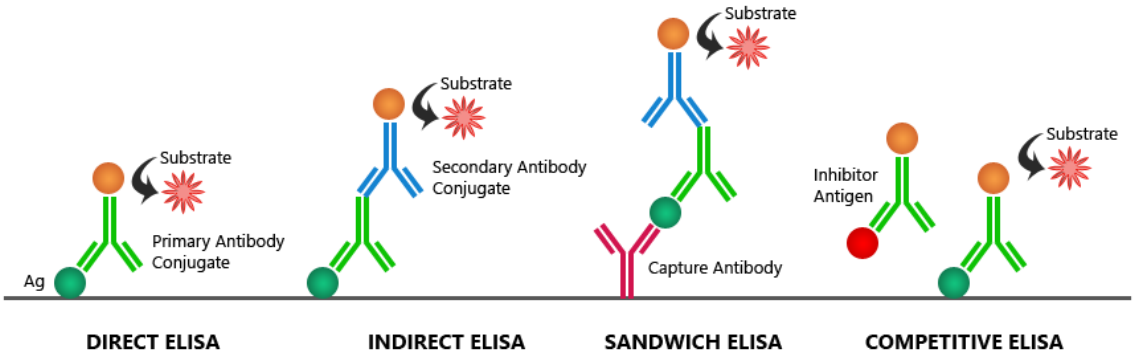


Figure 4. ELISA principles [18]

In all methods, the final step is the addition of a substrate to produce a colour change; the sample is then quantified with spectrophotometry. It is possible to determine IA-2A, GADA and ZnT8A with commercial ELISA kits, but determining IAA with ELISA has not yet been successful. [16; 17.] The 2015 IASP workshop sensitivity and specificity values for ELISA (bridge): GADA 76–86% and 97–99%, IA-2A 58–68% and 96–100% (Figures 5, 6 and 7).

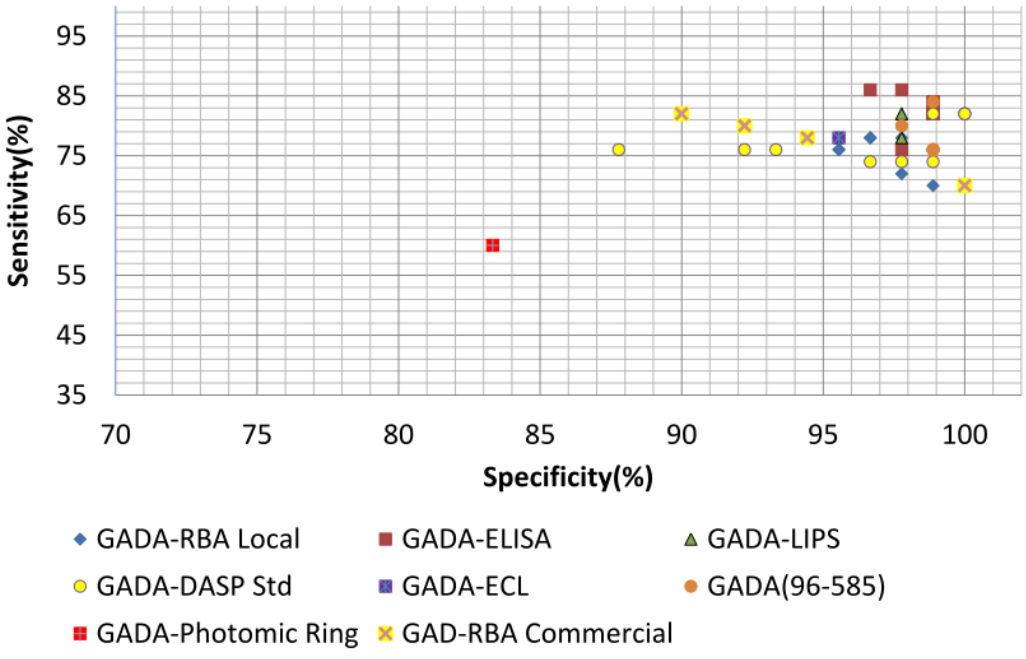


Figure 5. IASP 2015 workshop sensitivity vs. specificity for GADA.

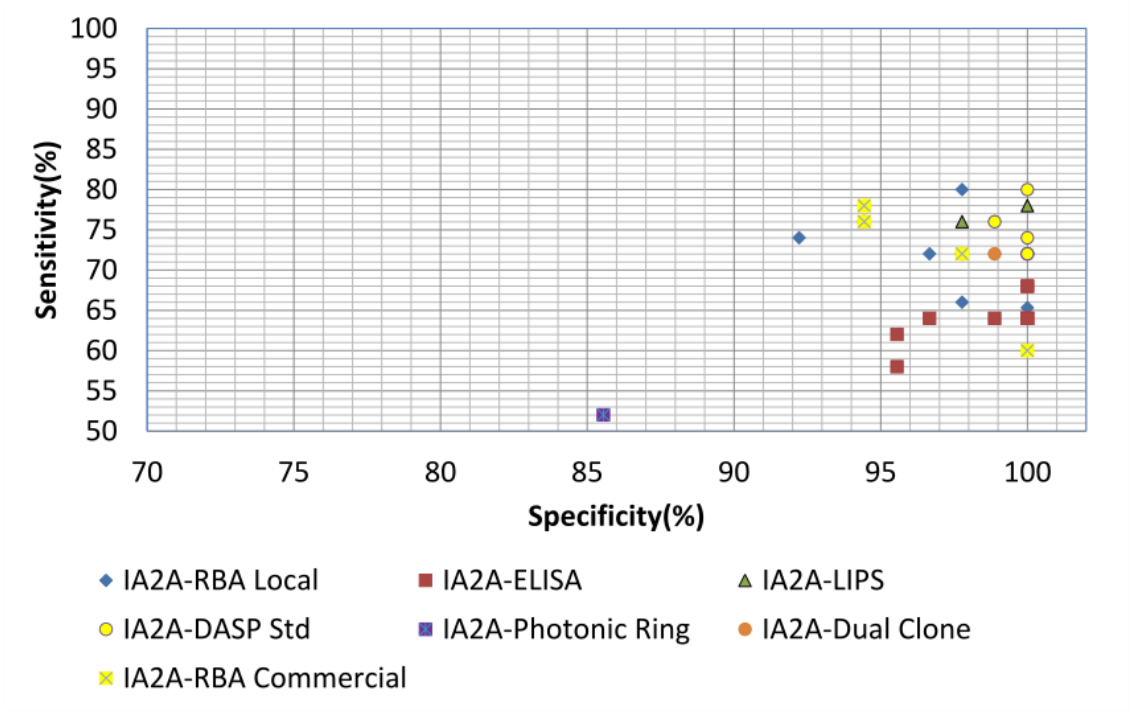


Figure 6. IASP 2015 workshop sensitivity vs. specificity for IA-2A.

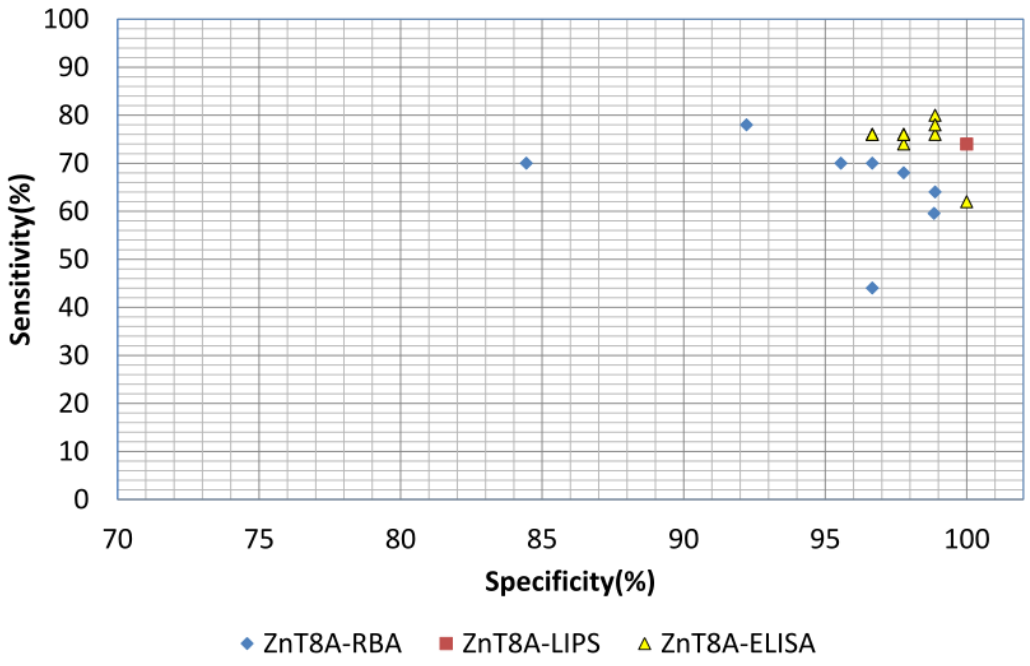


Figure 7. IASP 2015 workshop sensitivity vs. specificity for ZnT8A.

3.3 Electrochemiluminescence

Electrochemiluminescence (ECL) is a novel method used in the analysis of biological substances. It uses ruthenium-conjugated proteins instead of the common enzyme-conjugates. When a voltage is run through the sample, ruthenium ions undergo an oxidation-reduction reaction when in the presence of tripropylamine which then generates an ECL reaction. In a study by Steck AK. *et al.* [19] it was found that ECL could determine affinity to type 1 diabetes in those with a single antibody much more precisely than regular radioimmunoassays and it could be used to assess the risk of developing the disease much earlier than usual. Sensitivity and specificity values for ECL in the 2015 IASP workshop were: GADA 78% and 96%, IA-2A 72% 98%, IAA 60% and 98% (Figures 5 and 8). [17; 19].

