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Flow Cytometric microsphere-based immunoassay as a novel non-radiometric method for the detection of glutamic acid decarboxylase autoantibodies in type 1 Diabetes Mellitus

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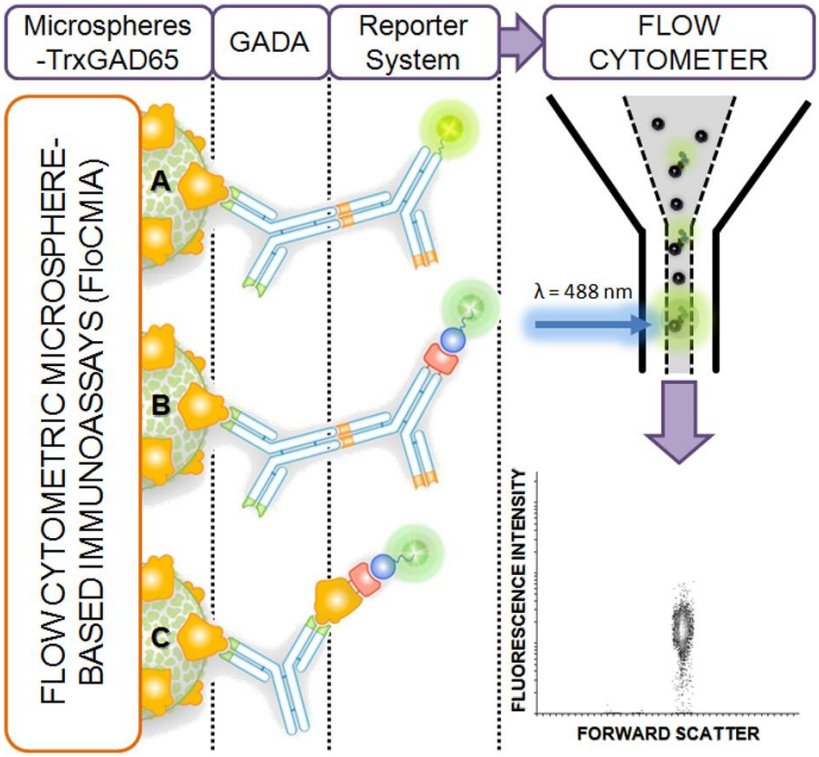
Flow Cytometric microsphere-based immunoassays (FloCMIA), involving polystyrene microspheres adsorbed with glutamic acid decarboxylase (GAD65) fused to thioredoxin (TrxGAD65), are used to assess GAD65 autoantibodies (GADA) in diabetic patients' sera.



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Abstract

The first measurable sign of arising autoimmunity in Type 1 Diabetes Mellitus is the detection of autoantibodies against beta-cell antigens, such as glutamic acid decarboxylase (GAD65). GAD65 autoantibodies (GADA) are usually measured by Radioligand Binding Assay (RBA). The aim of this work was to develop protocols of Flow Cytometric microsphere-based immunoassays (FloCMIA) which involved glutamic acid decarboxylase fused to thioredoxin (TrxGAD65) adsorbed on polystyrene microspheres. Detection of bound GADA was accomplished by the use of anti-human IgG-Alexa Fluor 488 (Protocol A), anti-human IgG-biotin and streptavidin-dichlorotriazinyl aminofluorescein (DTAF) (Protocol B) or TrxGAD65-biotin and streptavidin-DTAF (Protocol C). Serum samples obtained from 46 patients assayed for routine autoantibodies at Servicios Tecnológicos de Alto Nivel (STAN-CONICET) were analyzed by RBA, ELISA and three alternative FloCMIA designs. Protocol C exhibited the highest specificity (97.8%) and sensitivity (97.4%) and a wide dynamic range (1.00-134.40 SDs). Samples obtained from 40 new-onset diabetic patients were also analyzed to further evaluate the performance of protocol C. The latter protocol showed a sensitivity of 58.6% and a prevalence of 47.5%. Two patients resulted positive only by FloCMIA protocol C and its SDs were higher than RBA and ELISA, showing a significantly wide dynamic range. In conclusion, FloCMIA proved to be highly sensitive and specific, requiring a low sample volume; it is environmentally adequate, innovative and it represents a cost-effective alternative to traditional GADA determination by RBA and/or ELISA; making it applicable to most medium-complexity laboratories.

