Abstract

Multiplex Antibody-Detection by Agglutination-PCR (ADAP) assay was compared to singleplex standard radiobinding assays (RBA) to detect autoantibodies against insulin (IAA), GAD65 (GADA), islet antigen-2 (IA-2A), ZnT8 (ZnT8A) and tissue transglutaminase (TGA). Serum samples from 272 (114F/158M), 15-73 years of age healthy controls and 227 (109F/118M) newly diagnosed type 1 diabetes children, 1-11 years of age, were analyzed in both assay systems.The original WHO standard 97/550 and in-house reference standards for RBA were compared to ADAP. The ADAP and RBA generated parallel reference standards in all assays except TGA. Lower detection limits were observed in the ADAP assay for GADA,IAA and ZnT8A, markedly for TGA, but not for IA-2A. The Receiver Operating Characteristics (ROC) curve AUC analyses for pairwise comparison of ADAP with RBA showed no difference for GADA (n.s.), ADAP greater AUC for IAA ($p=0.005$), RBA greater AUC for IA-2A ($p=0.0004$) and ZnT8A ($p<0.0001$) while ADAP TGA had a greater AUC compared to both RBA TGA-IgG ($p<0.0001$) and TGA-IgA ($p<0.0001$) . These data suggest that the ADAP and RBA assays are comparable with equal performance for GADA, better ADAP performance for IAA while the RBA showed better performance in both IA-2A and ZnT8A associated with greater heterogeneity in autoantibody levels. The simultaneous analysis of 5 different autoantibodies by ADAP in sample volume reduced to only 4 μL and at an increased lower detection limit in all assays except IA-2A makes the ADAP automated autoantibody assay a distinct advantage for high throughput screening.

Keywords islet autoantibodies, radiobinding assay, agglutination-PCR, type 1 diabetes, celiac disease, insulin, glutamic acid decarboxylase, islet antigen-2, Zinc transporter 8, tissue transglutaminase

Introduction

Autoantibodies are strong and widely used biomarkers for the risk of autoimmune type 1 diabetes (T1D) and celiac disease (CD). Two or more autoantibodies against either insulin (IAA), glutamic acid decarboxylase 65 (GADA), islet antigen- 2 (IA-2A) or ZnT8 transporter (ZnT8A) predict that 70% of these subjects will progress to clinical onset of diabetes within 10 years (Ziegler et al., 2013; Liu et al., 2014; Pöllänen et al., 2020; Vehik et al., 2020). Two or more autoantibodies without dysglycemia and symptoms mark Stage 1 T1D while stage 2 is marked by dysglycemia but still no symptoms of T1D (Insel et al., 2015). Autoantibodies against tissue transglutaminase (TGA) predict CD (Björck et al., 2010; Liu et al., 2014; Andrén Aronsson et al., 2016; Maglio and Troncone, 2020) and TGA levels inform stages of intestinal inflammation (Montén et al., 2016) and advice when to perform a diagnostic biopsy (Agardh et al., 2015). T1D and CD share the HLA-DR3-DQ2 genetic risk haplotypes (Hagopian et al., 2017) and the two diseases are associated as about 10% of newly diagnosed T1D are at risk of also developing CD, often within 5 years of the initial diagnosis of T1D (Larsson et al., 2008; Gyllenberg et al., 2012; Cerqueiro Bybrant et al., 2018; Kurppa et al., 2018) . It is therefore important to analyze all five autoantibodies simultaneously since subjects at risk (Ziegler et al., 2020) and newly diagnosed T1D patients would benefit from being correctly classified with T1D (Carlsson et al., 2020). Furthermore, a 5-plex assay would enable T1D patients evaluated for CD risk (Hagopian et al., 2017) at the same time to minimize sample volume consumption.

Up until recently islet and celiac disease autoantibodies have been detected primarily by radioimmunoassays (RIA) or radiobinding assays (RBA). IAA are based on RIA with C14-monoiodinated ^{125}I-insulin in standard assay separating antibody-bound from free ^{125}I-insulin with Sepharose Protein A (Williams et al., 1997) or a mixture of Sepharose Protein A and G (Williams et al., 2006). IAA has been standardized in multiple workshops with mixed results (Bingley et al., 2003; Schlosser et al., 2010) and there is yet an international standard to be developed to harmonize levels between laboratories. GADA, IA-2A and ZnT8A as well as TGA are determined in RBA (Grubin et al., 1994; Bonifacio et al., 2010), two-sided ELISA (Brooking et al., 2003; Amoroso et al., 2016), chemiluminescence (CLIA) (Gu et al., 2011), ECL (Miao et al., 2013; Steck et al., 2016), lateral flow immunoassays (LFIA) (Kikkas et al., 2013; Kikkas et al., 2014) or luciferase immunoprecipitation system (LIPS) (Burbelo et al., 2008; Burbelo et al., 2010). The RBA are based on labelling the autoantigen with ^{35}S-methionine by *in vitro*

transcription translation, separating antibody bound from free antigen with Sepharose Protein A (Grubin et al., 1994). A WHO standard (97/550) was developed to harmonize levels of GADA and IA-2A between laboratories (Mire-Sluis et al., 2000) and further advanced by harmonization (Bonifacio et al., 2010). Multiplex assays have been attempted by RBA either by mixing ^{35}S-methionine labelled GAD65 with ^{3}H-leucine labelled IA-2A (Kawasaki et al., 1997). A multiplex assay to include IAA is of particular challenge. In double sided ELISA, a double GADA and IA-2A test was developed (Chen et al., 2005) followed by a three-screen multiplex assay (Amoroso et al., 2016; Ziegler et al., 2016).

More recently, a non-radioactive autoantibody assay was developed for antibodies against HIV using Antibody Detection by Agglutination-PCR (ADAP) assay to detect specific virus antibodies (Tsai et al., 2018). This assay system was adapted to simultaneously detect IAA, GADA and IA-2A (Cortez et al., 2020). In recent the Islet Autoantibody Standardization Program (IASP), the ADAP achieved the highest sensitivity of GADA and IA-2A assays tested and a top-tier performance for IAA (Cortez et al., 2020). While automated systems are yet to be developed for RIA and RBA, an ultrasensitive and high-throughput automated assay based on the Hamilton Microlab ADAP STAR automated liquid-handling platform was established (Karp et al., 2020). It would be of considerable interest to expand multiple islet autoantibody analysis to include not only IAA, GADA, IA-2A and ZnT8A (both R and W at position 325) as well as TGA. In the present study, we therefore tested a novel 5-plex assay for the simultaneous detection of IAA, GADA, IA-2A and ZnT8A as biomarkers of T1D and TGA for CD. In this study, we compared the 5-plex ADAP assay with RBA in reference standards for each autoantibody as well as in 227 newly diagnosed T1D children and 272 healthy controls.

Materials and Methods

Subjects

Serum samples from controls represented 272 (114F/158M), 15-73 years of age schoolchildren and blood donors. A total of 227 (109F/118M) newly diagnosed type 1 diabetes (T1D) children, 1-11 years of age, were randomly selected from newly diagnosed T1D patients, previously analysed by RBA and RIA (for IAA) in the Better Diabetes Diagnosis study (Delli et al., 2012; Andersson et al., 2014).

Reference standards

The following RBA reference standards were used:

1. IAA reference: the reference standard used throughout the years is from an IAA high titre type 1 diabetes serum (Andersson et al., 2011; Nilsson et al., 2013; Kanatsuna et al., 2015). The IAA reference standard, included in every run, is used in a six-step, doubling dilution.
2. GADA reference: Reference standard 673 is the original serum for the WHO standard 97/550 (Mire-Sluis et al., 2000). Reference standard 622 is an in-house reference standard diluted and adjusted to 97/550. Reference standard PEO is a serum from a GADA high titre patient with Stiff Person Syndrome (Hansson et al., 2011). It shows parallel dilution to 97/550.
3. IA-2A reference: Reference standard 673 (97/550) is also used for IA-2A. In order not to deplete 673, reference standard 272, a serum from a newly diagnosed type 1 diabetes patients (male) was adjusted to parallel the doubling dilution of 673 to be used in in-house routine.
4. ZnT8A reference: The reference standard 3003 for the triple mix RBA for the three ZnT8 (ZnT8-RWQ amino acid variants at position 325) autoantibody variants (Vaziri-Sani et al., 2011) is a high-titre type 1 diabetes serum reactive with all three variants and subjected to a six-step doubling dilution.
5. TGA references: the reference standard for IgA-TGA is from a patient with CD and positive for IgA-TGA only while the another patient serum is reference standard specific for IgG-TGA.

Antigen preparation for the Antibody Detection by Agglutination-PCR (ADAP) assay

The synthesis of islet cell autoantigen conjugates for insulin, GAD65 and IA-2 were described in detail previously (Cortez et al., 2020). The DNA barcoded autoantigens were stored at 4°C for short-term usage or aliquoted for long-term storage at -80°C. The ZnT8 conjugate of equimolar concentrations of ZnT8R (arginine at position 325) and ZnT8W (tryptophan at position 325) was similarly prepared. ZnT8 and tTG proteins were purchased from Baltymas and Diarect, respectively. Installation of DNA barcodes on ZnT8 and tTG were similar to previous reports (Cortez et al., 2020) where the proteins were first activated with sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) and reacted with thiolated DNA barcodes that had been reduced with dithiothreitol (DTT). Excess DNA were removed by size-exclusion column and the DNA-barcoded autoantigen were characterized by gel-electrophoresis and UV-VIS absorption.

Antibody Determination with Agglutination by PCR (ADAP)

Previously, we have reported the ADAP method for detection of three islet autoantibodies (Cortez et al., 2020). In addition, we have described an automated Hamilton MicroLab STAR system to carry out the ADAP assay (Karp et al., 2020). Herein, we further expanded the previous work to include a total of 5 autoantibodies in the ADAP assay (IAA, GADA, IA2A, ZnT8A and TGA) and conducted the 5-plex testing on a modified version of the Hamilton MicroLab STAR to achieve full automation. Briefly, 4 µL of serum samples were incubated with 8 µL of DNA-barcoded autoantigens at 37°C for 30 min. If present in the specimens, autoantibodies would agglutinate the autoantigens into a dense immune-complex. Then, 4µL of mixtures were aspired and mixed with 116 µL of ligation mixtures, where nearby DNA in the dense immunecomplex would be ligated to form a full-length DNA amplicon. Next, 25 µL of mixture above were further mixed with 25 µL of PCR amplification mixtures containing primers for all 5 autoantibodies for a total of 13 PCR cycles using On Deck Thermocycler (ODTC, Inheco, Martinsried, Germany). The amplified products were then aspired to 384 well plates that each well contained the cognate primer pairs for each autoantibodies to achieve specific quantification on a real-time quantitative PCR (RT-qPCR). In this study, the qPCR ready plates were transferred automatically to the Roche Lightcycler 480 System II (Roche Diagnostics International AG, Rotkreuz, Switzerland) to enable a full sample-to-answer solution.

IAA radioimmunoassay

A two-step approach was used to measure IAA by radioimmunoassay as described (Delli et al., 2012).

Noncompetitive method: serum samples (7 μL) were added to duplicate wells in a 96-well Nunc V96 MicroWell™ (Nunc A/S, Roskilde, Denmark) microplates , and 36 μL ^{125}I-insulin (PerkinElmer, Boston, MA) with an activity of 60,000 cpm/well added, then incubated for 48 hours at 4°C on a shaker. After incubation, 42 μL were transferred to MultiScreen HTS-DV Plates (Millipore AB, Solna, Sweden) containing 50 μl 40% Protein A-Sepharose which was washed three times in Antigen Buffer (Antigen Buffer (150 mmol/L NaCl, 20 mmol/L TrisHCl (pH 7.4), 0,15% v/v Polysorbate 20, 0,1 % w/v bovine serum albumin) at 4 C the day before in a Micro-Plate Strip Washer (BioTek ELx50; BioTek Instruments, Bedfordshire, U.K.). The radioactivity was measured in a beta counter (Wallac Micro Beta Trilux; PerkinElmer).

Competitive method: Positive samples for IAA were further analyzed in a competitive assay using cold insulin to displace non-specifically bound ^{125}I-insulin. Serum samples (7 μl) were added to four wells on a 96-well plate. To these wells, 36μL ^{125}I-insulin (60,000 cpm/well) was added in addition to 0.072 IU (or 2 IU/mL) unlabeled insulin (Actrapid; Novo Nordisk) added to the last two wells. The plates were incubated and examined under the same conditions as in the noncompetitive method.

IAA levels were calculated as relative in-house units and were related to positive controls. Positivity for IAA was 1.0 relative units. The competitive method was used to identify non-specific binding or false positive binding in the noncompetitive assay.

Our assays showed an intra-assay CV of 6.0% and an inter assay CV of 13.2%.

Our laboratory participated in the IASP 2018 workshop and showed a workshop sensitivity of 26% and specificity of 100%.

GADA and IA-2A radiobinding assays

Recombinant GAD65 and IA-2 were labeled with ^{35}S-methionine (GE Healthcare Life Sciences, Amersham, U.K.) by in vitro-coupled transcription and translation in the TNT SP6 coupled reticulocyte lysate system (Promega, Southampton, U.K.) (Grubin et al., 1994; Delli et al., 2012). Full-length human GAD65 cDNA was subcloned into the pTNT vector (Promega) (pThGAD65)(Hansson et al., 2011). The intracellular domain (aa 603–980) of IA-2 was used in

the IA-2ic cDNA cloned into the pSP64 Poly(A) vector (Promega)(Payton et al., 1995) as kindly made available by Michael Christie (School of Life Sciences, University of Lincoln, Lincoln, UK).