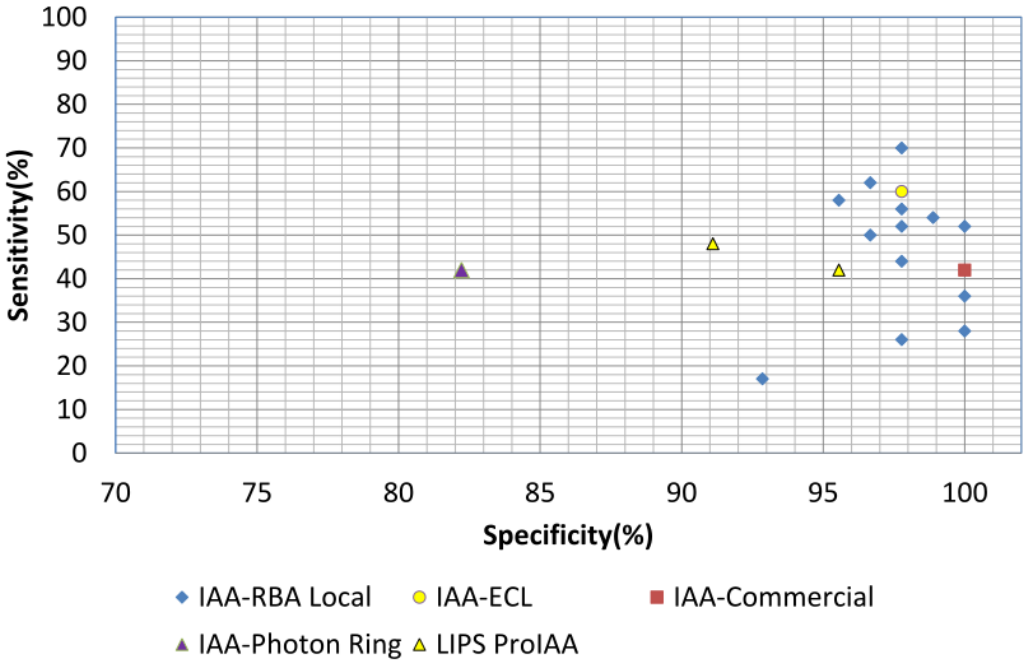


Figure 8. IASP 2015 workshop sensitivity vs. specificity for IAA

4 Truncated GAD65 (96–585) and GAD65 N-, M- and C-Epitopes

GAD autoantibodies are sensitive, widely used markers of islet autoimmunity and type 1 diabetes, and are used in diagnosis and prediction of diabetes, and in recruitment to intervention studies [14]. GAD autoantibodies are, however, found also in many healthy subjects without increased risk of progression to diabetes [16; 23; 24]. Recent international autoantibody standardization workshops have revealed that there are differences between RBA and ELISA in recognition of GAD in healthy control subjects. Further characterization revealed that control sera which were positive by radiobinding assay, but negative by ELISA recognized N-terminal (amino acids 1–95) epitope of GAD65 protein [16]. Studies by Williams *et al.* [20] showed that specificity of GADA assay was more specific when N-terminally truncated GAD65 (96–585) was used, while the sensitivity in patients was the same with both labels. It was suggested that truncated GAD65 should be used, especially when screening healthy family members for intervention trials [20].

It has been determined that the predilection to developing type 1 diabetes is related to an immune response against the epitopes in M- and C-terminals [22; 20]. Immune response against N-terminal has a minimal impact: it leads to non-specific binding of the GAD65 autoantibodies, which is not relevant to the pathogenesis of type 1 diabetes [20]. In sibling studies, it was determined that, while the initial response was simultaneously directed against the M- and C-terminals of the molecule, in some cases it later spread to the N-terminal [20]. In a study on GAD65 epitopes by Hoppu *et al.* [21] it was found that the antibodies were most abundant towards the M-epitope (94%) followed by C-epitope (83%) in the initial samples in diabetic and unaffected subjects. In a follow-up the ratios remained the same, with highest response directed towards M-epitope, followed by C-epitope. N-epitope response remained low (22%) in both study groups [21].

5 Materials and Methods

5.1 Subjects

The Finnish Pediatric Diabetes Register

The Finnish Pediatric Diabetes Register has been collecting data and blood samples from children with type 1 diabetes before the age of 16 years and their first-degree family members since June 2002. By 2016 there were over 8000 children with type 1 diabetes and over 13000 family members in the registry and it covers over 90% of the children with type 1 diabetes in Finland. In total, 101 samples with varying concentrations of autoantibodies were selected, concentrating on GAD65 autoantibodies near the cut off. Median age of the subjects was 9.15 years, with minimum age being 1.3 years and maximum 16.2 years. The samples were previously analyzed for ICA, IAA, GADA, IA-2A and ZnT8A and were now assayed for truncated GAD65 (96–585) autoantibodies and GAD65 N-, M- and C-epitope specificities.

DIABIMMUNE

The DIABIMMUNE study aimed at assessing the role of environmental factors in pathogenesis of type 1 diabetes, allergies and other immune-mediated diseases, focusing on the role of hygiene. Samples were collected from Finland, Estonia and Russian Karelia into two cohorts: the birth cohort and the young children cohort (YCC). Children in the YCC were recruited from the general population at the age of three years (36 months) and followed-up until the age of five (60 months). The children who were positive either for type 1 diabetes autoantibodies or allergen specific antibodies were invited also to four-year (48 months) visit. In total, there were over 3600 children in the YCC and the samples were collected to analyze type 1 diabetes, celiac disease, autoantibodies, allergies, T cell regulatory function, infections, epigenetics and gut microbiome. The samples selected for this study were from subjects in the YCC who tested positive for GADA at some point during DIABIMMUNE.

These samples were used to answer two questions: can specificity be improved without losing sensitivity with N-truncated GADA and does epitope specificity differ in children whose immune response does not spread from GADA to other antigens compared to those whose autoimmune response spread to other antigens, and possibly progress to type 1 diabetes. In total, 203 samples from 83 subjects were analyzed.

5.2 Methods

All samples were analysed for GAD65 antibodies, truncated GAD65 (96–585) and GAD-epitope autoantibodies with a radiobinding assay, as described previously [21]. Three different GAD65/GAD67 chimeras were used to analyse epitope-specific antibodies: 1. (GAD65 1–95/GAD67 102–593) to measure amino-terminal antibodies (N-epitope auto-antibodies), 2. (GAD65 1–101/GAD65 96–444/GAD67 453–593) to determine the middle PLP binding domain specific autoantibodies (M-epitope autoantibodies) and 3. (GAD67 1–453/GAD65 445–585) to determine carboxy-terminal autoantibodies (C-epitope auto-antibodies). The results were expressed in relative units (RU) based on a standard curve constructed from a dilution of a pool of highly positive samples. The cut-off limit for anti-body positivity (5.36 RU) was set at the 99th percentile of 373 non-diabetic Finnish chil-dren and adolescents. The cut-off limit for positivity for GAD epitopes was set at the 99th percentile in 104 non-diabetic young Finnish subjects and were 0.86 RU for N-epitope, 1.51 RU for M-epitope and 1.59 RU for C-epitope.

All analyses followed the following procedure: 2 µl of samples, standards and controls were incubated at +4 ˚C overnight in 96-well plates with shaking (300 RPM) with 20 000 CPM of 35S-labelled antigen diluted in 50 µl of TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween-20 (Amresco Inc., Ohio, USA), pH 7.4). ^{35}S-methionine (CAT NEG709A; Perkin Elmer, USA) labelled antigens were produced with TNTCoupled Reticulocyte Ly-sate system (CAT L1170; Promega, Madison, WI, USA).

After incubation, Protein A Sepharose CL-4B (PAS; 17-0963, GE Healthcare) + TBST solution was prepared in to a conical tube, with a concentration of 10 µl of PAS per well. Prior to making the solution, PAS was washed by centrifuging the solution with at least 4x its volume of TBST in 1000 RPM for 5 minutes. After centrifuging, the supernatant was aspirated. The required volume of TBST was calculated for 40 µl per well plus the

volume of ethanol lost during aspiration, roughly 20%. The PAS solution was then pipetted on the plates with the samples. Plates were then incubated in a mixer for a minimum of 1 hour in +4 ˚C and 1000 RPM. After incubation, the plates were washed with TBST, centrifuged for 3 minutes in +4 ˚C and 2000 RPM, after centrifugation the supernatant was aspirated. This process was repeated for 5 times, then the plates were incubated for 30 minutes to 1 hour in +4 ˚C and 1000 RPM.

Afterwards, the samples were transferred on to the liquid scintillation plates with the scintillation liquid (Optiphase Supermix; Perkin Elmer, USA; CAT 1200-439 for 1x5l) and then the plates were incubated while protected from light in room temperature and 900 RPM. Finally, the bound activity on the plates was measured by using a liquid scintillation counter (1450 MicroBeta Trilux; Perkin Elmer Life Sciences Wallac, Turku, Finland). The results were calculated using a MultiCalc software (Perkin Elmer Life Sciences Wallac) and compared to a standard curve constructed from serial dilutions of nine highly positive standard samples and one negative sample.

5.3 Data Analysis

The data of Diabetes Register and the DIABIMMUNE were not normally distributed (Histograms shown in Appendix 1.) Differences in the frequencies of autoantibodies were tested with Chi-squared statistics. The Mann-Whitney *U*-test was used to compare RU levels of autoantibodies. Correlations were analysed using the Spearman's non-parametric correlation. The statistical analyses were performed with IBM SPSS Statistics, version 25. For all analyses, *P* value of 0.05 was considered significant. Venn diagrams were drawn with http://bioinformatics.psb.ugent.be/webtools/Venn/ and edited with InkScape software version 0.92.2, diagrams were drawn with Microsoft Excel 2015.

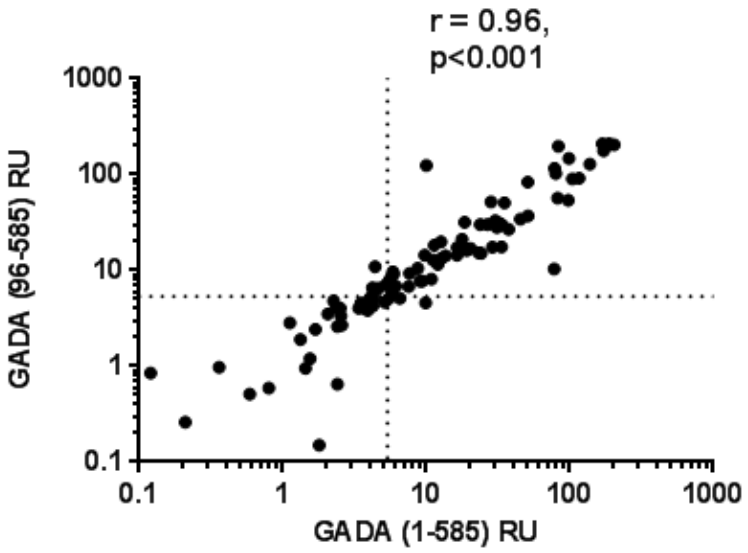
6 Results

6.1 Children with Newly Diagnosed Type 1 Diabetes

Type 1 diabetes children diagnosed under 16 years of age with varying concentrations of GAD65 autoantibodies were selected for this study and analysed for truncated GAD65 (96–585) autoantibodies.