Key words

Flow Cytometry, Diabetes Mellitus, glutamic acid decarboxylase autoantibody, immunoassay

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1. Introduction

Type 1 Diabetes Mellitus (T1DM) is classified as an organ-specific T-dependent autoimmune disease since it is caused by the autoimmunity-mediated destruction of pancreatic islet beta cells, leading to absolute dependence of exogenous insulin to control hyperglycemia [1]. The clinical presentation of this disease is preceded by an asymptomatic period in which detection of autoantibodies against a wide variety of beta-cell antigens is the first measurable sign of arising autoimmunity. There are four major autoantibodies that support diagnosis of T1DM [2, 3]: insulin/proinsulin autoantibodies (IAA/PAA), glutamic acid decarboxylase 65 kDa isoform autoantibodies (GADA), insulinoma-associated tyrosine phosphatase 2 autoantibodies (IA-2A) and Zinc transporter isoform 8 autoantibodies (ZnT8A). About 98% of children and adolescents with new-onset T1DM and approximately 10% of adult-onset diabetic patients present at least one of these autoantibodies [4, 5], thus the possibility to detect these markers facilitates both the prediction of T1DM in children and a more precise classification of adult-onset diabetic individuals.

The above mentioned autoantibodies are usually measured by Radioligand Binding Assay (RBA) [3, 6-8], which is considered the reference method. However, this methodology requires radioactive tracers employing [^{125}I] or [^{35}S]-Methionine, making this method environmentally inappropriate, expensive and limited to authorized laboratories. In order to replace radiometric fluid-phase assays such as RBA, solid phase tests such as Enzyme-Linked ImmunoSorbent Assays (ELISA) have been developed in numerous designs [9, 10 , 11-13]. Nevertheless, ELISAs are not as sensitive and specific as RBA which leads to misdiagnosis of a considerable proportion of patients. Despite this drawback, ELISA is more frequently employed than RBA for the determination of GADA , and hence many kits or in-house designs are used. These approaches are feasible when a handy source of glutamic acid decarboxylase 65 kDa isoform (GAD65) is available for immobilization to microplates (i.e, recombinant protein obtained from modified prokaryotic cells). In this sense, we have previously described the expression of human GAD65 in *Escherichia coli* as a soluble and properly folded fusion protein with thioredoxin (TrxGAD65) [14]. This method has allowed us to determine the presence of GADA by ELISA[12], with a similar format to that of Palomer et al [15]. This assay depends on the ability of divalent autoantibodies to form a bridge between immobilized TrxGAD65 and biotinylated TrxGAD65 present in the fluid phase. However, this in-house design did not overcome the disadvantages of solid phase assays since it was reported 52% sensitivity and 94.6% specificity in the Diabetes Autoantibody Standardization Program (DASP) 2009.

Even though Flow Cytometry (FC) has primarily been designed for multiparametric cellular analyses, this methodology can be employed to detect any particle in the lower micron range by laser light scattering. Inert microspheres of different sizes are considered suitable solid supports for antibody-antigen interactions [16]. In fact, FC has been historically considered an alternative analytical platform for the development of immunoassays [17] applicable to detection and quantification of soluble analytes using fluorochrome-conjugated antibodies. Moreover, FC-based methods have been proved advantageous for the simultaneous detection of multiple analytes [18].

The aim of the present work was to develop highly sensitive and quasi-quantitative alternative protocols of Flow Cytometric microsphere-based immunoassays (FloCMIA) for the detection of GADA.