In both assays, duplicate of 2.5 µl serum samples were diluted in 60 µL of labeled antigen (about 25,000 cpm) diluted in Antigen Buffer (150 mmol/L NaCl, 20 mmol/L TrisHCl (pH 7.4), 0,15% v/v Polysorbate 20, 0,1 % w/v bovine serum albumin) in 96-well plates (Nunc V96 MicroWell™, Nunc A/S, Roskilde, Denmark). The plates were incubated at 4°C overnight on a plate shaker at 300 rpm. After incubation, 50 µL were transferred to MultiScreen HTS-DV Plates (Millipore AB, Solna, Sweden) containing 50 µL 20% Protein A-Sepharose washed three times in Antigen Buffer at 4°C the day before. The coating plates were washed six times in Washing Buffer (150 mmol/L NaCl, 20 mmol/L TrisHCl (pH 7.4) with 0,15% (v/v) Polysorbate 20) with a 405^{TM}LS Microplate Strip Washer (Biotek Instruments, Inc. Winooski, VT, USA) and then dried for 5 min before 50 µL Optiphase Super mix scintillation cocktail was added to each well and the Sepharose-bound radioactivity counted in a 1450 MicroBeta® TriLux β-counter (PerkinElmer). GADA and IA-2A levels were expressed as units per milliliter (U/mL) derived from the WHO standard 97/550 (Mire-Sluis et al., 2000).

Samples were considered positive if GAD65A levels were 35 U/mL and IA-2A levels 6 U/mL. The intra-assay CV for duplicates was 7% in the GADA and 11% in the IA-2A assay.

In the DASP 2018 workshop, our laboratory was among the top-ranking laboratories for GADA with a workshop sensitivity of 80% and specificity of 99% and the top-ranking laboratory for IA-2A with a workshop sensitivity of 60% and specificity of 99%.

ZnT8A radiobinding assays

Serum were analyzed for ZnT8 autoantibodies (ZnT8A)—arginine (ZnT8RA), tryptophan (ZnT8WA), and glutamine (ZnT8-QA)—in the radioligand binding assay described in detailed (Vaziri-Sani et al., 2011; Delli et al., 2012). The cDNA clone was donated by late John C. Hutton. Briefly, a clone with arginine was received and the other two COOH-terminal constructs of ZnT8 were prepared using a Phusion site-directed mutagenesis kit (Finnzymes Oy, Espoo, Finland). In this assay, duplicates of 5 µL serum samples diluted in 55 µL ^{35}S-methionine-labeled antigen were incubated over night at 4°C on a plate shaker at 300 rpm followed by protein A-Sepharose 4B capture (Amersham Biosciences, Uppsala, Sweden) as described above. Antibody-bound radioactivity was counted in the 1450 MicroBeta Tri-Lux

Microplate Scintillation-Luminescence Counter (PerkinElmer). Levels of autoantibodies were estimated from the 3003 reference standard.

The three ZnT8A assays showed comparable precision as intra-assay CV was 5.5% for ZnT8RA, 5.3% for ZnT8WA, and 4.9% for ZnT8QA and reproducibility as inter-assay CV was 13.8% for ZnT8RA, 6.7% for ZnT8WA, and 11.0% for ZnT8QA.

In the Islet Autoantibody Standardization Program (IASP) 2018 workshop, our laboratory showed a workshop sensitivity of 52% and specificity of 100% for ZnT8RA, 50% and 100% for ZnT8WA, and 38% and 100% for ZnT8QA, respectively.

TGA radiobinding assay

Both IgA-tissue transglutaminase (tTG) and IgG-tTG were assessed with a radioligand-binding assay as described elsewhere (Agardh et al., 2006; Björck et al., 2010). Briefly, human tTG was synthesized by in vitro transcription translation in the presence of 20 µCi ^{35}S-methionine (Perkin Elmer) as described (Grubin et al., 1994) . The IgA-tTG antigen- antibody complexes were isolated with either 10% goat anti-human IgA agarose (Sigma, St Louis, MO) or 30% Protein A-Sepharose 4B (Zymed Laboratories Inc, San Francisco, CA). The relative amount of TGA was expressed as U/mL calculated from 4 reference standards. Cut-off levels for a positive result was calculated using quantile-quantile plots from 398 healthy blood donors and set at 16 U/mL for IgA-tTG and 4 U/mL for IgG-tTG, respectively (Agardh et al., 2006; Björck et al., 2010). Borderline zones between a weakly and moderately positive value were set between 16 and 26 U/mL for IgA-tTG and at 4 to 6.5 U/mL for IgG-tTG, respectively.

Statistical analysis

PRISM (version 8.1.1) and XLSTAT software (version 2018.1) were used for data and statistical analysis. ΔCt is a logarithmic parameter. (For instance, consider a sample of ΔCt value 2 and another sample of ΔCt of 4, their amplicon quantities differ by 4 fold rather than 2 fold). Two-tailed P values with an alpha of 0.05 were used as the cut off for significance. Islet autoantibody distributions were evaluated graphically for normality using quantile-quantile (Q-Q) normality plots. The Q-Q normality plots was used to show the autoantibody distribution (y axis) against the quantiles of a standard normal distribution (x-axis). In both linear and log transformed autoantibody levels, the Q-Q plot of control subjects often advice on an accurate cut off levels to discriminate negative from positive samples.

Pearson's correlation explored the relationship between log levels of autoantibodies in the ADAP and RBA, respectively.

The Receiver Operating Characteristics (ROC) plot (Zweig and Campbell, 1993) was used as the graphical representation of all sensitivity-specificity pairs to depict the overlap between the two distributions by plotting the sensitivity vs 1-specificity for the complete range of decision thresholds. The analysis of the ROC plots was carried out in MedCalc® Software Ltd: https://www.medcalc.org/features/roccurves.php to include area under the curve (AUC) with standard error (SE) and 95% confidence interval (CI) and also comparing ADAP with RBA in the different autoantibodies (Hanley and McNeil, 1982; DeLong et al., 1988). If P is less than the conventional 5% ($P<0.05$), the conclusion is that the two compared areas are significantly different.

Youden's J statistics was used as a measure of the ROC curve (Fluss et al., 2005) and to illustrate the effectiveness of the different autoantibodies detected by ADAP and RBA.

The ROC curves generated by ADAP and RBA, respectively, were considered as correlated ROC curves and nonparametric z statistics were used to compare the two methods (DeLong et al., 1988).

Results

ADAP analysis of RBA reference standards

The multiplex ADAP automated assay was tested in coded reference standards used in the single plex RBA analyses of IAA (Supplement figure 1), GADA (Supplement figure 2), IA-2A (Supplement figure 3), ZnT8A (Supplement figure 4) and TGA (Supplement figure 5).

The RBA IAA reference standard in ADAP was superimposable to the RBA and negative in the other reference standards (Supplement figure 1). The exception was the ZnT8A reference standard, which showed IAA in both assays with a lower detection limit in the ADAP.

The analysis of GADA in the ADAP method (Supplement figure 2) resulted in next to linear reference standards of the six-step doubling dilutions for the 673 WHO standard (panel A), the 622 in-house reference standard (panel C), ZnT8A reference standard (panel E) and PEO (Panel F). The other RBA reference standards were GADA negative in both

assays. The ADAP method detected GADA at an increased lower detection limit in all three GADA positive reference standards.

IA-2A was readily detected by ADAP (Supplement figure 3) in the 673 WHO standard, superimposable on the RBA (panel A), GADA RBA reference standard 622, positive in the ADAP but negative for RBA (Panel C), ADAP parallel but with a higher detection limit than RBA in the RBA IA-2A in-house reference standard (Panel D), ADAP, but not RBA, detected IA-2A in the ZnT8A reference standard (Panel E). Both RBA and ADAP were negative in the remaining RBA reference standards.

ZnT8A detected by RBA and ADAP were superimposable (Supplement figure 4, Panel E). The triple mix RBA ZnT8A standard showed a linear relationship to the 5-fold doubling dilutions which was replicated in the ADAP method. The ADAP had a lower detection limit (Panel E). The RBA and ADAP analyses of ZnT8A were negative in the remaining RBA reference standards.

The RBA reference standards for both TGA-IgG and TGA-IgA were linear on a logarithmic scale to the 10-fold doubling dilution standard (Supplement figure 5). However, the ADAP assay deviated from linearity in both RBA TGA-IgG (Panel G) and TGA-IgA (Panel H) reference standards. The two RBA for TGA-IgG and TGA-IgA as well as the ADAP assay also detected TGA in the RBA IA-2A 272 reference standard (Panel D). Again, the ADAP TGA assay showed a binding curve indicating a much lower detection limit compared to the RBA.

ADAP analysis of T1D and healthy controls serum samples.

Coded serum samples from healthy controls and newly diagnosed type 1 diabetes patients were analyzed for each autoantibody in the 5-plex ADAP and in each individual RBA using the respective in-house reference standards and cut off levels, respectively, for IAA (Figure 1), GADA (Figure 2), IA-2A (Figure 3), ZnT8A (Figure 4) and TGA (Figure 5).

IAA

The IAA quantile-quantile (Q-Q) plots showed comparable distribution between ADAP and RBA in both patients and controls (Figure 1, panels A and B). However, the RBA tended to detect a larger number of low binding control sera and a smaller number of low binding patient sera. This was well reflected in the ROC analysis, which showed a larger AUC in the ADAP compared to the RBA ($p=0.048$;Table 1). The two ROC curves differed ($p=0.005$) and the ADAP detected few IAA in controls and identified an increased number of weakly and

moderately positive T1D samples compared to the RBA (Panel C). Assuming an effective diagnostic specificity of 97% in both assays, the diagnostic sensitivity of IAA in ADAP would be 83% compared to 40% in the RBA. Nevertheless, the two assays correlated (Spearman r 0,8354; 95% CI 0,7898-0,8718; p<0.0001) illustrating that the ADAP detected more samples scored as positive compared to the RBA. A limited number of control samples were positive in the RBA but negative in the ADAP (Panel D).

GADA

The Q-Q plot for GADA revealed in ADAP compared with RBA an increased distribution of ADAP positive samples among the T1D patients and a decreased GADA positivity in healthy controls (Figure 2, panels A and B). However, the ROC analysis revealed that there was no difference between the two methods (difference between areas was 0.000153, p=0.9858; Table 1 and Pancel C). Assuming an effective diagnostic specificity of 96% in both assays, the diagnostic sensitivity of GADA in ADAP would be 91% compared to 84% in the RBA. The two assays were well correlated (Spearman r 0,9491; 95% CI 0,9338-0,9609; p<0.0001) illustrating that the ADAP scored more samples positive for GADA than the RBA (Panel D). The RBA scored 7 control samples positive for GADA but negative in the ADAP assay (Panel D).

IA-2A

The IA-2A Q-Q plots showed that the ADAP IA-2A signal were essentially zero until about the 70th percentile of the control sera (Figure 3, panel A). The distribution of the control sera in the RBA slowly rising levels until a deviation at the 98th percentile (Panel B). In addition, the ADAP linear IA-2A levels in the patients (Panel A) differed from the RBA non-linear increase despite plotted on a log scale (Panel B). The ROC AUC values were different between the ADAP and the RBA (p=0.0004, Table 1 and Panel C). The most effective diagnostic specificity was 99% in the RBA representing a diagnostic sensitivity of 91%. Using a diagnostic specificity of 99% also in ADAP would yield a diagnostic sensitivity of 82%. The correlation between the two assays was significant (Spearman r 0,7186; 95% CI 0,6468-0,7777; p<0.0001), however, the distribution of binding levels showed that the RBA found as many as 19 samples positive while they were negative in the ADAP as compared to 7 samples positive in the ADAP but negative in the RBA (Panel D). It is also noted that the levels of samples positive in both ADAP and RBA were more heterogenous.

ZnT8A

The ZnT8A Q-Q plots showed that the ADAP ΔCt distribution remained close to zero up to the 90th percentile in the healthy controls compared to the slowly rising U/mL levels in the RBA (Figure 4, Panel A). The T1D patient sera showed ADAP ΔCt signals at about the 30th percentile compare to the 40th percentile in the RBA. The ADAP detect more controls with high ΔCt values compared to the RBA which made the ROC to differ in appearance and the AUC values to differ (Panel C). The ADAP had a lower AUC value than the RBA (p=0.0004, Table 1 and Panel C). The most effective diagnostic specificity was 97% in the RBA representing a diagnostic sensitivity of 90%. Using a diagnostic specificity of 97% also in ADAP would yield a diagnostic sensitivity of 65%. The ADAP and RBA ZnT8A correlated (Spearman's r 0,7493; 95% CI 0,6839 – 0,8027; p<0.0001). However, as can been seen the samples scored double positive showed a significant heterogeneity in levels (Panel D). Furhtermore, there was a significant number of serum samples being above cut-off in the ADAP but below in the RBA. Similarly, there were more than 20 samples above cut-off in the RBA but negative in the ADAP (Panel D).

TGA

As TGA is used to screen newly diagnosed T1D children for the risk of Celiac Disease and all samples were analyzed for both TGA detected in ADAP and in both TGA-IgG and TGA-IgA (Figure 5). It is understood that this analysis is to evaluated the frequency of TGA in newly diagnosed T1D, not as a predictive marker of CD in the general population. The Q-Q plot showed slowly increasing TGA ΔCt values in both controls and T1D patients (Figure 5, Panel A). In contrast, TGA-IgG showed increasing levels that paralleled the controls and TGA-IgA detected increasing levels in the patients before the controls. The ROC curves differed markedly comparing ADAP with both TGA-IgG (Panel B) and TGA-IgA (Panel C). The ADAP had a had higher AUC values compared to both TGA-IgG (p<0,0001) and TGA-IgA (p<0.0001) (Table 1). The most effective diagnostic specificity was 90% in the ADAP assay representing a diagnostic sensitivity of 66%. Using a diagnostic specificity of 90% also in TGA-IgG and TGA-IgA would yield a diagnostic sensitivity of 15% and 30%, respectively. The ADAP correlated to TGA-IgG (Spearman's r 0,5524; 95% CI 0,4518 – 0,6391; p<0.0001). High level ADAP and RBA TGA tended to be concordant (Panels D and E).