There was good correlation between the levels of GADA (1–585) and GADA (96–585) (r = 0.96, P < 0.00; Figure 9A). The autoantibody levels and frequencies near the cut-off value (5.36 RU) are represented in Figure 9B.

A



B

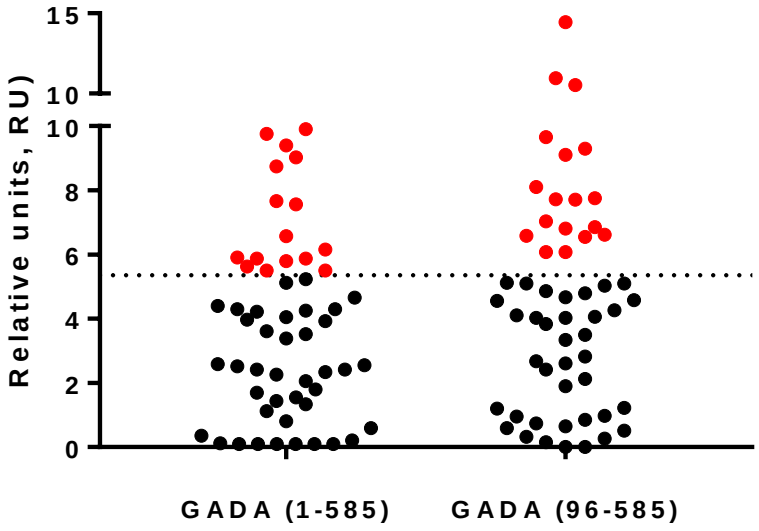
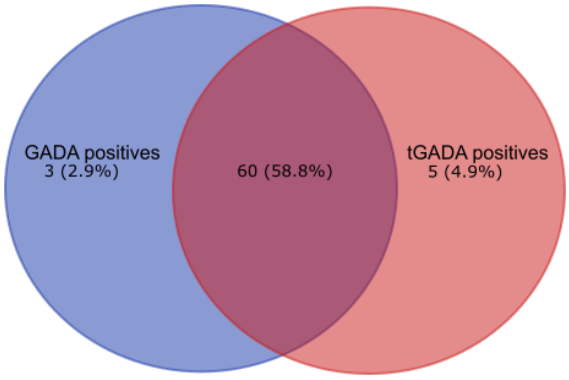


Figure 9. (A) Correlation diagram of GADA (1–585) and GADA (96–585) results for 101 newly diagnosed patients. Low positive GADA results (<=10 RU) and the corresponding truncated GADA results (B) show that altogether two more type 1 diabetic patients tested positive for tGAD (96–585) compared to GADA (1–585). Threshold for both assays is 5.36 and is represented with dotted lines. Results over the cut-off value are shown by red dots.

Out of 101 subjects, 63 (62%) were positive for GADA (1–585) and 65 (64%) were positive for GADA (96–585). Sixty (59%) patients showed positivity for both GADA (1–585) and GADA (96–585) (Figure 10A). There was no statistical difference in frequency of positivity between assays (*P* = 0.77). Majority of the subjects were positive only for M-epitope (12.7%), with N-epitope (5.9%) and C-epitope (5.9%) showing almost equal numbers of positives (Figure 10B). Out of 63 GADA (1–585) positives, 11 subjects (17%) were positive for N-epitope, 25 subjects (40%) were positive for M-epitope, and 18 subjects (29%) were positive for C-epitope. While majority of the subjects showed positivity for both M- and C-epitopes; 4 subjects (3.9%) showed positivity for all three epitopes, only 1 subject (0.98%) showed positivity for both N- and C-epitopes and no subjects were positive for both M- and N-epitopes (Figure 10B) nor in the frequencies of autoantibodies (*P* = 0.77). Twenty-eight (44%) subjects did not show positivity for any epitope and out of that group 2 subjects (3.1%) were negative for truncated GADA (96–585).

A



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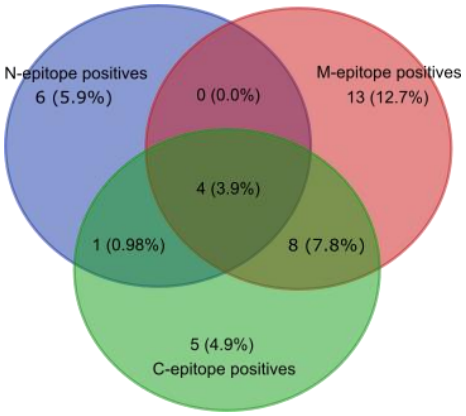


Figure 10. Venn diagram showing the frequency of GADA and truncated GADA results (A) in newly diagnosed type 1 patients. Two more patients were found to be positive for tGADA (96–585) that for GADA (1–585), but the difference was not significant *(P* = 0.77*).* Venn diagram showing N-, M-, and C-epitope responses (B) in newly diagnosed type 1 diabetes patients

6.2 DIABIMMUNE Children

Out of over 3600 DIABIMMUNE children (the YCC), 83 children were previously found to be GADA (1–585) positive in at least one sampling point (3, 4 and 5 years). All time points (202 samples) of these children were analysed for truncated GAD65 (96–585) autoantibodies. There was statistically significant correlation between GADA (1–585) and GADA (96–585) levels (r = 0.63, P < 0.001; Figure 11). There was, however, significant difference in frequencies of GADA (1–585) and tGADA (96–585) positivity (P < 0.001).

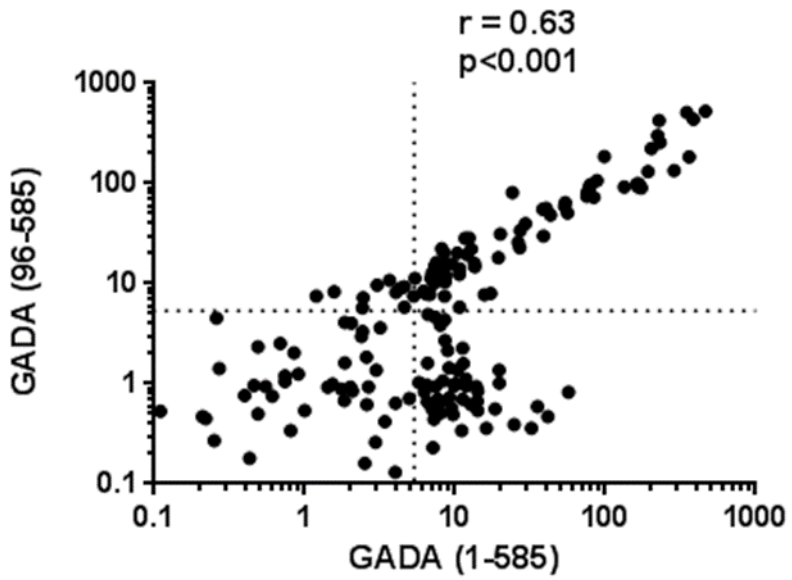


Figure 11. Correlation of GADA (1–585) and GADA (96–585) results in 202 serum samples from 83 DIABIMMUNE children. Threshold for both assays is 5.36 RU and is shown in the diagram with dotted lines.

Out of 202 samples in 83 subjects, 120 (59%) were positive for GADA (1–585) and 82 (41%) were positive for GADA (96–585). In total 60 samples out of 202 had discrepancies in GADA and tGADA results. Those positive only for tGADA (11 samples) were all low positive (5.74 – 10.73 RU), as were most of solely GADA positive (49 samples). Out of 60 samples, 5 had autoantibody levels above 20 RU. Seventy-one (35%) samples showed positivity for both GADA (1–585) and GADA (96–585; Figure 12). GADA levels (1-585) were significantly higher than tGADA (96–585) levels $P = 0.037$.

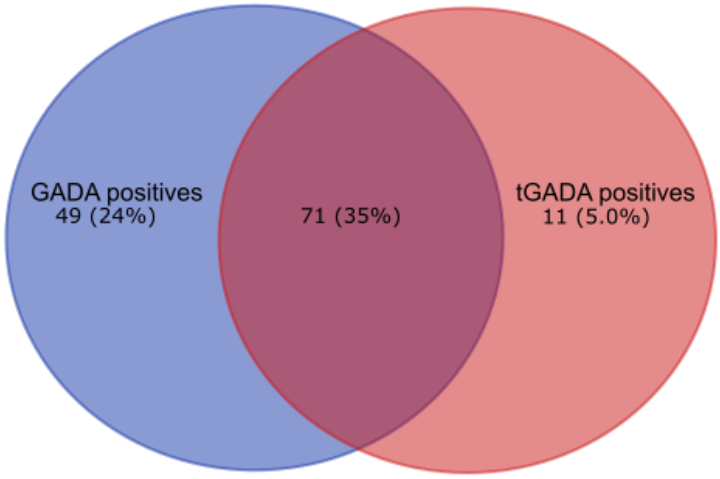


Figure 12. Venn diagram of GADA (1–585) and GADA (96–585) positive DIABIMMUNE subjects.

Correlation between GADA (1–585) and GADA (96–585) results at 36 months of age is shown in Figure 13. When all samples are included in analysis, the correlation was significant ($r = 0.63$, $P < 0.001$). It increased to 0.88 when GADA positive, but tGADA negative results (24) were abolished from the analysis. Correlation of GADA (1-585) and tGADA (96–585) was very good for multiple autoantibody positive and T1D patients ($r = 0.93$) at 36 months, and excellent at 48 and 60 months of age ($r = 0.97$ and 0.99, respectively).