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2. Experimental

2.1. Human sera collection

Blood samples were collected from patients after overnight fasting and the corresponding sera were stored at -20ºC until assayed. Forty six sera showing a wide range of GADA positivity by RBA were selected from the samples collected in our laboratory during routine autoantibodies determination (Servicios Tecnológicos de Alto Nivel, STAN-CONICET). Besides, 40 sera from children and adolescents with newly diagnosed T1DM (diagnosed according to WHO criteria [19]) were also collected. The protocols were approved by the Ethical Committees of the J. P. Garrahan Pediatric Hospital of Buenos Aires. Written consent from all participants involved in this study and parental consent in the case of minors, was obtained.

Normal human control sera were obtained from healthy subjects without personal or family history of autoimmune disease. The collection of serum samples was approved by the Ethics Committee of the Clinical Hospital José de San Martín, University of Buenos Aires.

2.2. Detection of GADA by RBA

2.2.1. Production of [^{35}S]-GAD65 tracer

The tracer [^{35}S]-GAD65 was obtained by *in vitro* transcription/translation of cDNA encoding the human GAD65 using a rabbit reticulocyte lysate system (Promega, Madison, WI, USA) in the presence of [^{35}S]-Methionine (New England, Nuclear, Boston, MA, USA), according to the manufacturer´s instructions. Translation products were diluted in RBA buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4, 0.1% V/V Tween 20) and applied to a PD10 column (Pharmacia-LKB Biotechnology, Uppsala, Sweden) in order to remove free [^{35}S]-Methionine.

2.2.2. RBA protocol

RBA was performed as previously described [7] with minor modifications [11]. Briefly, 2.5 µL of human sera were incubated overnight at 4°C with 10,000 cpm of [^{35}S]-GAD65 in a final volume of 60 µL in RBA buffer. After that, 50 µL of protein A-Sepharose 4B FF (GE Healthcare Biosciences, Uppsala, Sweeden) in RBA buffer (2:3) were added to isolate immunocomplexes, and incubated 2 h at room temperature on an end-over-end shaker. Subsequently, samples were allowed to settle and the supernatants were discarded. Pellets were washed three times with 200 µL of RBA buffer and once with 200 µL of 0.2 M NaCl in RBA buffer. Finally, pellets were suspended in 100 µL of 1% w/V SDS and supernatants were carefully transferred to vials for scintillation counting (1 min/tube). Results for each sample were calculated as Bound% (B%) = 100 x (bound cpm/total cpm), and expressed as Standard Deviation scores (SDs) = (B% - B_c%) /SD_c where B_c% is the mean B% of control sera and SD_c its standard deviation. Twenty human control sera were included in each assay. Samples were considered positive when SDs > 3. This assay had 79.6% sensitivity and 98% specificity in the DASP 2007.

2.3. Detection of GADA by ELISA

2.3.1. Reagents

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Lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.0) was used in the TrxGAD65 expression protocol. Phosphate saline buffer (PBS, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.14 M NaCl, 2.7 mM KCl, and pH 7.4) was used for microplate coating. A 3% w/V skim milk in PBS (PBS-M) and PBS containing 0.05% V/V Tween 20 (PBS-T) were used as blocking solution and wash buffer, respectively. Sample or reagent dilutions were prepared in 3% w/V skim milk in 0.05% V/V PBS–Tween 20 (PBS-M-T). Avidin-HRP was purchased from Jackson ImmunoResearch Laboratories, Inc. and 3, 3', 5, 5'-tetramethyl-benzidine/H_2O_2 (TMB, GE Healthcare, Uppsala, Sweden) was employed as the chromogenic substrate.