Conclusions

The ADAP multiplex assay for IAA, GADA and IA-2A was previously reported in 7 different cohorts with high diagnostic sensitivity, specificity as well as correlation, concordance and agreement (Cortez et al., 2020). The development of multiplex ADAP also to include ZnT8A and TGA in the present study further support the reliability and utility of the analysis approach that is based on the ADAP platform.

We first characterized the 5plex ADAP assay by analysing coded reference standards for theindividual RBA and showed acceptable linear ADAP autoantibody reference standard dilutions. In particular, the parallel reference standards for RBA and ADAP to detect IAA may in part be explained by the fact that this standard is from a T1D patient treated with insulin. Prior literature suggest that the insulin therapy can lead to epitope spreading and generate different IAA from clinical onset (Ludvigsson et al., 1988; Brooks-Worrell et al., 1999). The WHO 97/500 (Mire-Sluis et al., 2000) GADA and IA-2A reference standard in the RBA showed parallel IA-2A dilution curves while the ADAP dilution curve indicated increased detection limits suggesting an increased autoantibody complexity. Sample 622, an in-house RBA reference standard for GADA, but not for IA-2A, showed ADAP IA-2A detection. Similarly, sample 272, an in-house reference standard for IA-2A RBA showed poor IA-2A reactivity in ADAP. These observations underscore the complexity of having human sera as designated reference standards when different assay formats are used. The 673 (WHO 97/500) and 622 are patient sera obtained in large quantities after plasmapheresis was attempted to save residual beta cell function (Ludvigsson et al., 1983). These reference standards have served well on-going world-wide standardization attempts and were indeed used in the first ICA workshop (Gleichmann and Bottazzo, 1987). However, differences explained by not only the way autoantigens are prepared but also the detection of the immune complexes in RBA and ADAP should therefore be carefully evaluated before existing reference standards are employed in novel assay formats as demonstrated in the data shown in Figure 1. Further studies would be needed to identify reference standards suitable for both ADAP and RBA. ZnT8A is a unique example as subjects at risk may develop autoantibodies that are unique to any of the three amino acid residue variants, R, W and Q at position 325 (Vaziri-Sani et al., 2011; Delli et al., 2012). Indeed, the ZnT8A RBA reference standard is from a type 1 diabetes patient who had RBA autoantibodies to all three variants. As the ADAP assay showed different detection limit, care should be taken to select other patient sera for a suitable

ADAP ZnT8A reference standard. This will be of particular importance once ADAP reagents become available for all three variants in a multiplex design.

The difference between the RBA and ADAP in detecting TGA in the in-house reference sera for RBA IgG-TGA and IgA-TGA underscore the possibility of developing a highly sensitive and specific ADAP analysis for TGA. One approach will be to further dilute the present RBA standards and test for linearity on a log scale. Another approach would be to search for TGA positive sera from children with celiac disease autoimmunity (Agardh et al., 2005; Björck et al., 2010; Liu et al., 2014) to represent an equal proportion of TGA IgG and IgA. Further studies are also needed to determine to what extent the ADAP assay equally effective detect IgG and IgA immune complexes.

The data in analysis of real patient serum samples indicated differences between ADAP and RBA in the ability to detect IAA, GADA, IA-2A, ZnT8A and TGA, consistent with the differences observed using RBA reference standards. The ROC AUC analysis that did not differ between ADAP and RBA for GADA and IAA suggests that the two assay formats are comparable for these autoantibodies. However, as previously discussed (Cortez et al., 2020), it will be important to validate the ADAP assay in detecting islet autoimmunity prior to the clinical onset of type 1 diabetes. The first appearing islet autoantibody in longitudinal follow-up such as the TEDDY (Krischer et al., 2015; Vehik et al., 2020; Bonifacio et al., 2021), DIPP (Ilonen et al., 2013; Pöllänen et al., 2020) and the T1DI consortium (Anand et al., 2021) underscore the importance of sensitive IAA and GADA assays requiring the sample sparing multiplex ADAP assay. A reliable sensitive and specific assay is of particular importance for IAA, the incidence of which as the first appearing autoantibody is at 1-3 years of age.

The significant number of sera with low level positive for ADAP but negative for RBA from the T1D patient is of interest. Additionally, as the Q-Q plot analyses of GADA indicate both ADAP and RBA detected GADA in some serum samples from healthy individuals. It is possible that ADAP has a slightly lower detection of GADA in the healthy individuals. The ADAP assay therefore offer a unique opportunity to determine if these sera represent, for example, IgG3 subtype autoantibodies. It may be possible to determine IgG3-GADA or even IgG3-IA-2A as well as IgG3-ZnT8A, by preabsorbing the samples to protein A-Sepharose which does not bind IgG3.

The significantly better performance of RBA compared to ADAP to detect IA-2A is posing a particular challenge as the correlation between the two assays were lower for this

autoantibody. In contrast to ADAP IAA and ADAP GADA that show more samples positive in ADAP and negative in RBA, the opposite was true for RBA IA-2A. Above the respective assay cut-offs, the two assays correlated statistically, but inspecting the spread of the individual data in Figure 5b suggest an unexpected IA-2A heterogeneity in the T1D patient sera. This observation is of particular interest as the appearance of IA-2A as a second autoantibody seem to increase the risk for progression to T1D (Lundgren et al., 2015; Steck et al., 2015; Lundgren et al., 2017; Vehik et al., 2020). The IA-2A assay remains the one autoantibody assay where the RBA, but not the ADAP, detected high level IA-2A. It may indicate that some IA-2 -immunoglobulin complexes are not amendable to agglutination for ADAP detection. Alternatively, the two assays may be detecting distinct epitopes for IA-2A.

In contrast to IA-2A but similar to GADA, the ADAP method readily detected ZnT8A. The ROC AUC analysis showed better performance by RBA. This is important as recent data suggest that ZnT8A rarely is observed as the first appearing autoantibody but that in children with multiple autoantibodies the frequency and level of ZnT8A was associated with impaired glucose metabolism (Andersson et al., 2013).

The ADAP TGA assay showed markedly improved analytical sensitivity when analysing the IgA and IgG TGA RBA reference standards. This poses an unique opportunity to further develop highly sensitive TGA assay to detect celiac disease (CD) related autoantibodies at challenging sample matrices such as saliva, which usually has much lower autoantibody levels compared to serum samples.

Taken together, the 5-plex ADAP assay for the simultaneous detection of IAA, GADA, IA-2A, ZnT8A and TGA show comparable performance in autoantibody sensitivity and specificity at the time of clinical diagnosis of childhood T1D. The diagnostic sensitivity and specificity of IAA, GADA and ZnT8A, but not of IA-2A, was increased in ADAP compared to RBA. ADAP has the ability to detect additional positive samples. The clinical meaning of such enhanced sensitivity remains to be investigated when ADAP is subjected to longitudinal samples from individuals at increased genetic risk for T1D and followed from birth. The multiplex sample sparing autoantibody assay has the strength of simultaneous detection of four islet autoantibodies and of TGA. The latter biomarker indicates the risk for celiac disease autoimmunity as well as celiac disease which affects about 10% of subjects with type 1 diabetes.

Acknowledgements

The authors thank Charlotte Brundin and Ida Jönsson for expert technical assistance.

Funding

This work was supported by The Leona M. and Harry B. Helmsley Charitable Trust (grant number 2009-04078). AL was supported by the DiaUnion collaborative study, co-financed by EU Interreg ÖKS, Capital Region of Denmark, Region Skåne and the Novo Nordisk Foundation.

Competing interest

FJC, DG, DT, PVR, DS and CTT are employed by Enable Biosciences. FJC, DG, DT, PVR, DS and CTT are shareholders of Enable Biosciences. PVR and CTT are inventors of the ADAP patent licensed from University of California, Berkeley to Enable Biosciences. This does not alter our adherence to journal policies on sharing data and materials.

References

Agardh, D., Dahlbom, I., Daniels, T., Lörinc, E., Ivarsson, S.A., Lernmark, A. and Hansson, T., 2005, Autoantibodies against soluble and immobilized human recombinant tissue transglutaminase in children with celiac disease. J Pediatr Gastroenterol Nutr 41, 322-7.

Agardh, D., Lee, H.S., Kurppa, K., Simell, V., Aronsson, C.A., Jörneus, O., Hummel, M., Liu, E., Koletzko, S. and Group, T.S., 2015, Clinical features of celiac disease: a prospective birth cohort. Pediatrics 135, 627-34.

Agardh, D., Lynch, K., Brundin, C., Ivarsson, S.A., Lernmark, A. and Cilio, C.M., 2006, Reduction of tissue transglutaminase autoantibody levels by gluten-free diet is associated with changes in subsets of peripheral blood lymphocytes in children with newly diagnosed coeliac disease. Clin Exp Immunol 144, 67-75.

Amoroso, M., Achenbach, P., Powell, M., Coles, R., Chlebowska, M., Carr, L., Furmaniak, J., Scholz, M., Bonifacio, E., Ziegler, A.G. and Rees Smith, B., 2016, 3 Screen islet cell autoantibody ELISA: A sensitive and specific ELISA for the combined measurement of autoantibodies to GAD. Clin Chim Acta 462, 60-64.

Anand, V., Li, Y., Liu, B., Ghalwash, M., Koski, E., Ng, K., Dunne, J.L., Jönsson, J., Winkler, C., Knip, M., Toppari, J., Ilonen, J., Killian, M.B., Frohnert, B.I., Lundgren, M., Ziegler, A.G., Hagopian, W., Veijola, R., Rewers, M. and Group, T.D.S., 2021, Islet Autoimmunity and HLA Markers of Presymptomatic and Clinical Type 1 Diabetes: Joint Analyses of Prospective Cohort Studies in Finland, Germany, Sweden, and the U.S. Diabetes Care.

Andersson, C., Carlsson, A., Cilio, C., Cedervall, E., Ivarsson, S.A., Jonsdottir, B., Jönsson, B., Larsson, K., Neiderud, J., Lernmark, A., Elding Larsson, H. and Group, D.-I.S., 2013,

Glucose tolerance and beta-cell function in islet autoantibody-positive children recruited to a secondary prevention study. Pediatr Diabetes 14, 341-9.
Andersson, C., Kolmodin, M., Ivarsson, S.A., Carlsson, A., Forsander, G., Lindblad, B., Ludvigsson, J., Kockum, I., Marcus, C., Samuelsson, U., Ortqvist, E., Lernmark, A., Elding Larsson, H., Törn, C. and Group, B.D.D.S., 2014, Islet cell antibodies (ICA) identify autoimmunity in children with new onset diabetes mellitus negative for other islet cell antibodies. Pediatr Diabetes 15, 336-44.
Andersson, C., Larsson, K., Vaziri-Sani, F., Lynch, K., Carlsson, A., Cedervall, E., Jönsson, B., Neiderud, J., Månsson, M., Nilsson, A., Lernmark, A., Elding Larsson, H. and Ivarsson, S.A., 2011, The three ZNT8 autoantibody variants together improve the diagnostic sensitivity of childhood and adolescent type 1 diabetes. Autoimmunity 44, 394-405.
Andrén Aronsson, C., Lee, H.S., Koletzko, S., Uusitalo, U., Yang, J., Virtanen, S.M., Liu, E., Lernmark, Å., Norris, J.M., Agardh, D. and Group, T.S., 2016, Effects of Gluten Intake on Risk of Celiac Disease: A Case-Control Study on a Swedish Birth Cohort. Clin Gastroenterol Hepatol 14, 403-409.e3.
Bingley, P.J., Bonifacio, E. and Mueller, P.W., 2003, Diabetes Antibody Standardization Program: first assay proficiency evaluation. Diabetes 52, 1128-36.
Björck, S., Brundin, C., Lörinc, E., Lynch, K.F. and Agardh, D., 2010, Screening detects a high proportion of celiac disease in young HLA-genotyped children. J Pediatr Gastroenterol Nutr 50, 49-53.
Bonifacio, E., Weiß, A., Winkler, C., Hippich, M., Rewers, M.J., Toppari, J., Lernmark, Å., She, J.X., Hagopian, W.A., Krischer, J.P., Vehik, K., Schatz, D.A., Akolkar, B., Ziegler, A.G. and Group, T.S., 2021, An Age-Related Exponential Decline in the Risk of Multiple Islet Autoantibody Seroconversion During Childhood. Diabetes Care.
Bonifacio, E., Yu, L., Williams, A.K., Eisenbarth, G.S., Bingley, P.J., Marcovina, S.M., Adler, K., Ziegler, A.G., Mueller, P.W., Schatz, D.A., Krischer, J.P., Steffes, M.W. and Akolkar, B., 2010, Harmonization of glutamic acid decarboxylase and islet antigen-2 autoantibody assays for national institute of diabetes and digestive and kidney diseases consortia. J Clin Endocrinol Metab 95, 3360-7.
Brooking, H., Ananieva-Jordanova, R., Arnold, C., Amoroso, M., Powell, M., Betterle, C., Zanchetta, R., Furmaniak, J. and Smith, B.R., 2003, A sensitive non-isotopic assay for GAD65 autoantibodies. Clin Chim Acta 331, 55-9.
Brooks-Worrell, B.M., Nielson, D. and Palmer, J.P., 1999, Insulin autoantibodies and insulin antibodies have similar binding characteristics. Proc Assoc Am Physicians 111, 92-6.
Burbelo, P.D., Hirai, H., Issa, A.T., Kingman, A., Lernmark, A., Ivarsson, S.A., Notkins, A.L. and Iadarola, M.J., 2010, Comparison of radioimmunoprecipitation with luciferase immunoprecipitation for autoantibodies to GAD65 and IA-2beta. Diabetes Care 33, 754-6.
Burbelo, P.D., Hirai, H., Leahy, H., Lernmark, A., Ivarsson, S.A., Iadarola, M.J. and Notkins, A.L., 2008, A new luminescence assay for autoantibodies to mammalian cell-prepared insulinoma-associated protein 2. Diabetes Care 31, 1824-6.
Carlsson, A., Shepherd, M., Ellard, S., Weedon, M., Lernmark, Å., Forsander, G., Colclough, K., Brahimi, Q., Valtonen-Andre, C., Ivarsson, S.A., Elding Larsson, H., Samuelsson, U., Örtqvist, E., Groop, L., Ludvigsson, J., Marcus, C. and Hattersley, A.T., 2020, Absence of Islet Autoantibodies and Modestly Raised Glucose Values at Diabetes Diagnosis Should Lead to Testing for MODY: Lessons From a 5-Year Pediatric Swedish National Cohort Study. Diabetes Care 43, 82-89.