The frequency of tGADA positivity was higher at 36 months of age in children who developed multiple autoantibodies during the follow-up period compared to only single-GADA positive (78.9% vs 14.8%, *P* =< 0.001).

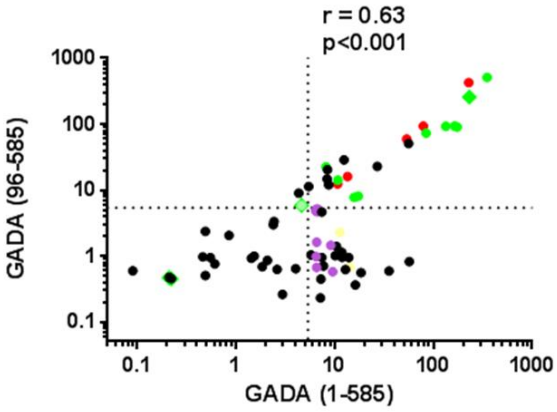


Figure 13. Plot of GADA (1-585) and tGADA (96-585) results at 36 months of age. DIABIMMUNE children, who progressed to type 1 diabetes are indicated in red points, those who developed multiple autoantibodies during the follow-up time, but have not progressed to diabetes are shown with green points. Black points represent children who are GADA and/or tGADA positive but have not developed other beta cell autoantibodies during the follow-up. Of these N-epitope positive samples are indicated with purple points. The dotted lines represent the cut-off points for positive values (5.56 RU).

Out of merely GADA (1–585) positive subjects, 12 (14%) were positive only at 36 months, 1 (1%) was positive only at 48 months and 7 (8%) were positive only at 60 months. Seven (8%) subjects were positive only at 36 and 48 months, 1 (1%) was positive at 48 and 60 months and none were positive at 36 and 60 months. Four (5%) subjects were positive at all time points (Figure 14).

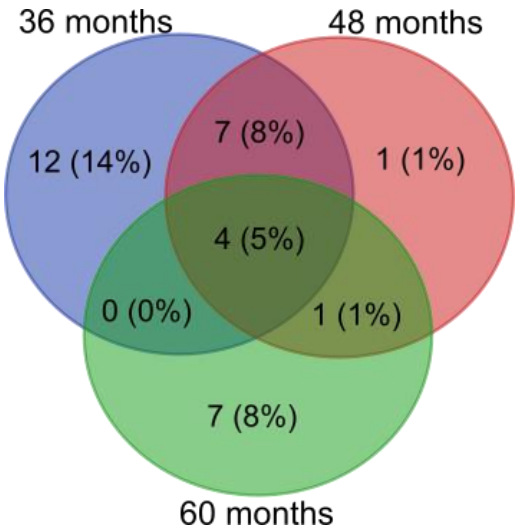


Figure 14. Venn diagram of GADA (1–585) positives, but tGADA (96-585) negative samples at different ages.

Of 83 subjects at 36 months, 46 (55%) were positive for GADA (1–585), twenty-four (29%) were positive for GADA (96–585), seven (8%) were positive for N-epitope, 12 (14%) were positive for M-epitope and 11 (13%) were positive for C-epitope. Out of 83 subjects at 60 months, 46 subjects (55%) were positive for GADA (1–585), thirty-four subjects (41%) were positive for GADA (96–585), eleven subjects (13%) were positive for N-epitope, 19 subjects (83%) were positive for M-epitope and 12 subjects (14%) were positive for C-epitope (Figure 15). Of 120 GADA positive samples, 57 were negative for all epitope antibodies. The majority of these (35 samples) had antibody level under 10 RU (media 8.67, range 5.43-27.07).

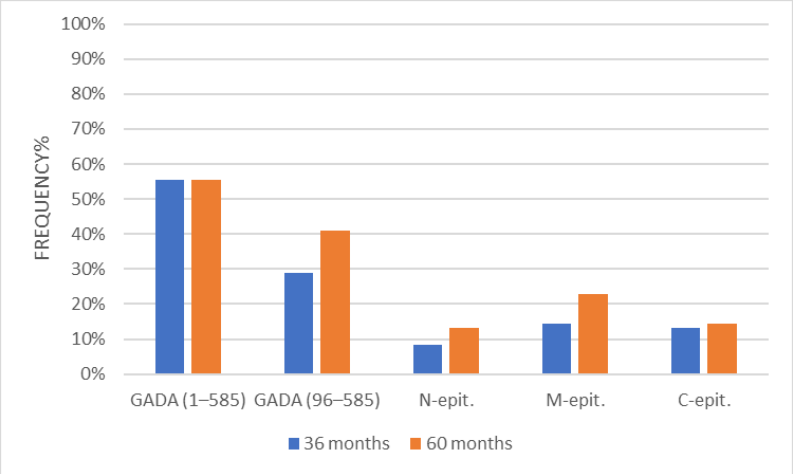


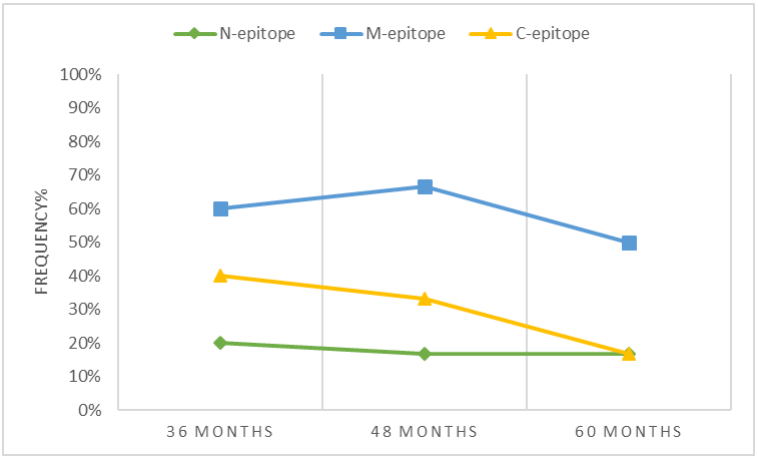
Figure 15. Frequencies of GAD65 (1-585), truncated GAD65 (96-585) and N-, M- and C epitope antibodies in the DIABIMMUNE children at the age of 36 and 60 months.

Altogether six children have progressed to type 1 diabetes up to now. Serum sample was available from five children at the age of 36 months, and from six children at 48 and 60 months of age. Twenty percent (20%) of these children were positive for N-epitope, 60% were positive for M-epitope and 40% were positive for C-epitope at the age of 36 months. At the age of 48 months, 17% were positive for N-epitope, 67% were positive for M-epitope and 33% were positive for C-epitope and 17% were positive for N-epitope, 50% were positive for M-epitope and 17% were positive for C-epitope at the age of 60 months (Figure 16A and Table 1). M-epitope was the most common among progressors, followed by C-epitope. N-epitope was positive only in one subject. Most subjects also showed positivity for multiple epitope and were multipositive (Table 1). The other antibodies besides GADA were: IAA (3 subjects at 36 months, 2 at 48 months and 2 at 60 months), IA-2A (4 subjects at 36 months and 5 at 48 and 60 months), ZnT8A (4 subjects at 36 and 48 months, 3 at 60 months) and ICA (5 subjects at 36 and 48 months, 4 at 60 months; Table 1).

GENDER	RISK	GADA at 36 months	tGADA at 36 months	Other autoantibodies at 36 months	Epitopes at 36 months	GADA at 48 months	tGADA at 48 months	Other autoantibodies at 48 months	Epitopes at 48 months	GADA at 60 months	tGADA at 60 months	Other autoantibodies at 60 months	Epitopes at 60 months
Progressors													
M	1					1	1	IAA, ICA	M	1	1	IAA, ICA	M
M	3	1	1	ZnT8A, ICA	N, M	1	1	IA-2A, ZnT8A, ICA	N, M, C	1	1	IA-2A, ZnT8A, ICA	N, M, C
F	3	1	1	IAA,IA-2A, ICA	M, C	1	1	IAA,IA-2A, ICA	M, C	1	1	IAA, Ia-2A	
F	1	1	1	IA-2A, ZnT8A, ICA	M, C	1	1	IA-2A, ZnT8A, ICA	M	1	1	IA-2A, ZnT8A, ICA	M
F	3	1	1	IAA,IA-2A, ZnT8A, ICA		0	0	IA-2A, ZnT8A		0	0	IA-2A, ZnT8A	
F	2	1	1	IAA,IA-2A, ZnT8A, ICA		1	1	IA-2A, ZnT8A, ICA		0	0	IA-2A, ICA	
Multipositives													
F	4	1	1	IAA	M, C	1	1		M, C	1	1	ZnT8A, ICA	M, C
F	0/1	1	1	IAA	M, C	1	1	IAA, ZnT8A, ICA	M, C				
F	0/1	1	1	IAA, IA-2A, ZnT8A, ICA		1	1	IAA, IA-2A, ZnT8A, ICA		1	0	IAA, IA-2A, ZnT8A, ICA	
F	0/1	1	1		M, C	1	1	ICA	M, C	1	1	ICA	M, C
F	3	1	1	ZnT8A, ICA	M, C								
M	2	1	1	IAA, IA-2A, ZnT8A, ICA	M, C	1	1	ZnT8A	M, C	1	1	IAA, ZnT8A	M, C
F	3	1	1	IAA, IA-2A, ZnT8A, ICA		1	1	IAA, IA-2A, ZnT8A, ICA	M	1	1	IA-2A, ZnT8A, ICA	M
M	0/1	1	1	ICA	M, C								
M	3	0	1							1	1	IAA, ZnT8A	M, C
M	0/1	0	0							1	1	ICA	M, C
F	0/1	0	0			0	0			1	1	IAA, ZnT8A, ICA	M, C
M	3	0	0			0	0			1	1	ICA	
F	0/1	0	0							1	1	IAA	M
F	1									1	1	IAA, ICA	M, C
M		1	1	IAA, ZnT8A, ICA	M,C								
Single-GADA positives at 36 months													
F	3	1	1		M, C	1	1			0	1		
F	0/1	1	0		N	0	0			0	0		
F	0	1	0		N								
M	2	1	1		M	0	1		N	0	1		N
F	0/1	1	1			1	1		M	0	1		
F	0/1	1	0		N	1	0		N				
M	0/1	1	0		N	1	0		N	1	0		N
F	0	1	0		N	0	0			0	0		
M	0/1	1	0			0	0		N	0	0		N, C
M	0/1	1	1			1	1		N, C	1	1		C
M	0/1	1	0		N	0	0			0	0		

Table 1. Summary Table of T1D progressors, multiple autoantibody positive subjects and those out of 30 single-GADA positive subjects who were positive for some epitope (For abbreviations, see Abbreviations list.)