Cerqueiro Bybrant, M., Grahnquist, L., Örtqvist, E., Andersson, C., Forsander, G., Elding Larsson, H., Lernmark, Å., Ludvigsson, J., Marcus, C., Carlsson, A. and Ivarsson, S.A., 2018, Tissue transglutaminase autoantibodies in children with newly diagnosed type 1 diabetes are related to human leukocyte antigen but not to islet autoantibodies: A Swedish nationwide prospective population-based cohort study. Autoimmunity 51, 221-227.

Chen, S., Willis, J., Maclean, C., Ananieva-Jordanova, R., Amoroso, M.A., Brooking, H., Powell, M., Collins, A., Bennett, S., Mitchell, S., Burne, P., Furmaniak, J. and Smith, B.R., 2005, Sensitive non-isotopic assays for autoantibodies to IA-2 and to a combination of both IA-2 and GAD65. Clin Chim Acta 357, 74-83.

Cortez, F.J., Gebhart, D., Robinson, P.V., Seftel, D., Pourmandi, N., Owyoung, J., Bertozzi, C.R., Wilson, D.M., Maahs, D.M., Buckingham, B.A., Mills, J.R., Roforth, M.M., Pittock, S.J., McKeon, A., Page, K., Wolf, W.A., Sanda, S., Speake, C., Greenbaum, C.J. and Tsai, C.T., 2020, Sensitive detection of multiple islet autoantibodies in type 1 diabetes using small sample volumes by agglutination-PCR. PLoS One 15, e0242049.

Delli, A.J., Vaziri-Sani, F., Lindblad, B., Elding-Larsson, H., Carlsson, A., Forsander, G., Ivarsson, S.A., Ludvigsson, J., Kockum, I., Marcus, C., Samuelsson, U., Örtqvist, E., Groop, L., Bondinas, G.P., Papadopoulos, G.K., Lernmark, Å. and Group, B.D.D.S., 2012, Zinc transporter 8 autoantibodies and their association with SLC30A8 and HLA-DQ genes differ between immigrant and Swedish patients with newly diagnosed type 1 diabetes in the Better Diabetes Diagnosis study. Diabetes 61, 2556-64.

DeLong, E.R., DeLong, D.M. and Clarke-Pearson, D.L., 1988, Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 44, 837-45.

Fluss, R., Faraggi, D. and Reiser, B., 2005, Estimation of the Youden Index and its associated cutoff point. Biom J 47, 458-72.

Gleichmann, H. and Bottazzo, G.F., 1987, Progress toward standardization of cytoplasmic islet cell-antibody assay. Diabetes 36, 578-84.

Grubin, C.E., Daniels, T., Toivola, B., Landin-Olsson, M., Hagopian, W.A., Li, L., Karlsen, A.E., Boel, E., Michelsen, B. and Lernmark, A., 1994, A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. Diabetologia 37, 344-50.

Gu, Y., Zhang, M., Chen, H., Wang, Z., Xing, C., Yang, H., Xu, X., Liu, Y., Zhou, Z., Yu, L., Hutton, J., Eisenbarth, G. and Yang, T., 2011, Discordant association of islet autoantibodies with high-risk HLA genes in Chinese type 1 diabetes. Diabetes Metab Res Rev 27, 899-905.

Gyllenberg, A., Asad, S., Piehl, F., Swanberg, M., Padyukov, L., Van Yserloo, B., Rutledge, E.A., McNeney, B., Graham, J., Orho-Melander, M., Lindholm, E., Graff, C., Forsell, C., Akesson, K., Landin-Olsson, M., Carlsson, A., Forsander, G., Ivarsson, S.A., Larsson, H., Lindblad, B., Ludvigsson, J., Marcus, C., Lernmark, A., Alfredsson, L., Olsson, T., Kockum, I., Group, S.C.D.S., Group, D.I.i.S.S. and group, B.D.D.S., 2012, Age-dependent variation of genotypes in MHC II transactivator gene (CIITA) in controls and association to type 1 diabetes. Genes Immun 13, 632-40.

Hagopian, W., Lee, H.S., Liu, E., Rewers, M., She, J.X., Ziegler, A.G., Lernmark, Å., Toppari, J., Rich, S.S., Krischer, J.P., Erlich, H., Akolkar, B., Agardh, D. and Group, T.S., 2017, Co-occurrence of Type 1 Diabetes and Celiac Disease Autoimmunity. Pediatrics 140.

Hanley, J.A. and McNeil, B.J., 1982, The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 143, 29-36.

Hansson, I., Lynch, K.F., Hallmans, G., Lernmark, Å. and Rolandsson, O., 2011, High-titer GAD65 autoantibodies detected in adult diabetes patients using a high efficiency expression vector and cold GAD65 displacement. Autoimmunity 44, 129-36.

Ilonen, J., Hammais, A., Laine, A.P., Lempainen, J., Vaarala, O., Veijola, R., Simell, O. and Knip, M., 2013, Patterns of β-cell autoantibody appearance and genetic associations during the first years of life. Diabetes 62, 3636-40.

Insel, R.A., Dunne, J.L., Atkinson, M.A., Chiang, J.L., Dabelea, D., Gottlieb, P.A., Greenbaum, C.J., Herold, K.C., Krischer, J.P., Lernmark, Å., Ratner, R.E., Rewers, M.J., Schatz, D.A., Skyler, J.S., Sosenko, J.M. and Ziegler, A.G., 2015, Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association. Diabetes Care 38, 1964-74.

Kanatsuna, N., Delli, A., Andersson, C., Nilsson, A.L., Vaziri-Sani, F., Larsson, K., Carlsson, A., Cedervall, E., Jönsson, B., Neiderud, J., Elding Larsson, H., Ivarsson, S.A., Törn, C., Fex, M. and Lernmark, Å., 2015, Doubly Reactive INS-IGF2 Autoantibodies in Children with Newly Diagnosed Autoimmune (type 1) Diabetes. Scand J Immunol 82, 361-9.

Karp, D.G., Cuda, D., Tandel, D., Danh, K., Robinson, P.V., Seftel, D., Tian, H., Pandori, M., Miller, K.W.P. and Tsai, C.T., 2020, Sensitive and Specific Detection of SARS-CoV-2 Antibodies Using a High-Throughput, Fully Automated Liquid-Handling Robotic System. SLAS Technol 25, 545-552.

Kawasaki, E., Yu, L., Gianani, R., Verge, C.F., Babu, S., Bonifacio, E. and Eisenbarth, G.S., 1997, Evaluation of islet cell antigen (ICA) 512/IA-2 autoantibody radioassays using overlapping ICA512/IA-2 constructs. J Clin Endocrinol Metab 82, 375-80.

Kikkas, I., Mallone, R., Larger, E., Volland, H. and Morel, N., 2014, A rapid lateral flow immunoassay for the detection of tyrosine phosphatase-like protein IA-2 autoantibodies in human serum. PLoS One 9, e103088.

Kikkas, I., Mallone, R., Tubiana-Rufi, N., Chevenne, D., Carel, J.C., Créminon, C., Volland, H., Boitard, C. and Morel, N., 2013, A simple and fast non-radioactive bridging immunoassay for insulin autoantibodies. PLoS One 8, e69021.

Krischer, J.P., Lynch, K.F., Schatz, D.A., Ilonen, J., Lernmark, Å., Hagopian, W.A., Rewers, M.J., She, J.X., Simell, O.G., Toppari, J., Ziegler, A.G., Akolkar, B., Bonifacio, E. and Group, T.S., 2015, The 6 year incidence of diabetes-associated autoantibodies in genetically at-risk children: the TEDDY study. Diabetologia 58, 980-7.

Kurppa, K., Laitinen, A. and Agardh, D., 2018, Coeliac disease in children with type 1 diabetes. Lancet Child Adolesc Health 2, 133-143.

Larsson, K., Carlsson, A., Cederwall, E., Jönsson, B., Neiderud, J., Jonsson, B., Lernmark, A., Ivarsson, S.A. and Group, S.S., 2008, Annual screening detects celiac disease in children with type 1 diabetes. Pediatr Diabetes 9, 354-9.

Liu, E., Lee, H.S., Aronsson, C.A., Hagopian, W.A., Koletzko, S., Rewers, M.J., Eisenbarth, G.S., Bingley, P.J., Bonifacio, E., Simell, V., Agardh, D. and Group, T.S., 2014, Risk of pediatric celiac disease according to HLA haplotype and country. N Engl J Med 371, 42-9.

Ludvigsson, J., Binder, C. and Mandrup-Poulsen, T., 1988, Insulin autoantibodies are associated with islet cell antibodies; their relation to insulin antibodies and B-cell function in diabetic children. Diabetologia 31, 647-51.

Ludvigsson, J., Heding, L., Liedén, G., Marner, B. and Lernmark, A., 1983, Plasmapheresis in the initial treatment of insulin-dependent diabetes mellitus in children. Br Med J (Clin Res Ed) 286, 176-8.

Lundgren, M., Lynch, K., Larsson, C., Elding Larsson, H. and group, D.P.i.S.s., 2015, Cord blood insulinoma-associated protein 2 autoantibodies are associated with increased risk of type 1 diabetes in the population-based Diabetes Prediction in Skåne study. Diabetologia 58, 75-8.

Lundgren, M., Steed, L.J., Tamura, R., Jonsdottir, B., Gesualdo, P., Crouch, C., Sjöberg, M., Hansson, G., Hagopian, W.A., Ziegler, A.G., Rewers, M.J., Lernmark, Å., Toppari, J., She, J.X., Akolkar, B., Krischer, J.P., Haller, M.J., Elding Larsson, H. and Group, T.S., 2017, Analgesic antipyretic use among young children in the TEDDY study: no association with islet autoimmunity. BMC Pediatr 17, 127.

Maglio, M. and Troncone, R., 2020, Intestinal Anti-tissue Transglutaminase2 Autoantibodies: Pathogenic and Clinical Implications for Celiac Disease. Front Nutr 7, 73.

Miao, D., Guyer, K.M., Dong, F., Jiang, L., Steck, A.K., Rewers, M., Eisenbarth, G.S. and Yu, L., 2013, GAD65 autoantibodies detected by electrochemiluminescence assay identify high risk for type 1 diabetes. Diabetes 62, 4174-8.

Mire-Sluis, A.R., Gaines Das, R. and Lernmark, A., 2000, The World Health Organization International Collaborative Study for islet cell antibodies. Diabetologia 43, 1282-92.

Montén, C., Bjelkenkrantz, K., Gudjonsdottir, A.H., Browaldh, L., Arnell, H., Naluai, Å. and Agardh, D., 2016, Validity of histology for the diagnosis of paediatric coeliac disease: a Swedish multicentre study. Scand J Gastroenterol 51, 427-33.

Nilsson, A.L., Vaziri-Sani, F., Andersson, C., Larsson, K., Carlsson, A., Cedervall, E., Jönsson, B., Neiderud, J., Elding Larsson, H., Ivarsson, S.A. and Lernmark, Å., 2013, Relationship between Ljungan virus antibodies, HLA-DQ8, and insulin autoantibodies in newly diagnosed type 1 diabetes children. Viral Immunol 26, 207-15.

Payton, M.A., Hawkes, C.J. and Christie, M.R., 1995, Relationship of the 37,000- and 40,000-M(r) tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512). J Clin Invest 96, 1506-11.

Pöllänen, P.M., Ryhänen, S.J., Toppari, J., Ilonen, J., Vähäsalo, P., Veijola, R., Siljander, H. and Knip, M., 2020, Dynamics of Islet Autoantibodies During Prospective Follow-Up From Birth to Age 15 Years. J Clin Endocrinol Metab 105.

Schlosser, M., Mueller, P.W., Törn, C., Bonifacio, E., Bingley, P.J. and Laboratories, P., 2010, Diabetes Antibody Standardization Program: evaluation of assays for insulin autoantibodies. Diabetologia 53, 2611-20.