A



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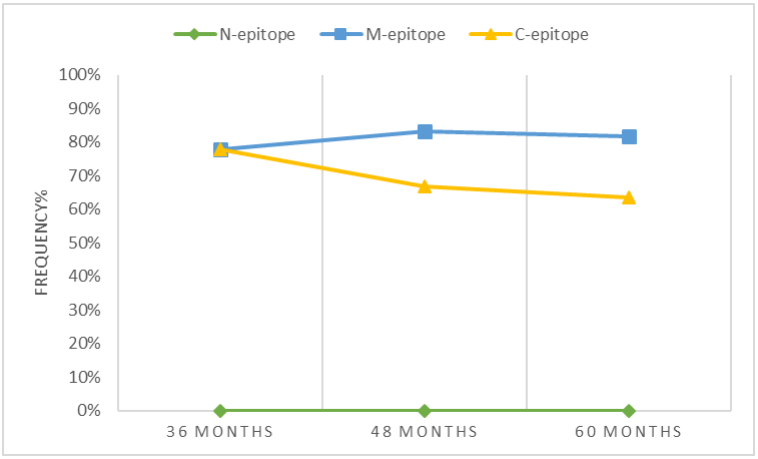

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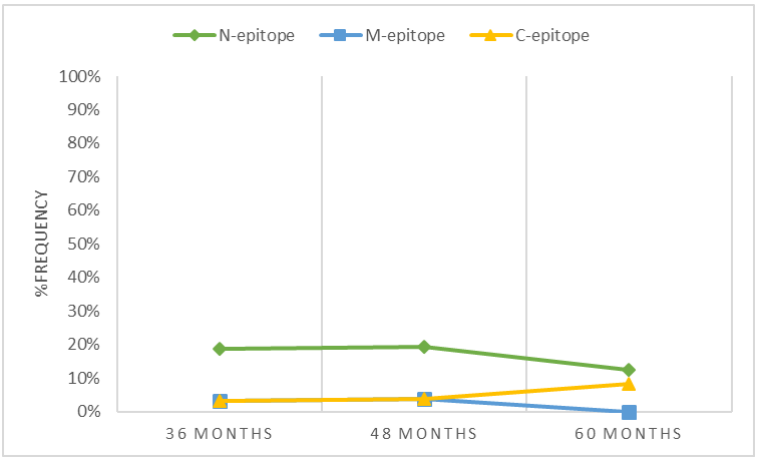

Figure 16. Frequencies of antibodies to N-terminal (green line), middle (M, blue line) and C-terminal parts of GAD65 protein at 36, 48 and 60 months of age in T1D progressors (A), multiple autoantibody positive (B) and single-GADA/tGADA positive children (C).

In total, 15 children developed multiple autoantibodies during the follow-up time. Out of 14 samples at the age of 36 months, nine were GADA (1–585) positive and of those, seven (78%) were positive both for M- and C-epitopes; 8 were positive for multiple autoantibodies (Figure 16B, Table 1). Out of 8 samples at 48 months, 6 were GADA (1–585) positive and of those, 5 (83%) were positive for M-epitope and 4 (67%) were positive for C-epitope; 5 were multiple autoantibody positive (Figure 16B, Table 1). Out of 11 GADA (1–585) positive samples at 60 months, 9 (82%) were positive for M-epitope and 7 (64%) were positive for C-epitope. Seven subjects out of nine were positive for both M- and C-epitopes (Figure 16B and Table 1). No subject showed positivity for N-epitope at any point. The other antibodies recognized were: IAA (6 subjects at 36 months, 3 at 48 months and 6 at 60 months), IA-2A (3 subjects at 36 months, 2 at 48 months and 2 at 60 months), ZnT8A (5 subjects at 36 months, 4 at 48 months and 6 at 60 months) and ICA (6 subjects at 36 months, 4 at 48 months and 7 at 60 months; Table 1).

Of 32 subjects positive for GADA (1-585) at 36 months, 8 were positive for tGADA (96-585), but autoimmunity did not spread to other beta-cell antigens during the follow-up time. Those positive for any epitope are shown in Table 1 and Figure 16C. Out of 32 children, 6 (19%) were positive for N-epitope and 1 (3%) was positive for both M- and C-epitopes at the age of 36 months. Out of 26 samples at 48 months, 10 were positive for GADA (1–585), 7 were positive for tGADA, 5 (38%) were positive for N-epitope and 1 (8%) was positive for both M- and C-epitopes. Of 24 samples at 60 months, 5 were positive for GADA (1–585), 4 were positive for tGADA, 3 (13%) were positive for N-epitope, 2 (8%) were positive for C-epitope and no sample was positive for M-epitope (Figure 16C, Table 1).

The proportions of epitope frequencies in different type 1 risk classes are shown in Figure 17. Out of nineteen samples from eight children with protective HLA risk class 0, three samples (16%) were positive for N-epitope, 1 sample (5%) was positive for M-epitope and no samples had positivity for C-epitope. Of 89 samples from 36 subjects with protective/no risk HLA risk class 0/1, seventeen samples (19%) were positive for N-epitope, 13 samples (15%) were positive for M-epitope and 12 samples (13%) were positive for C-epitope. In 25 samples from nine children with HLA risk class 1 (no risk), two samples (8%) were positive for N-epitope, 7 samples (28%) were positive for M-epitope and 2 samples (8%) were positive for C-epitope. Out of 23 samples from nine children with HLA risk class 2 (slightly increased risk), two samples (9%) were positive for N-epitope,

5 samples (22%) were positive for M-epitope and 3 samples (13%) were positive for C-epitope.

Out of 44 samples from 18 children with moderate or high with HLA risk class 3 (17 subjects) or 4 (one subject), three samples (7%) were positive for N-epitope, 12 samples (27%) were positive for M-epitope and 9 samples (20%) were positive for C-epitope (Figure 19). For further analysis, the children were divided to two HLA risk groups: group 1. protective or no risk genotypes (risk classes 0–1, 53 subjects) and 2. increased risk genotypes (risk classes 2–4, 29 subjects). All samples from children were included to the analysis. There was no statistical difference in GADA (1–585) positivity between the groups (p=0.799), but tGADA (96–585) positivity was significantly more common in the increased risk group 2 (60% vs. 29.5%, $P < 0.001$). N-epitope positivity was more common in the group 1 than in the group 2 (17.1% vs 7.1%, $P = 0.051$), while the M- and C-epitope positivity was more common in the increased risk group 2 ($P = 0.028$ and $P = 0.043$).

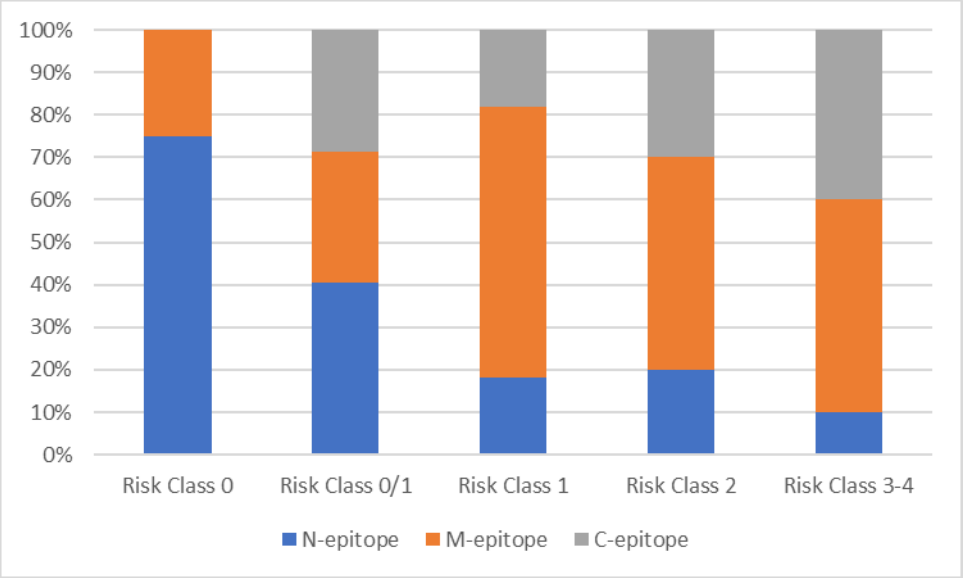

Figure 17. The proportions of epitope frequencies according to type 1 diabetes HLA risk class (0 = protective genotype, 0/1 = protective or no risk, 1 = no risk, 2 = slightly increased risk, 3 = moderate risk, 4 = high risk).

7 Discussion

In a previous study on N-truncated GADA by Williams *et al.* [16] in 2015, the sensitivity between GADA (1–585) and GADA (96–585) was identical (80%) in recently diagnosed type 1 diabetes patients with excellent correlation between the two methods (r = 0.99, P < 0.001). We had chosen 101 newly diagnosed type 1 diabetic patients from The National Diabetes Register with varying concentrations of GAD65 autoantibodies, concentrating in samples near the cut-off value, and analysed them for N-terminally truncated GAD65 (tGADA, 96-585) and N-, M-, C-epitope autoantibodies. The correlation between GADA and tGADA assays was very good (r = 0.96, P < 0.001) and there was no significant difference in positivity between assays (P = 0.77).

However, GADA (96–585) assay recognised two more type 1 diabetic patients compared to GADA assay (Figure 10A). The three subjects which were positive for GADA (1–585) but negative for truncated GADA (96–585) were low GADA (1–585) positive (5.50–9.90 RU; Figure 9B), and they were multiple autoantibody positive samples; these subjects would not have remained autoantibody negative. Out of the six newly diagnosed subjects who were only N-epitope positive, 4 were positive for both GADA (1–585) and tGADA (96–585), one was positive only for GADA and one was negative for both. Most probably the immune response was partly directed to GAD67, but this was not analyzed. Otherwise, the epitope immune responses followed a similar trend established in a study by Hoppu *et al.* [21] where most of the immune response in progressors was directed towards M- and C-epitopes.