Steck, A.K., Fouts, A., Miao, D., Zhao, Z., Dong, F., Sosenko, J., Gottlieb, P., Rewers, M.J., Yu, L. and Group, T.S., 2016, ECL-IAA and ECL-GADA Can Identify High-Risk Single Autoantibody-Positive Relatives in the TrialNet Pathway to Prevention Study. Diabetes Technol Ther 18, 410-4.

Steck, A.K., Vehik, K., Bonifacio, E., Lernmark, A., Ziegler, A.G., Hagopian, W.A., She, J., Simell, O., Akolkar, B., Krischer, J., Schatz, D., Rewers, M.J. and Group, T.S., 2015, Predictors of Progression From the Appearance of Islet Autoantibodies to Early Childhood Diabetes: The Environmental Determinants of Diabetes in the Young (TEDDY). Diabetes Care 38, 808-13.

Tsai, C.T., Robinson, P.V., Cortez, F.J., Elma, M.L.B., Seftel, D., Pourmandi, N., Pandori, M.W. and Bertozzi, C.R., 2018, Antibody detection by agglutination-PCR (ADAP) enables

early diagnosis of HIV infection by oral fluid analysis. Proc Natl Acad Sci U S A 115, 1250-1255.

Vaziri-Sani, F., Delli, A.J., Elding-Larsson, H., Lindblad, B., Carlsson, A., Forsander, G., Ivarsson, S.A., Ludvigsson, J., Marcus, C. and Lernmark, A., 2011, A novel triple mix radiobinding assay for the three ZnT8 (ZnT8-RWQ) autoantibody variants in children with newly diagnosed diabetes. J Immunol Methods 371, 25-37.

Vehik, K., Bonifacio, E., Lernmark, Å., Yu, L., Williams, A., Schatz, D., Rewers, M., She, J.X., Toppari, J., Hagopian, W., Akolkar, B., Ziegler, A.G., Krischer, J.P. and Group, T.S., 2020, Hierarchical Order of Distinct Autoantibody Spreading and Progression to Type 1 Diabetes in the TEDDY Study. Diabetes Care 43, 2066-2073.

Williams, A.J., Bingley, P.J., Bonifacio, E., Palmer, J.P. and Gale, E.A., 1997, A novel micro-assay for insulin autoantibodies. J Autoimmun 10, 473-8.

Williams, A.J., Norcross, A.J., Chandler, K.A. and Bingley, P.J., 2006, Non-specific binding to protein A Sepharose and protein G Sepharose in insulin autoantibody assays may be reduced by pre-treatment with glycine or ethanolamine. J Immunol Methods 314, 170-3.

Ziegler, A.G., Haupt, F., Scholz, M., Weininger, K., Wittich, S., Löbner, S., Matzke, C., Gezginci, C., Riethausen, S., Beyerlein, A., Zillmer, S., Amoroso, M., Coles, R., Powell, M., Furmaniak, J., Smith, B.R., Winkler, C., Bonifacio, E. and Achenbach, P., 2016, 3 Screen ELISA for High-Throughput Detection of Beta Cell Autoantibodies in Capillary Blood. Diabetes Technol Ther 18, 687-693.

Ziegler, A.G., Kick, K., Bonifacio, E., Haupt, F., Hippich, M., Dunstheimer, D., Lang, M., Laub, O., Warncke, K., Lange, K., Assfalg, R., Jolink, M., Winkler, C., Achenbach, P. and Group, F.d.S., 2020, Yield of a Public Health Screening of Children for Islet Autoantibodies in Bavaria, Germany. JAMA 323, 339-351.

Ziegler, A.G., Rewers, M., Simell, O., Simell, T., Lempainen, J., Steck, A., Winkler, C., Ilonen, J., Veijola, R., Knip, M., Bonifacio, E. and Eisenbarth, G.S., 2013, Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA 309, 2473-9.

Zweig, M.H. and Campbell, G., 1993, Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 39, 561-77.

Table 1 Receivers Operating Characteristics and correlations between RBA and ADAP.

	RBA	**ADAP**	**Difference**
Controls	273	273	
Type 1 diabetes patients	227	227	
IAA			
ROC AUC	0,885	0,934	0,0485
95% confidence interval	0,854-0,912	0,909-0,954	0,0147-0,0823
Z statistics	23,500	36,317	2,810
Significance level (area 0.5)	<0.0001	<0.0001	P=0,0050
Youden index J	0.7080	0,7989	
Diagnostic Sensitivity	40%	83%	
Diagnostic Specificity	97%	97%	
GADA			
ROC AUC	0,963	0,963	0,000153
95% confidence interval	0,943-0,978	0,943-0,978	-0,0167-0,0170
Z statistics	55,372	49,283	0,0178
Significance level (area 0,5)	<0.0001	<0,0001	P=0,9858
Youden index J	0,8651	0,8672	
Diagnostic Sensitivity	84%	91%	
Diagnostic Specificity	96%	96%	
IA-2A			
ROC AUC	0,986	0,955	0,0319
95% confidence interval	0,972-0,995	0,932-0,971	0,0141-0,0496
Z statistics	95,509	51,874	3,512
Significance level (area 0,5)	<0.0001	<0.0001	P=0.0004
Youden index J	0,9185	0,7791	
Diagnostic Sensitivity	91%	82%	
Diagnostic Specificity	99%	99%	
ZnT8A			
ROC AUC	0,974	0,892	0,0827
95% confidence interval	0,956-0,986	0,861-0,918	0,0524-0,113
Z statistics	61,095	26,376	5,353
Significance level (area 0,5)	<0,0001	<0,0001	P<0.0001
Youden index	0,8727	0,6456	
Diagnostic Sensitivity	90%	65%	
Diagnostic Specificity	97%	97%	
TGA - IgG			
ROC AUC	0,608	0,816	0,208
95% confidence interval	0,563-0,651	0,779 -0,849	0,158-0,258
Z statstics	4,256	15,452	8,164
Significance level (area 0,5)	P<0,0001	<0,0001	P<0,0001
Youden index	0,1890	0,5575	
Diagnostic Sensitivity	10%	66%	
Diagnostic Specificity	90%	90%	

TGA-IgA			
ROC AUC	0,622	0,816	0,194
95% confidence interval	0,578-0,664	0,779 -0,849	0,149-0,239
Z statstics	6,608	15,452	8,446
Significance level (area 0,5)	P<0.0001	<0,0001	P<0,0001
Youden index	0,2249	0,5575	
Diagnostic Sensitivity	30%	62%	
Diagnostic Specificity	90%	90%	

Figure legends.

Figure 1. IAA analyzed in ADAP (ΔCt) and RBA (U/mL), respectively, in newly diagnosed type 1 diabetes children (n=227)(black symbols) and healthy controls (n=273)(grey symbols). The Q-Q plots reveal lover detection level of patient sera in ADAP compared to RBA. The dotted lines are the cut offs in the respective local reference standards (panel a). The correlation between ADAP and RBA with dotted line cut offs, reveal zones where both ADAP and RBA score negative or one of the assays, but not the other, would score positive samples (panel b). The ROC curves for ADAP (solid blue line) and RBA (green dotted line) illustrate differences between the two assays as summarized in Table 1 (panel c).

Figure 2. GADA analyzed in ADAP (ΔCt) and RBA (U/mL), respectively, in newly diagnosed type 1 diabetes children (n=227)(black symbols) and healthy controls (n=273)(grey symbols). The Q-Q plots reveal lover detection level of patient sera in ADAP compared to RBA. The dotted lines are the cut offs in the respective local reference standards (panel a). The correlation between ADAP and RBA with dotted line cut offs, reveal zones where both ADAP and RBA score negative or one but not the other would score positive samples (panel b). The ROC curves for ADAP (solid blue line) and RBA (green dotted line) illustrate differences between the two assays as summarized in Table 1 (panel c).

Figure 3. IA-2A analyzed in ADAP (ΔCt) and RBA (U/mL), respectively, in newly diagnosed type 1 diabetes children (n=227)(black symbols) and healthy controls (n=273)(grey symbols). The Q-Q plots reveal lover detection level of patient sera in ADAP compared to RBA. The dotted lines are the cut offs in the respective local reference standards (panel a). The correlation between ADAP and RBA with dotted line cut offs, reveal zones where both ADAP and RBA score negative or one but not the other would score positive samples (panel b). The ROC curves for ADAP (solid blue line) and RBA (green dotted line) illustrate differences between the two assays as summarized in Table 1 (panel c).

Figure 4. ZnT8A analyzed in ADAP (ΔCt) and RBA (U/mL), respectively, in newly diagnosed type 1 diabetes children (n=227)(black symbols) and healthy controls (n=273)(grey symbols). The Q-Q plots reveal lover detection level of patient sera in ADAP compared to RBA. The dotted lines are the cut offs in the respective local reference standards (panel a).

The correlation between ADAP and RBA with dotted line cut offs, reveal zones where both ADAP and RBA score negative or one but not the other would score positive samples (panel b). The ROC curves for ADAP (solid blue line) and RBA (green dotted line) illustrate differences between the two assays as summarized in Table 1 (panel c).

Figure 5. IgG-TGA and IgA-TGA analyzed in ADAP (ΔCt) and RBA (U/mL), respectively, in newly diagnosed type 1 diabetes children (n=227)(black symbols) and healthy controls (n=273)(grey symbols). The Q-Q plots reveal lover detection level of patient sera in ADAP compared to RBA. The dotted lines are the cut offs in the respective local reference standards (panel A). The correlation between ADAP and RBA with dotted line cut offs, reveal zones where both ADAP and RBA score negative or one but not the other would score positive samples (panel B). The ROC curves for ADAP (solid blue line) and RBA (green dotted line) illustrate differences between the two assays as summarized in Table 1 (Panels C, D and E).

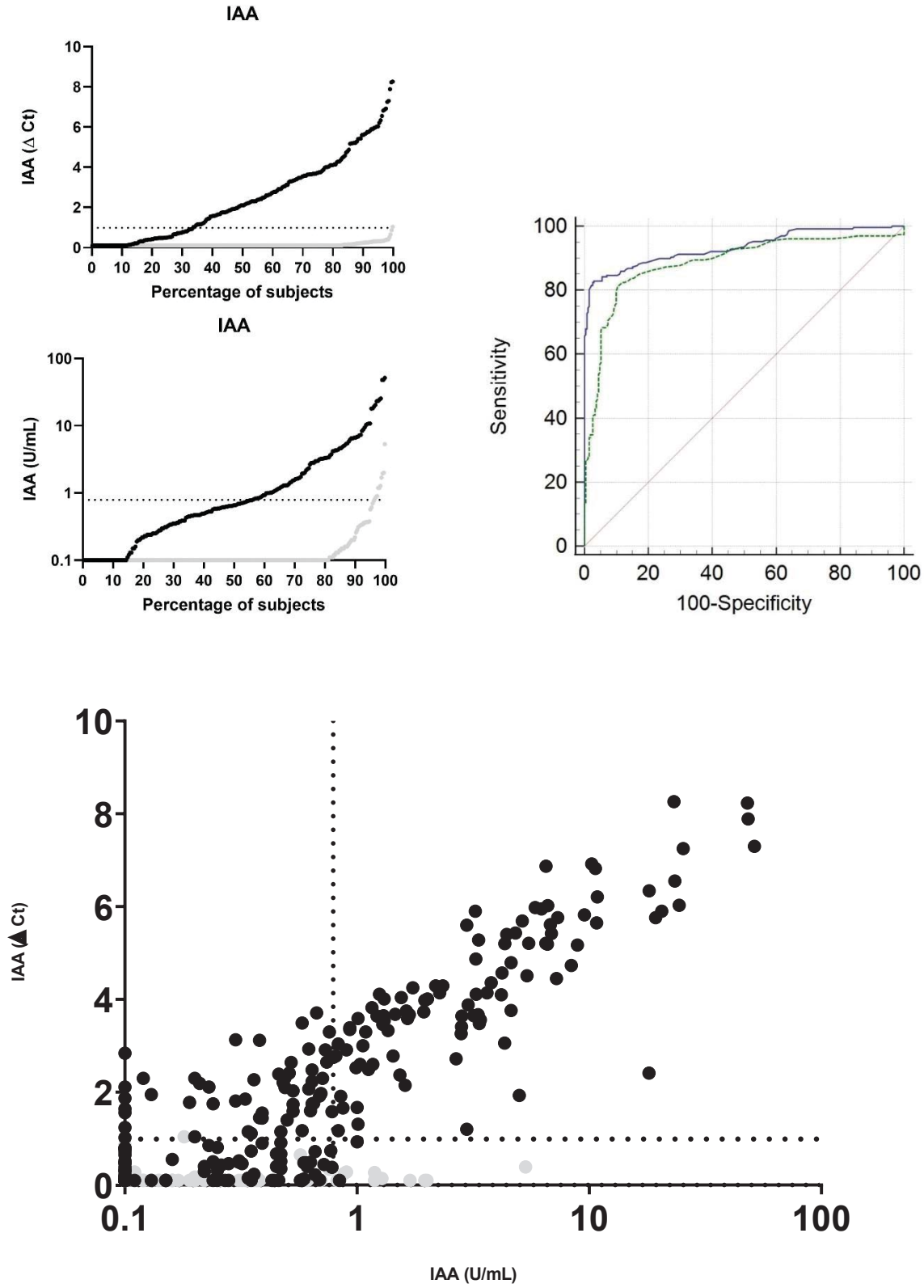

IAA
IAA (Δ Ct)
Percentage of subjects
IAA
IAA (U/mL)
Percentage of subjects
Sensitivity
100-Specificity
IAA (Δ Ct)
IAA (U/mL)

Figure 1

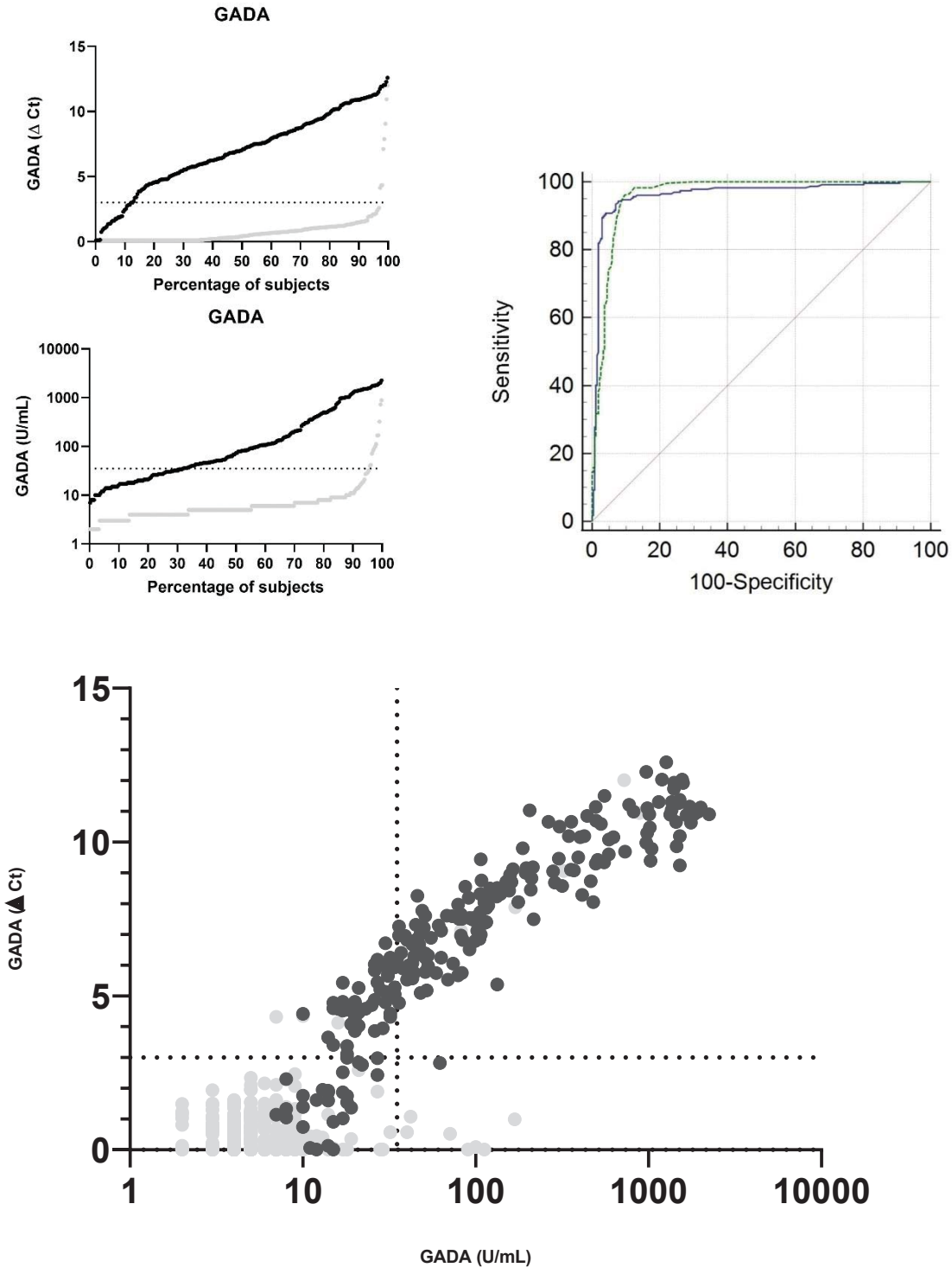

GADA
GADA (Δ Ct)
Percentage of subjects
GADA
GADA (U/mL)
Percentage of subjects
Sensitivity
100-Specificity
GADA (Δ Ct)
GADA (U/mL)

Figure 2

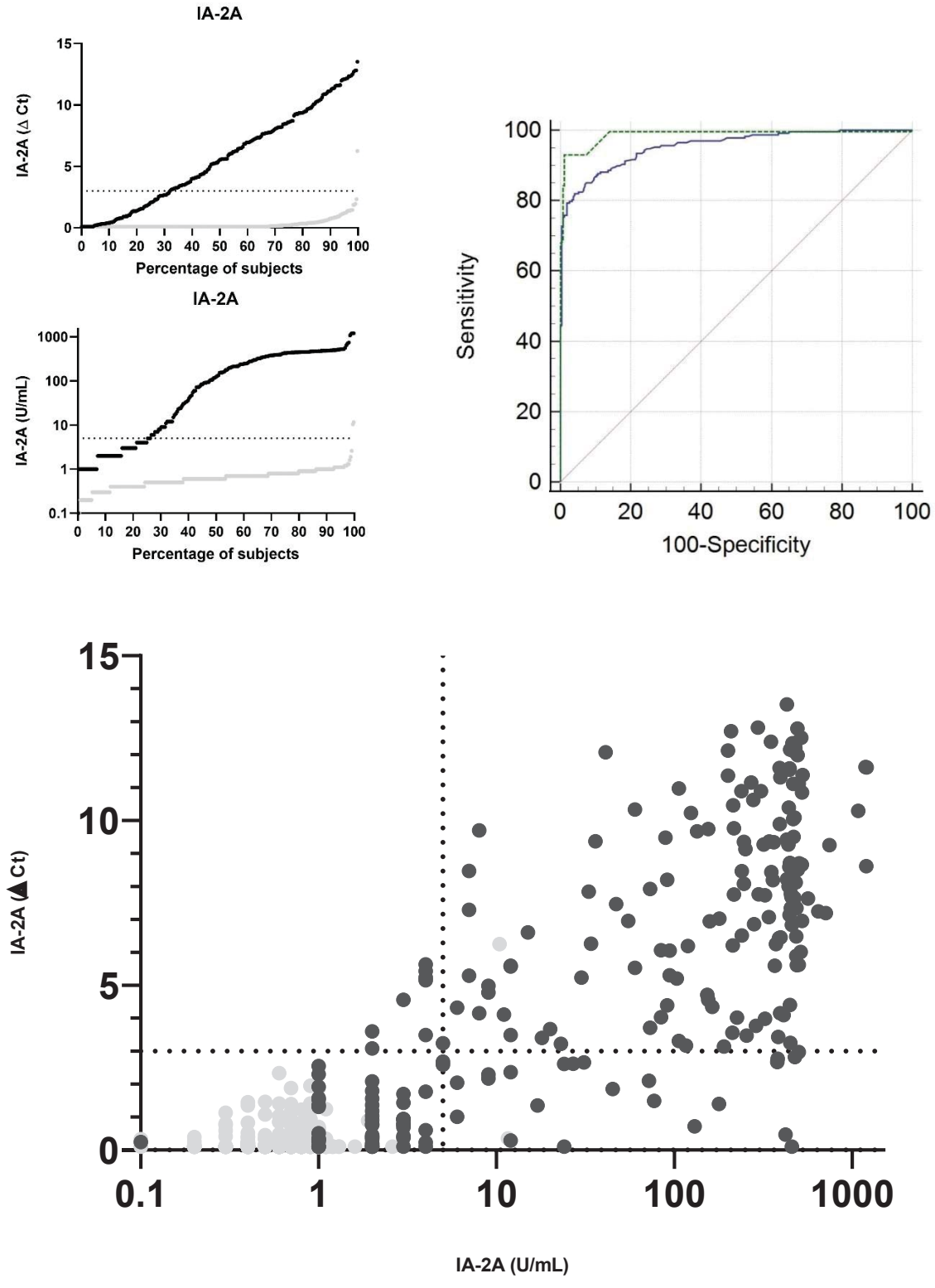

IA-2A
IA-2A (Δ Ct)
Percentage of subjects
IA-2A
IA-2A (U/mL)
Percentage of subjects
Sensitivity
100-Specificity
IA-2A (Δ Ct)
IA-2A (U/mL)

Figure 3

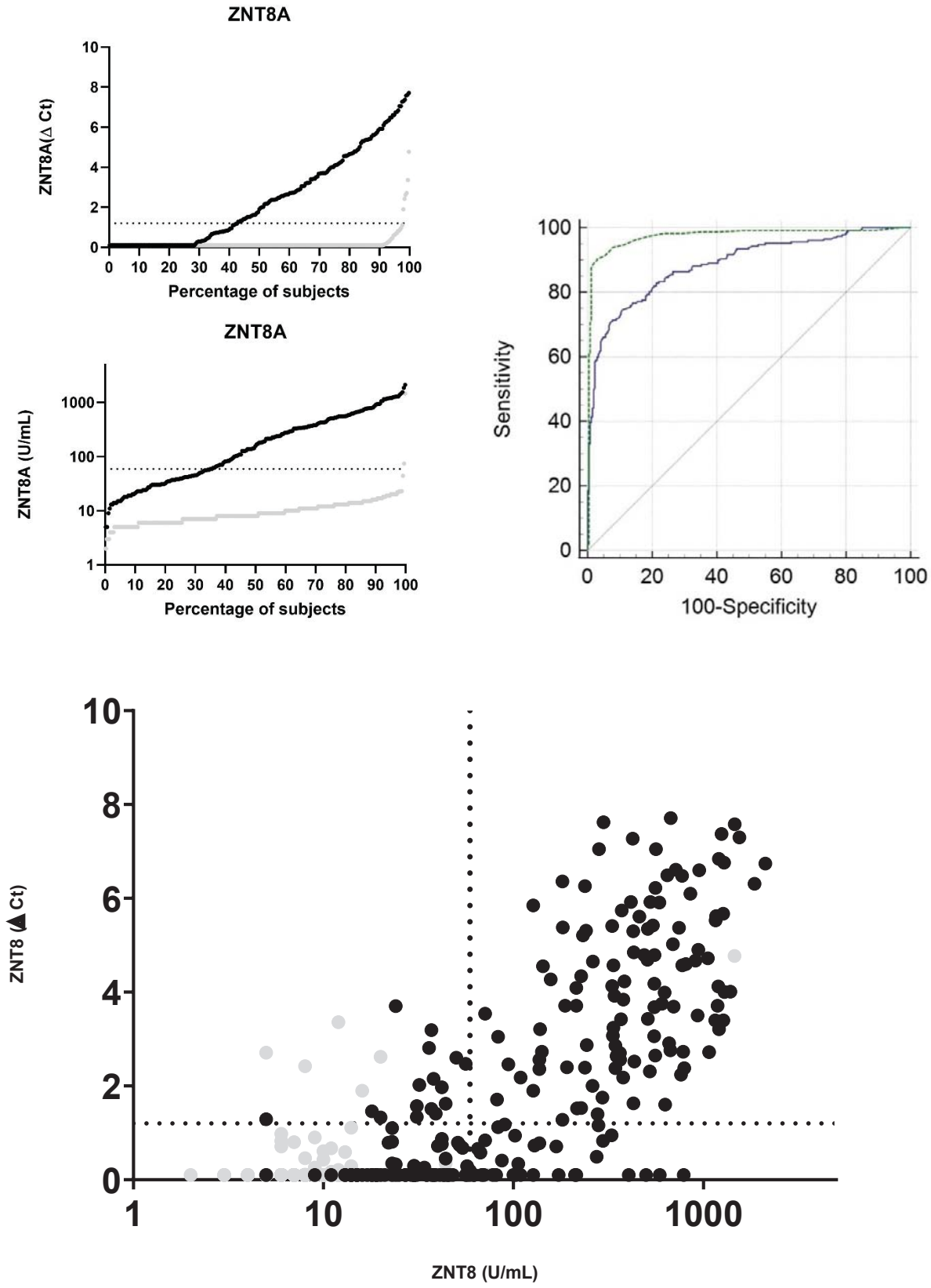
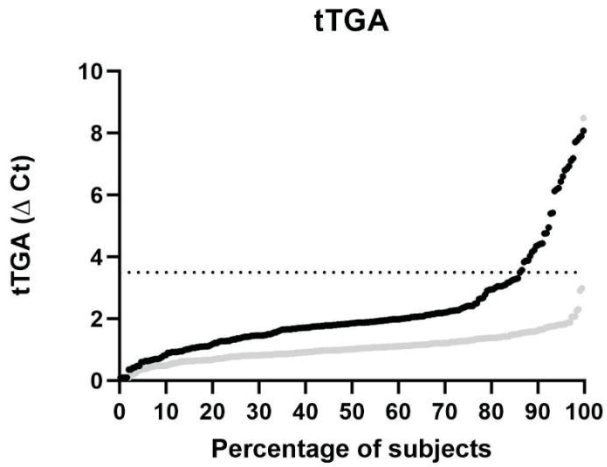
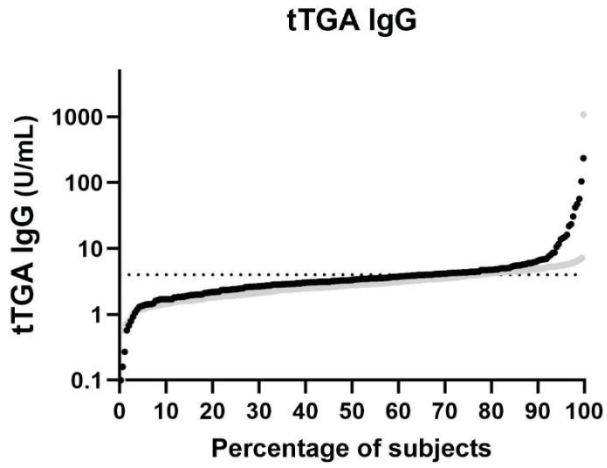