The correlation in DIABIMMUNE subjects was not as good, though still significant, when compared to the newly diagnosed type 1 diabetes subjects (r = 0.96 v. r = 0.63; Figures 10A and 12). Correlation of GADA and tGADA in first degree relatives of type diabetes patients according to study by Williams *et al.* [16] was 0.96, although many sera in the study were also GADA positive, but tGADA negative. The reason for this could be that our DIABIMMUNE children represent general population, they are not selected for type 1 diabetes risk genotypes. Correlation improved significantly when we analyzed T1D and multiple autoantibody positive subjects and abolished samples of merely GADA positive subjects (r = 0.93 at 36 months, r = 0.97 at 48 months and r = 0.99 at 60 months). There was significant difference in frequency of positive samples between GADA and tGADA assays (58.3% and 39.7%, P < 0.001) and in the autoantibody levels (P = 0.037).

The frequency of truncated GADA (96–585) was higher at 36 months of age in children who developed to multiple autoantibody positive during the follow-up time compared to single GADA positive children ($P < 0.001$), while the frequency was not significantly different for GADA ($P = 0.13$). This would indicate that tGADA predicts multipositivity and T1D better than GADA (1–585). This is in line with results by Williams *et al.* [16], who showed that autoantibodies to truncated GAD65 in first degree relatives are more closely associated with diabetes risk than autoantibodies against full-length GAD65.

The epitope frequencies showed some variation between subjects: frequencies of M- and C-epitopes were much higher in multiple autoantibody positive subjects and in those who later progressed to type 1 diabetes, whereas subjects who were only GADA (1–585) positive showed autoantibody response only towards N-epitope. Almost none of the subjects showed epitope spreading: in all cases, except two who showed epitope spreading towards the C-epitope, the immune response was simultaneous and did not later spread to other epitopes. However, the first samples were taken at the age of three years, and many subjects were already multipositive at that point, which means that we did not have access to samples at the earliest stage of beta cell immunity. Further, the last sample was taken at the age of five years, and possible further epitope spreading could not be analyzed. Our results followed a similar trend with those acquired in studies by Ronkainen *et al.* and Hoppu *et al.* [22; 21] where the immune response was focused on M-epitope in progressors and multiple autoantibody positive subjects, followed by C-epitope with very few showing positivity towards N-epitope.

The epitope development in HLA phenotype risk classes showed that no type 1 diabetes risk -subjects (risk classes 0-1) had much higher frequencies of N-epitope, and elevated risk classes (risk classes 2-4) had increased frequencies of M- and C-epitopes. Truncated GADA (96–585) positivity was also more common in HLA phenotype risk groups 2–4, when compared to those with no risk/protective phenotype ($p<0.001$), while there was no significant difference in frequency of GADA positivity between risk groups 1 (risk classes 0–1) and 2 (risk classes 2–4) ($P = 0.799$). There is some discrepancy in epitope assay sensitivities; many subjects did not recognize any epitope or had values close to cut-off, but at the same time had high response to GADA. The epitope assays are either not sensitive enough, antigenic epitopes are hindered, and/or the responses were towards GAD67, which our assays did not specify.

In conclusion, tGADA (96–585) assay recognizes T1D positives as well as GADA (1–585) assay. In those children who are at a risk of developing multiple autoantibodies and T1D in the future, the tGADA assay is more specific since it leaves out those GADA positives whose immune response is only directed towards the N-epitope, which does not seem to be related to an increased risk of developing multipositivity or T1D. This is important especially when autoantibody positive participants are chosen for follow-up studies, such as intervention trials.

References

1 Atkinson M, Eisenbarth G, Michels A. Type 1 diabetes, *Lancet* 2014; 383; 69–82

2 Pociot F, Lernmark Å. Genetic risk factors for type 1 diabetes. *Lancet* 2016; 387; 2331–39

3 Lampasona V, Liberati D. Islet Autoantibodies. *Curr Diab Rep* 2016; 53

4 Insel RA, Dunne JL, Atkinson MA, Chiang JL, Dabelea D, Gottlieb PA, Greenbaum CJ, Herold KC, Krischer JP, Lernmark Å, Ratner RE, Rewers MJ, Schatz DA, Skyler JS, Sosenko JM, Ziegler AG. Staging Presymptomatic Type 1 Diabetes: A Scientific Statement of JDRF, the Endocrine Society, and the American Diabetes Association. *Diabetes Care* 2015; 38(19): 1964–1974

5 Kostic AD, Gevers D, Siljander H, Vatanen T, Hyötyläinen T, Hämäläinen AM, Peet A, Tillmann V, Pöhö P, Mattila I, Lähdesmäki H, Franzosa EA, Vaarala O, de Goffau M, Harmsen H, Ilonen J, Virtanen SM, Clish CB, Orešič M, Huttenhower C, Knip M, on behalf of the DIABIMMUNE Study Group, Xavier RJ. The Dynamics of the Human Infant Gut Microbiome in Development and in Progression toward Type 1 Diabetes. *Cell Host & Microbe* 2015; 17(2): 260–273

6 Oikarinen S, Tauriainen S, Hober D, Lucas B, Vazeou A, Sioofy-Khojine A, Bozas E, Muir P, Honkanen H, Ilonen J, Knip M, Keskinen P, Saha MT, Huhtala H, Stanway G, Bartsocas C, Ludvigsson J, Taylor K, Hyöty H. Virus Antibody Survey in Different European Populations Indicates Risk Association Between Coxsackievirus B12 and Type 1 Diabetes. *Diabetes* 2014; 63(2): 655–662

7 Knip M, Virtanen SM, Åkerblom HK. Infant Feeding and the risk of type 1 diabetes. *The American Journal of Clinical Nutrition* 2010; 91(5): 1506S–1513S

8 Vaarala O, Ilonen J, Ruohtula T, Pesola J, Virtanen SM, Härkönen T, Koski M, Kallioinen H, Tossavainen O, Poussa T, Järvenpää AL, Komulainen J, Lounamaa R, Åkerblom HK, Knip M. Removal of Bovine Insulin From Cow's Milk Formula and Early Initiation of Beta-Cell Autoimmunity in the FINDIA Pilot Study. *Arch Pediatr Adolesc Med* 2012; 166(7): 608–614

9 Knip M, Åkerblom HK, Becker D, Dosch HM, Dupre J, Fraser W, Howard N, Ilonen J, Krischer JP, Kordonouri O, Lawson ML, Palmer JP, Savilahti E, Vaarala O, Virtanen SM. Hydrolyzed Infant Formula and Early β-Cell Autoimmunity. *JAMA* 2014; 311(22): 2279–2287

10 Knip M, Åkerblom HK, Al Taji E, Becker D, Bruining J, Castano L, Danne T, de Beaufort C, Dosch H-M, Dupre J, Fraser WD, Howard N, Ilonen J, Konrad D, Kordonouri O, Krischer JP, Lawson ML, Ludvigsson J, Madacsy L, Mahon JL, Ormisson A, Palmer JP, Pozzilli P, Savilahti E, Serrano-Rios M, Songini M, Taback S, Vaarala O, White NH, Virtanen SM, Wasikowa R. Effect of Hydrolyzed Infant Formula vs Conventional Formula on Risk of Type 1 Diabetes. *JAMA*. 2018; 319(1): 38–48

11 Nejentsev S, Koskinen S, Sjöroos M, Reijonen H, Scwartz EI, Kovalchuck L, Sochnev A, Adojaan B, Podar T, Knip M, Simell O, Koskenvuo M, Åkerblom HK, Ilonen J. Distribution of insulin-dependent diabetes mellitus (IDDM)-related HLA alleles correlates with the difference in IDDM incidence in four populations of the Eastern Baltic region. *Tissue Antigens* 1998; 52; 473–477

12 Pöllänen PM, Lempainen J, Laine AP, Toppari J, Veijola R, Vähäsalo P, Ilonen J, Siljander H, Knip M. Characterisation of rapid progressors to type 1 diabetes among children with HLA-conferred disease susceptibility. *Diabetologia* 2017; 60; 1284–1293

13 Vatanen T, Kostic AD, d'Hennezel E, Siljander H, Franzosa EA, Yassour M, Kolde R, Vlamakis H, Arthur TD, Hämäläinen AM, Peet A, Tillmann V, Uibo R, Mokurov S, Dorshakova N, Ilonen J, Virtanen SM, Szabo SJ, Porter JA, Lähdesmäki H, Huttenhower C, Gevers D, Cullen TW, Knip M, on behalf of the DIABIMMUNE Study Group, Xavier RJ. Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell* 2016; 165(4): 842–853

14 Knip M, Siljander H, Ilonen J, Simell O, Veijola R. Role of humoral beta-cell autoimmunity in type 1 diabetes. *Pediatric Diabetes* 2016; 17(Suppl. 22): 17–24

15 Fenalti G, Law RH, Bickle AM, Langendorf C, Tuck K, Rosado CJ, Faux NG, Mahmood K, Hampe CS, Banga JP, Wilce M, Schmidberger J, Rossjohn J, El-Kabbani O, Pike RN, Smith AI, Mackay IR, Rowley MJ, Whisstock JC. GABA production by glutamic acid decarboxylase is regulated by a dynamic catalytic loop. *Nat Struct Mol Biol* 2007; 14; 280–286

16 Williams AJK, Lampasona V, Schlosser M, Mueller PW, Pittman DL, Winter WE, Akolkar B, Wyatt R, Brigatti C, Krause S, Achenbach P, and Participating Laboratories. Detection of Antibodies Directed to the N-Terminal Region of GAD Is Dependent on Assay Format and Contributes to Differences in the Specificity of GAD Autoantibody Assays for Type 1 Diabetes. *Diabetes* 2015; 64; 3239–3246

17 Wadhwa M, Knezevic I, Kang HN, Thorpe R. Immunogenicity assessment of biotherapeutic products: An overview of assays and their utility. *Biologicals* 2015; 43(5): 298–306

18 Boster Biological Technology. ELISA Fundamental Principle, How ELISA Works - Immunoassays. https://www.bosterbio.com/protocol-and-troubleshooting/elisa-principle. Retrieved 11.1.2018.