ZNT8A
ZNT8A(Δ Ct)
Percentage of subjects
ZNT8A
ZNT8A (U/mL)
Percentage of subjects
Sensitivity
100-Specificity
ZNT8 (Δ Ct)
ZNT8 (U/mL)

Figure 4





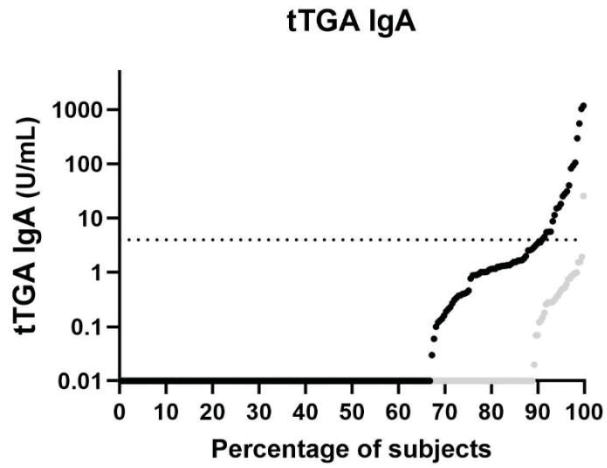
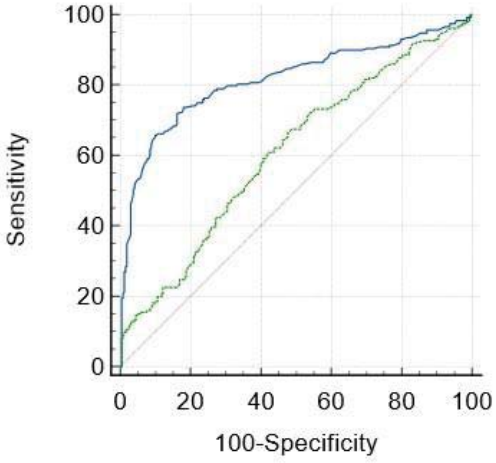
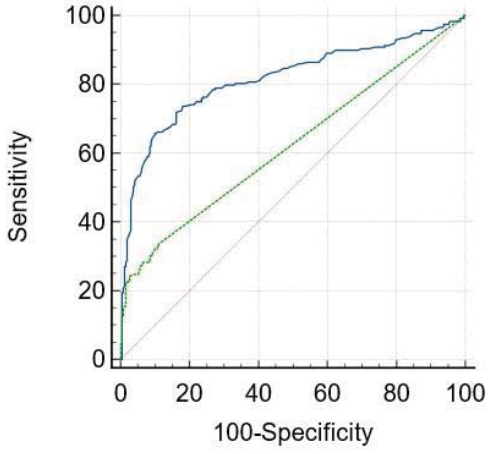


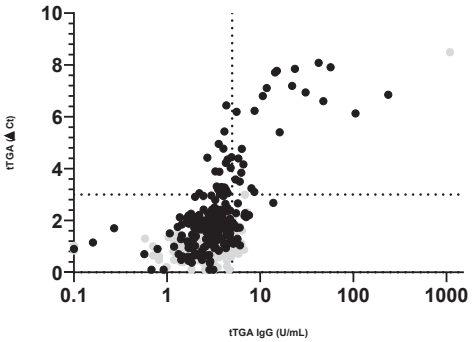
Figure 5, panel A



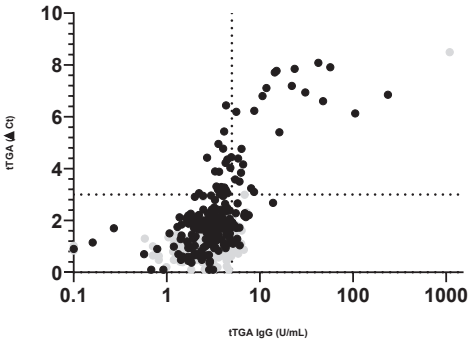
Panel B: TGA-IgG



Panel C: TGA-IgA



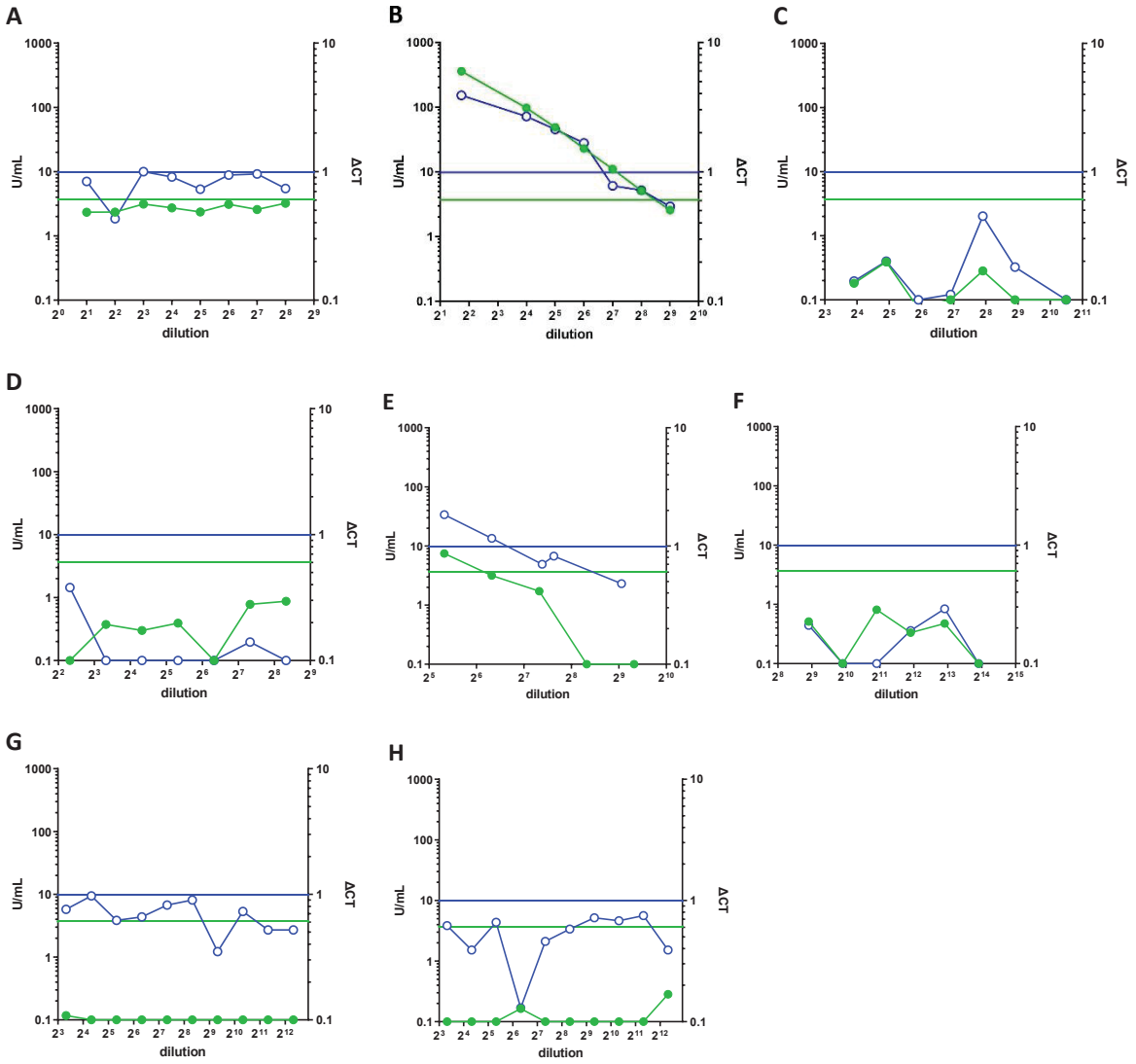
Panel D: TGA-IgG



Panel E: TGA-IgA

Figure 5 Panels B-E

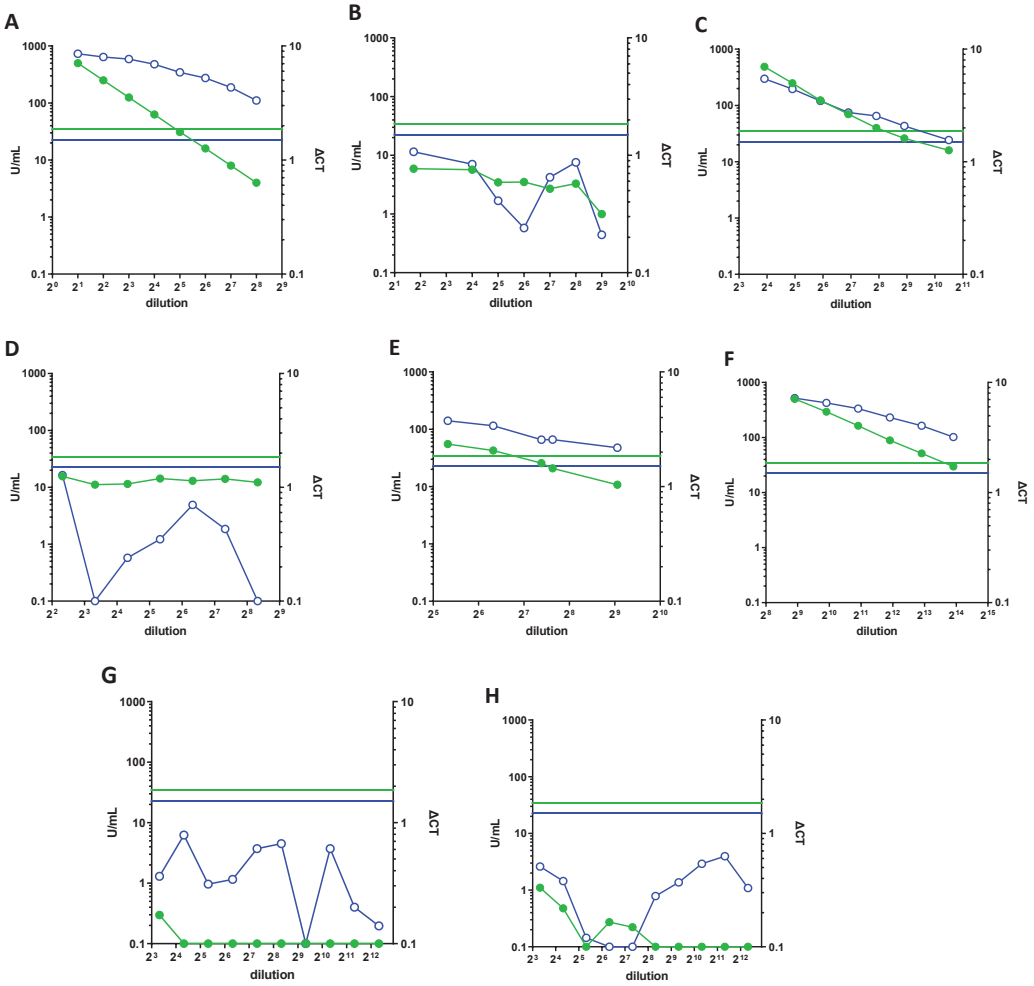
Supplement Figure 1 - IAA



Supplement Figure 1. Autoantibodies against insulin (IAA) were analyzed by ADAP (blue symbols) and RBA (green symbols), respectively. The data in the different panels represents: A. standard 673 (WHO standard 97/550) negative for IAA in both assays; B. IAA RBA reference standard showed parallel dilution in the ADAP analysis; C. RBA GADA and IA-2A reference standard 622 was negative for IAA in both assays; D.RBA IA-2A 272 reference standard was negative for IAA in both assays; E. ZnT8A 3003 reference standard was negative for IAA in RBA while the ADAP detected low level IAA; F. RBA GADA PEO reference standard was negative for IAA in both assays; G. RBA TGA IgG reference standard was negative for IAA in both assays; and H. RBA TGA IgA reference standard was negative for IAA in both assays.