19 Steck AK, Fouts A, Miao D, Zhao Z, Dong F, Sosenko J, Gottlieb P, Rewers MJ, Yu L, and the TrialNet Study Group. ECL-IAA and ECL-GADA Can Identify High-Risk Single Autoantibody-Positive Relatives in the TrialNet Pathway to Prevention Study. *Diabetes Technology & Therapeutics* 2016; 18(7): 410–414

20 Williams AJK, Lampasona V, Wyatt R, Brigatti C, Gillespie KM, Bingley PJ, Achenbach P. Reactivity to N-Terminally Truncated GAD_{65}(96-585) Identifies GAD Autoantibodies That Are More Closely Associated With Diabetes Progression in Relatives of Patients With Type 1 Diabetes. *Diabetes* 2015; 64; 3247–3252

21 Hoppu S, Ronkainen MS, Kulmala P, Åkerblom HK, Knip M, The Childhood Diabetes in Finland Study Group. GAD65 antibody isotypes and epitope recognition during the prediabetic process in siblings of children with type 1 diabetes. *Clin Exp Immunol* 2004; 136; 120–128

22 Ronkainen MS, Hoppu S, Korhonen S, Simell S, Veijola R, Ilonen J, Simell O, Knip M. Early epitope- and isotype-specific humoral immune responses to GAD65 in young children with genetic susceptibility to type 1 diabetes. *Eur J Endocrinol* 2006; 155; 633–642

23 Schlosser M, Mueller PW, Achenbach P, Lampasona V, Bingley PJ, and Participating Laboratories. Diabetes Antibody Standardization Program: First evaluation of assays for autoantibodies to IA-2β. *Diabetes Care* 2011; 34(11): 2410–2412

24 Schlosser M, Mueller PW, Törn C, Bonifacio E, Bingley PJ, Participating Laboratories. Diabetes Antibody Standardization Program: evaluation of assays for insulin autoantibodies. *Diabetologia* 2010; 53(12): 2611–2620

25 Simmons K, Michels AW. Lessons from Type 1 Diabetes for Understanding Natural History and Prevention of Autoimmune Disease. *Rheumatic Disease Clinics* 2014; 40(4): 797–811

26 Savola K 2000: Role of IA-2 Antibodies in Clinical and Preclinical Type 1 Diabetes. Academic Dissertation. University of Oulu. Faculty of Medicine. Department of Paediatrics.

27 Simmons K, Michels AW. Type 1 diabetes: A predictable disease. *World Journal of Diabetes* 2015; 6(3): 380–390

Histograms

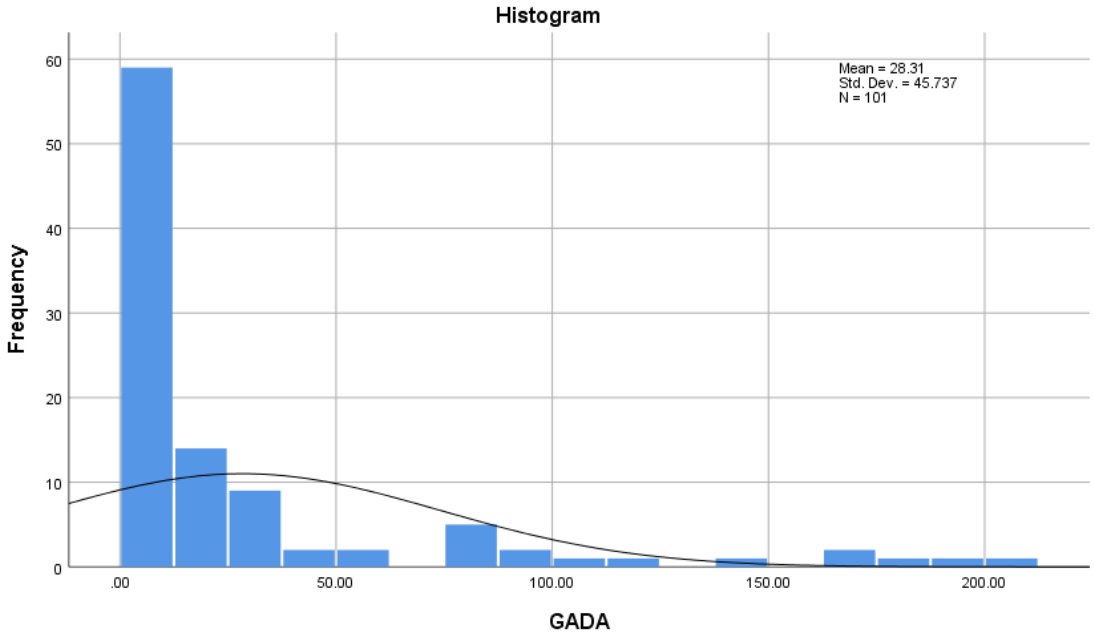

Figure 1 Histogram of GADA in total number of subjects, with distribution curve.

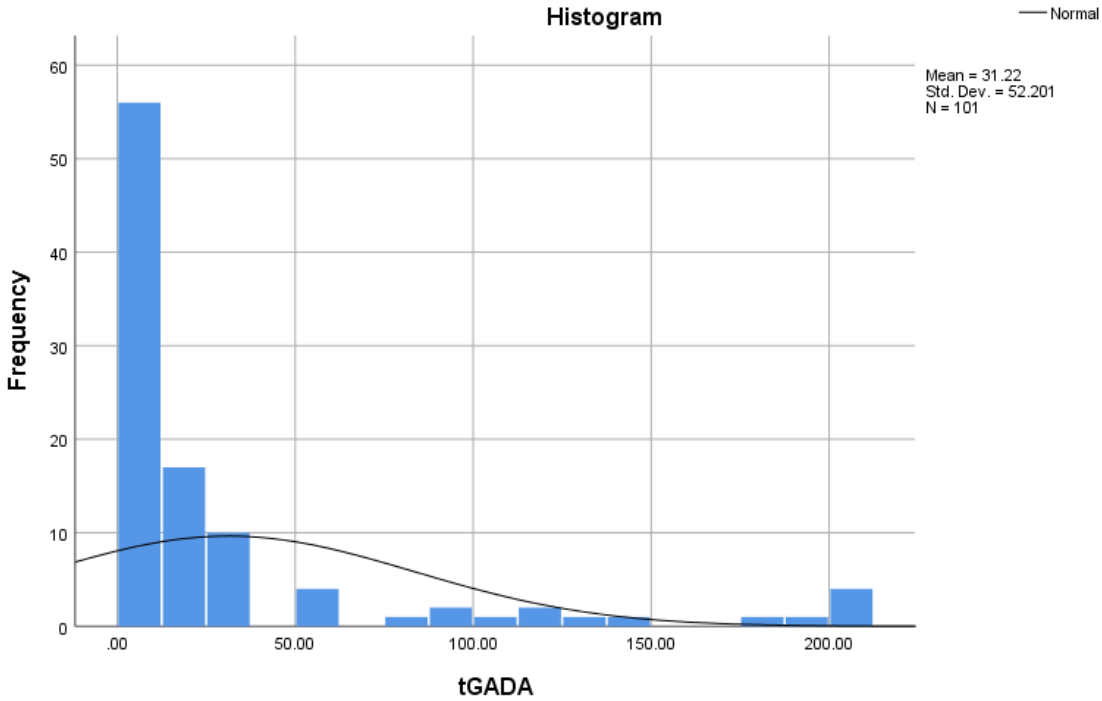

Figure 2 Histogram of tGADA in total number of subjects, with distribution curve.

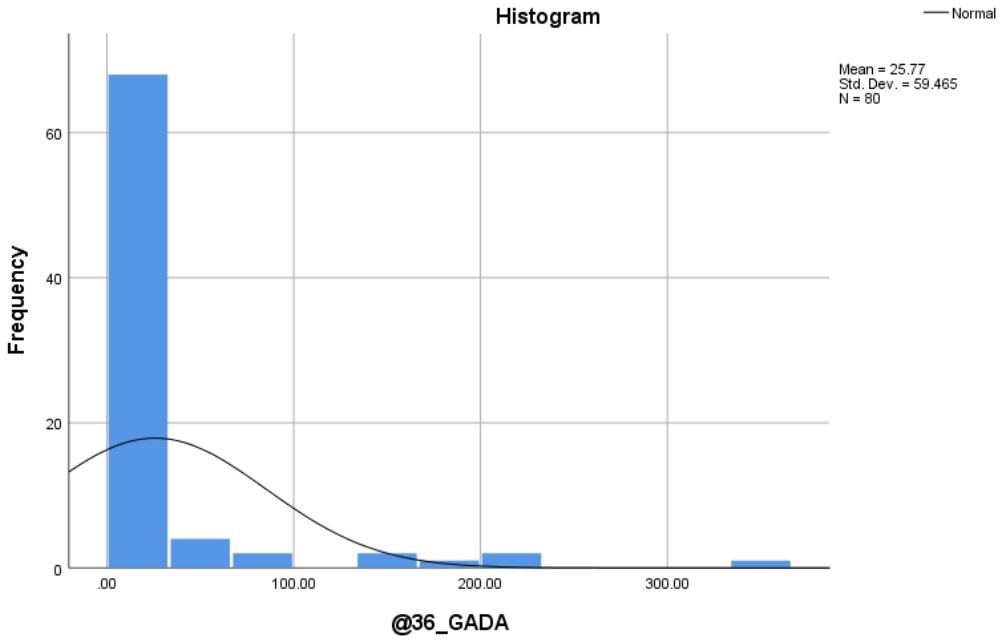

Figure 3 Histogram of GADA in 36 month subjects, with distribution curve.

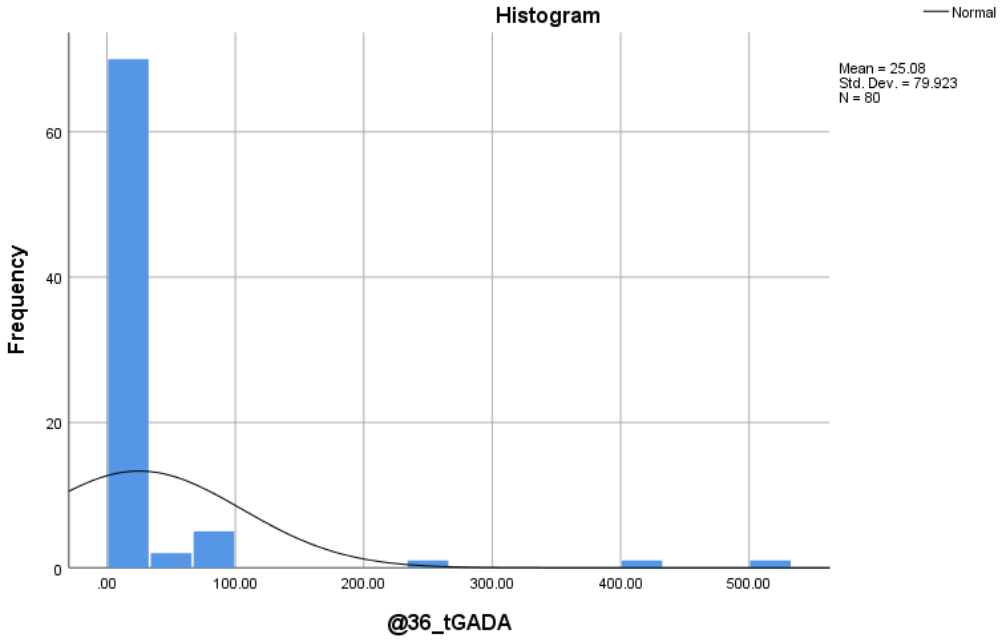

Figure 4 Histogram of tGADA in 36 month subjects, with distribution curve.

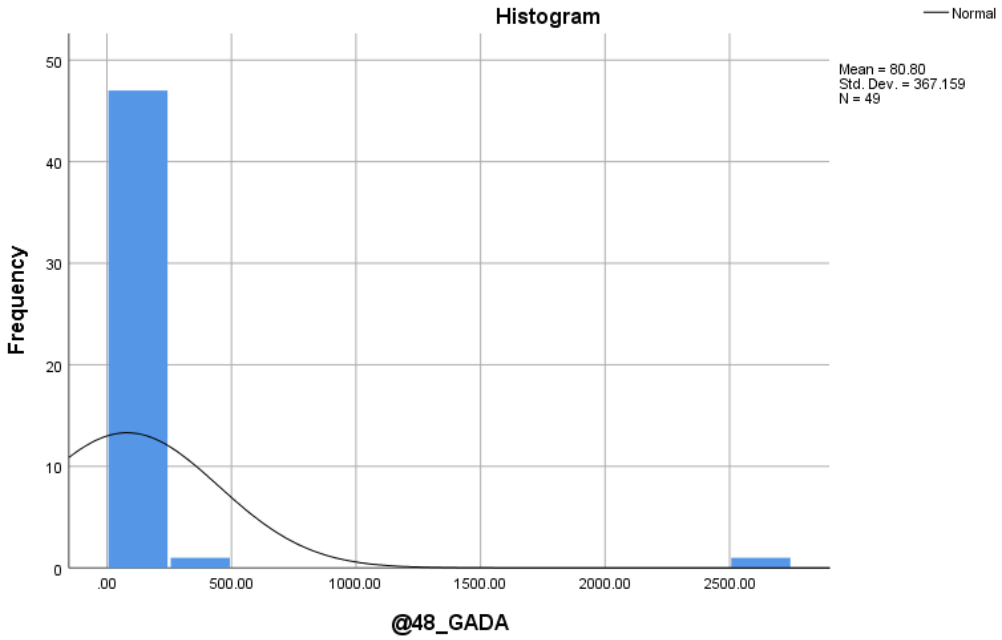

Figure 5 Histogram of GADA in 48 month subjects, with distribution curve.

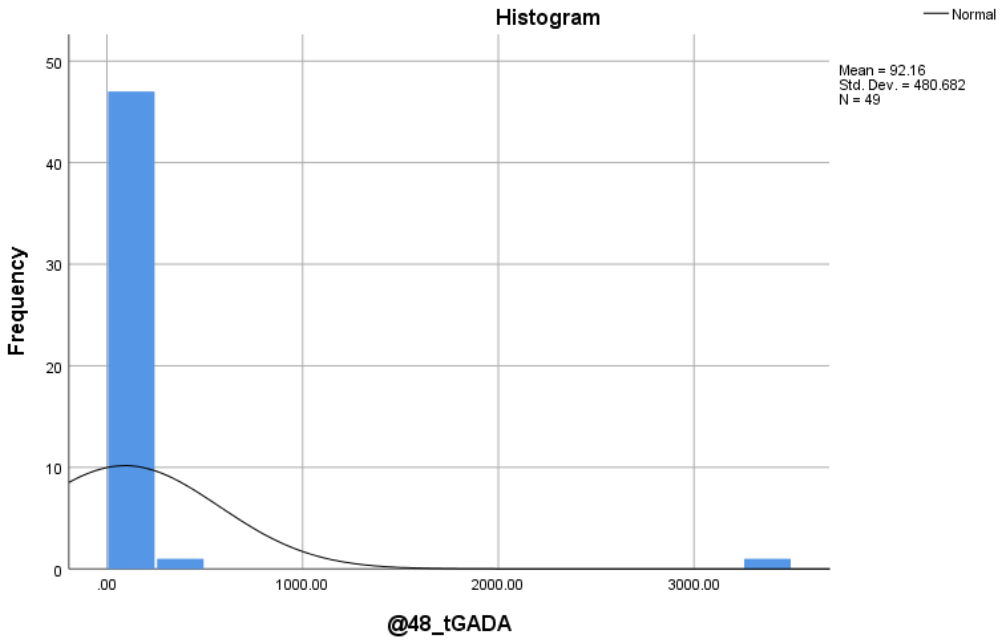

Figure 6 Histogram of tGADA in 48 month subjects, with distribution curve.

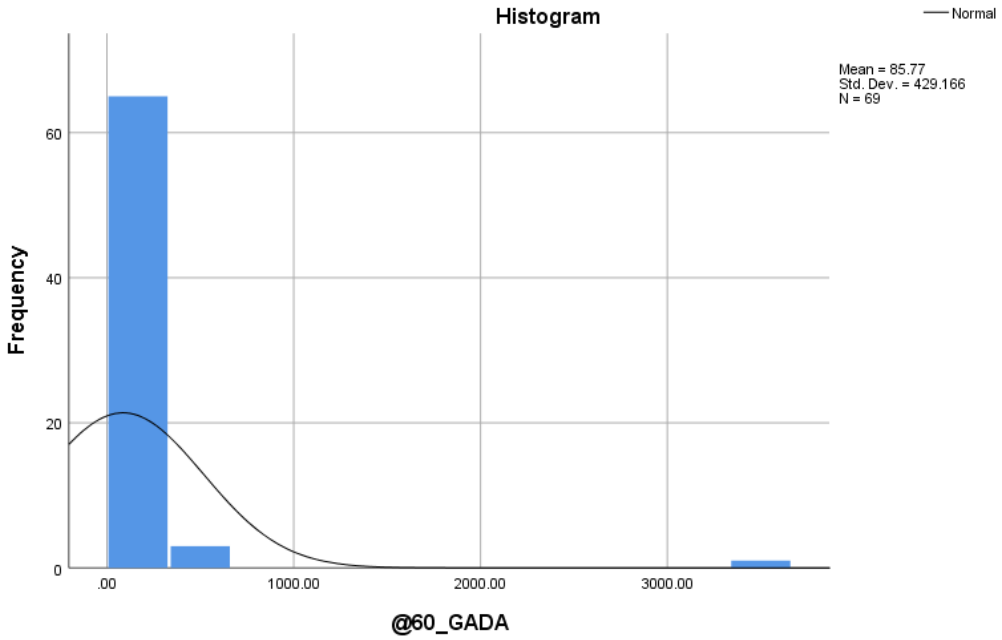

Figure 7 Histogram of GADA in 60 month subjects, with distribution curve.

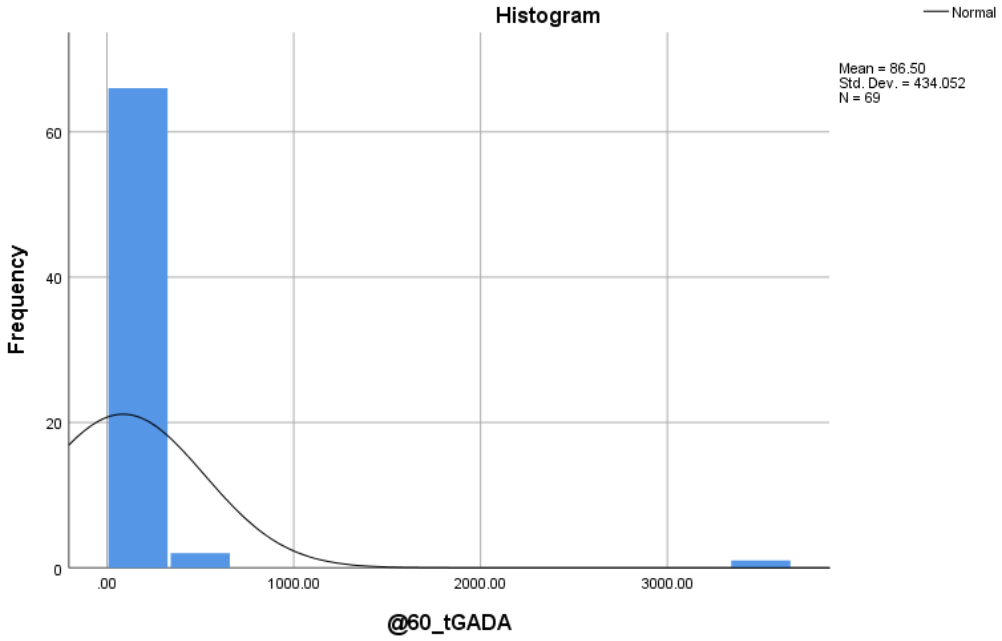

Figure 8 Histogram of tGADA in 60 month subjects, with distribution curve.