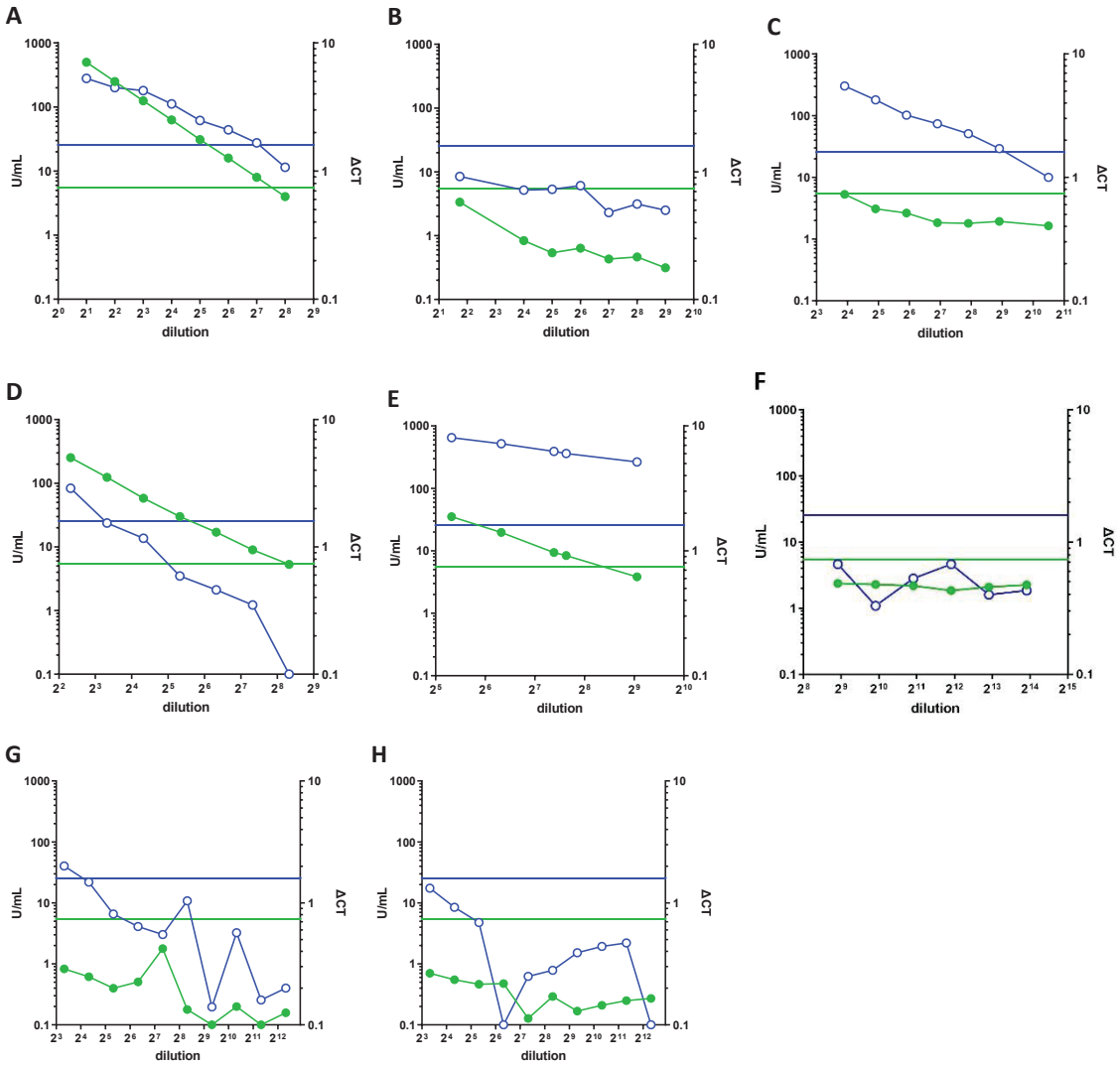
A=673, B=IAA std, C=622, D=272, E=3003, F=PEO, G=TGA IgG std, H=TGA IgA std

Supplement Figure 2 – GADA



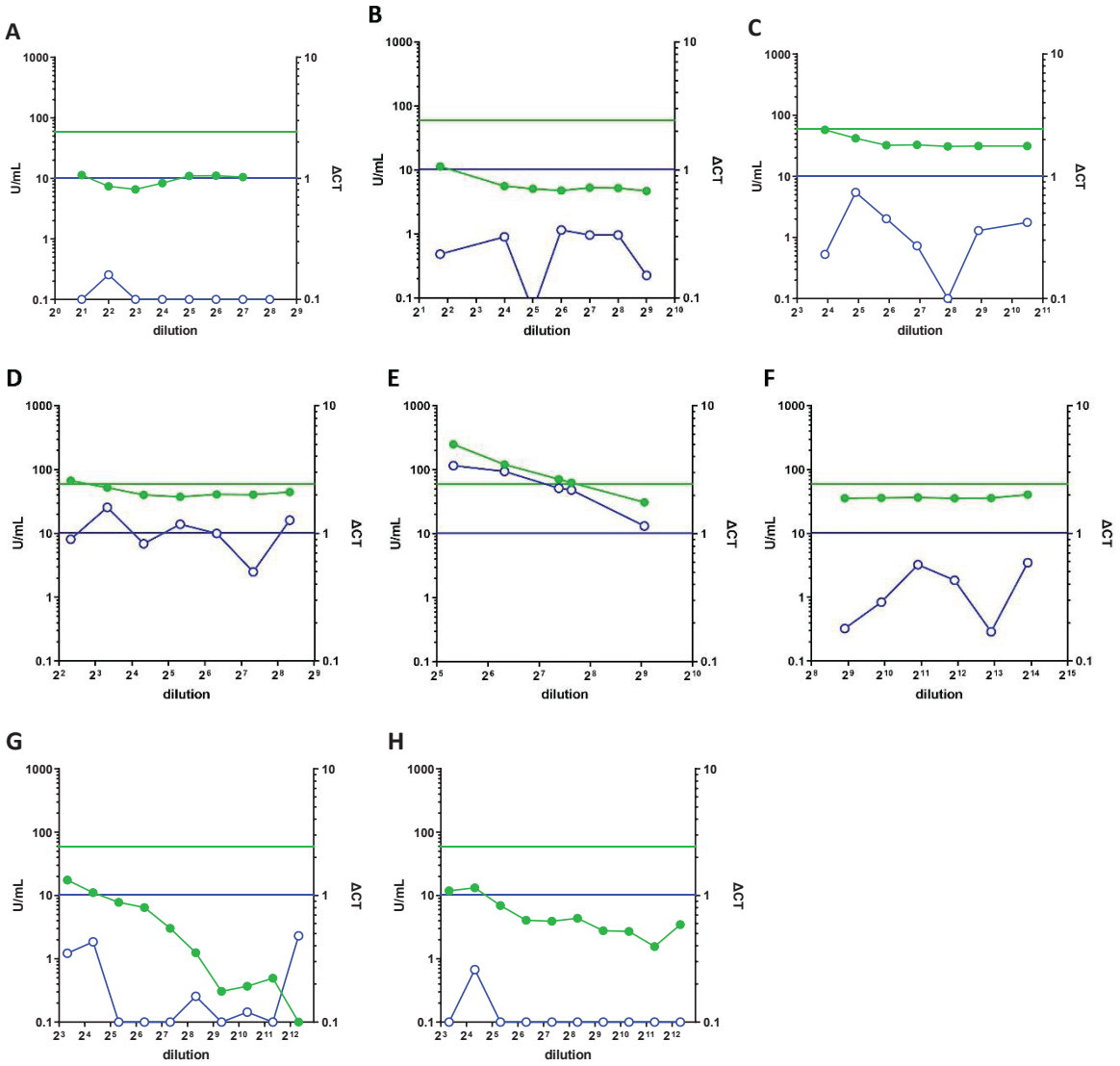
Supplement Figure 2. Autoantibodies against glutamic acid decarboxylase (GADA) were analyzed by ADAP (blue symbols) and RBA (green symbols), respectively. The data in the different panels represents: A. standard 673 (WHO standard 97/550) positive for GADA in both assays; B. IAA RBA reference standard was negative in both assays; C. RBA GADA reference standard 622 was positive for GADA in both assays; D. RBA IA-2A 272 reference standard was negative for GADA in both assays; E. ZnT8A 3003 reference standard was positive for GADA in both assays; F. RBA GADA PEO reference standard was positive for GADA in both assays; G. RBA TGA IgG reference standard was negative for GADA in both assays; and H. RBA TGA IgA reference standard was negative for GADA in both assays. The horizontal lines represent autoantibody cut off in the respective assay.

Supplement figure 3 – IA-2A

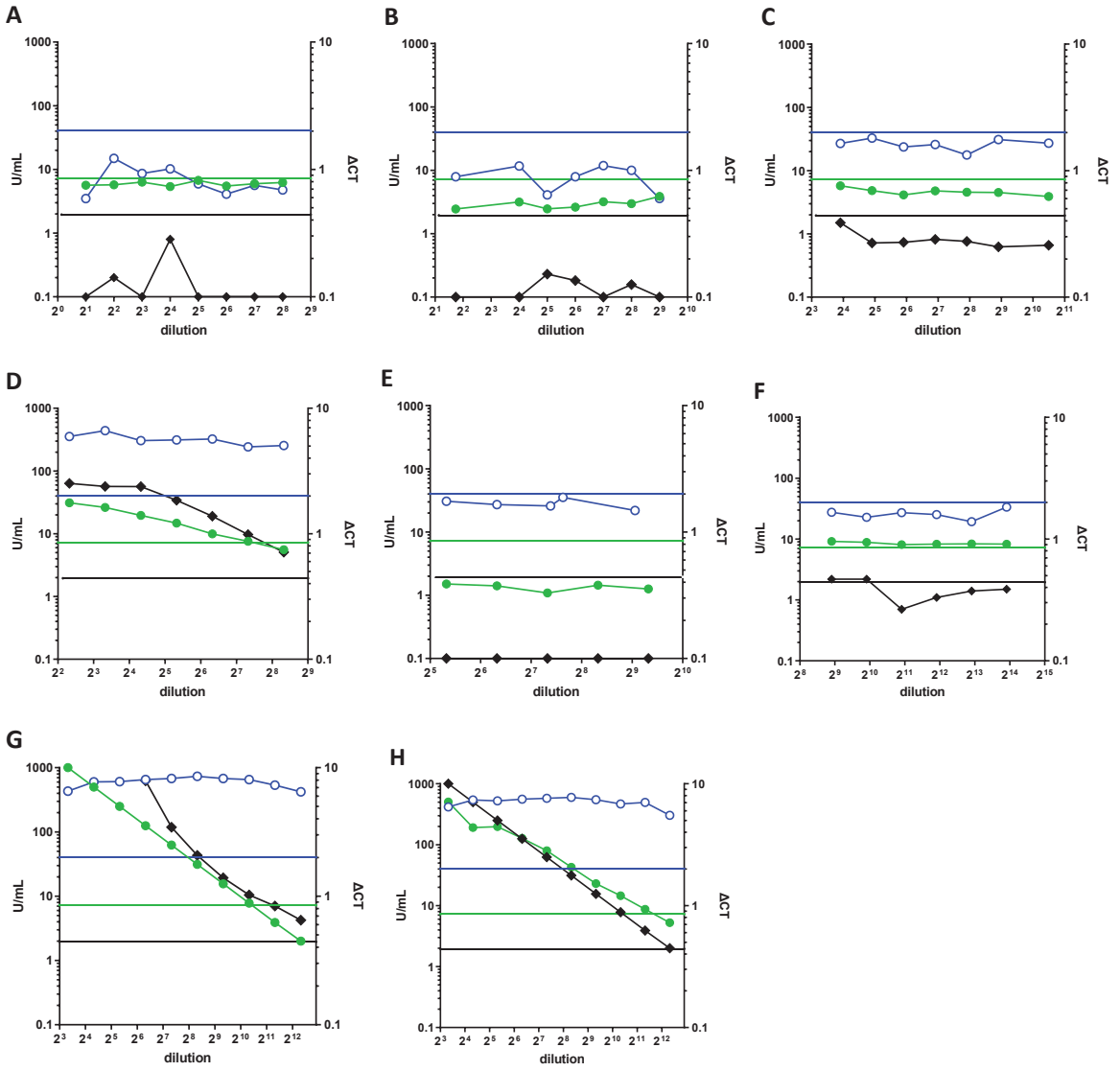


Supplement Figure 3. Autoantibodies against islet antigen-2 (IA-2A) were analyzed by ADAP (blue symbols) and RBA (green symbols), respectively. The data in the different panels represents: A. standard 673 (WHO standard 97/550) positive for IA-2A in both assays; B. IAA RBA reference standard was negative in both assays; C. RBA GADA reference standard 622 was positive for IA-2A in the ADAP but negative in the RBA in both assay; D. RBA IA-2A 272 reference standard was positive for IA-2A in both assays; E. ZnT8A 3003 reference standard was positive for IA-2A in both assays, ADAP more so than RBA; F. RBA GADA PEO reference standard was negative for IA-2A in both assays; G. RBA TGA IgG reference standard was negative for IA-2A in both assays; and H. RBA TGA IgA reference standard was negative for IA-2A in both assays. The horizontal lines represent autoantibody cut off in the respective assay.

Supplement Figure 4.



Supplement Figure 4. Autoantibodies against ZnT8 (ZnT8A) were analyzed by ADAP (blue symbols) and RBA (green symbols), respectively. The data in the different panels represents: A. standard 673 (WHO standard 97/550) negative for ZnT8A in both assays; B. IAA RBA reference standard was ZnT8A negative in both assays; C. RBA GADA reference standard 622 was negative for ZnT8A in both assay; D. RBA IA-2A 272 reference standard was negative for ZnT8A in both assays; E. ZnT8A 3003 reference standard was positive for ZnT8A in both assays, ADAP more so than RBA; F. RBA GADA PEO reference standard was negative for ZnT8A in both assays; G. RBA TGA IgG reference standard was negative for ZnT8A in both assays; and H. RBA TGA IgA reference standard was negative for ZnT8A in both assays. The horizontal lines represent autoantibody cut off in the respective assay.



Supplement Figure 5. Autoantibodies against tissue transglutaminase (TGA) were analyzed by ADAP (blue symbols), RBA TGA IgG (green symbols) and TGA IgA (black symbols), respectively. The data in the different panels represents: A. standard 673 (WHO standard 97/550) negative for TGA in all three assays; B. IAA RBA reference standard was TGA negative in all three assays; C. RBA GADA reference standard 622 was negative for TGA in all three assays; D. RBA IA-2A 272 reference standard was TGA positive for ADAP, weakly positive for RBA TGA IgA but negative for RBA TGA IgG; E. ZnT8A 3003 reference standard was negative for TGA in all three assays; F. RBA GADA PEO reference standard was negative forTGA in all three assays; G. RBA TGA IgG reference standard was positive for TGA in all three assays; and H. RBA TGA IgA reference standard was was positive for TGA in all three assays; ADAP more so than RBA. The horizontal lines represent autoantibody cut off in the respective assay.