2.3.2. Expression of TrxGAD65 in *Escherichia coli* and biotinylation

The fusion protein TrxGAD65 was expressed in *E. coli* as previously described [14]. Briefly, *E. coli* GI698 was transformed with pGAD65 (Trx). Bacteria were cultured at 30°C in 0.2% w/V casein amino acids, 0.5% w/V glucose, 1 mM $MgCl_2$ and 100 µg/mL ampicillin. Protein expression was induced with 2 mL of 10 mg/mL tryptophan overnight at 20°C. Bacteria from 200 mL culture were collected by centrifugation, suspended in 2 mL of lysis buffer and sonicated in the presence of 1 mM 2-mercaptoethanol (2ME) and protease inhibitors (0.1% w/V aprotinin and 2 mM phenylmethylsulfonyl fluoride) over crushed ice. After sonication, Triton X-100 was added to a final concentration of 0.1% V/V and incubated for 10 min at 0°C. The soluble intracellular fraction was then separated by centrifugation at 15,000 rpm for 10 min. The fusion protein was purified by affinity chromatography following the protocol previously described [20]. The resin was based on an agarose support covalently modified with phenylarsine oxide, which permitted the binding of proteins containing vicinal dithiol residues [21]. Bound proteins were eluted with two-column volumes of lysis buffer containing 100 mM 2ME. A typical TrxGAD65 preparation yielded 9 mg of 80-90% pure TrxGAD65 per Liter of culture. The fusion protein was stored in a mixture of 50% V/V glycerol, 0.2 mM pyridoxal 5-phosphate, 0.05% V/V Tween 20 and 0.1% w/V aprotinin. Two milliliters of the purified fusion protein were subjected to buffer exchange to PBS using a ZEBA desalt spin column (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturers' instructions. The desalted protein was then incubated for 2 h at 0°C with 0.32 mg of sulfo-NHS-biotin (Pierce Biotechnology, Rockford, IL, USA) and free biotin was removed on a new ZEBA desalt spin column. Storage conditions for TrxGAD65-biotin were the same as those for TrxGAD65.

2.3.3. ELISA protocol

The protocol employed was the same as that previously described [12] with minor modifications. Except when otherwise indicated, incubations were at room temperature, washing steps were performed with 200 µL of PBS-T and 50 µL/well were added in each incubation step. Polystyrene microplates (Maxisorp, NUNC, Roskilde, Denmark) were coated overnight at 4°C with TrxGAD65 purified preparation diluted 1/50 in PBS, washed 3 times with 200 µL of PBS, blocked for 1.5 h with 200 µL of PBS-M, and washed 5 times. Samples were added and microplates were incubated for 1 h. All samples and blanks were assayed in duplicate. After incubation with samples, plates were washed 5 times and TrxGAD65-biotin diluted 1/300 was added. After another 1 h incubation, plates were washed 5 times and bound TrxGAD65-biotin was detected by the addition of Avidin-HRP diluted 1/500. After 1 h incubation, microplates were washed 4 times plus 1 final washing

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step with 200 µL of PBS, TMB was added and incubated 15 min in the dark. The color reaction was stopped with 2 M H_2SO_4 and the oxidized substrate was measured at 450 nm in an ELISA plate reader Multiskan ex (Thermo electron corporation, Vantaa, Finland). Results were calculated as specific absorbance (A_s, mean of each sample minus the mean of blank wells), and expressed as SDs = (A_s - A_c) /SD_c, where A_c is the mean specific absorbance of control sera and SD_c its standard deviation. The cut-off value of the assay was SDs > 3.

2.4. Detection of GADA by FloCMIA

2.4.1. Passive adsorption of microspheres with TrxGAD65

2.4.1.1. Reagents

Isotonic buffered saline (IBS) (2.3 mM NaH_2PO_4, 14.2 mM Na_2HPO_4, 0.14 M NaCl, 3.8 mM KCl, 3.1 mM sodium azide) was used as buffer for passive adsorption. For blocking free sites a solution of 0.5% w/V bovine serum albumin in IBS (IBS-BSA) was employed. The washing and storage buffer was PBS containing 0.02% w/V sodium azide (PBS-SA). Polystyrene microspheres were purchased from Spherotech, Inc. (Lake Forest, IL, USA). These particles are prepared by using conventional emulsion polymerization process with styrene as the monomer and benzoyl peroxide as polymerization initiator without any cross-linking agent (making them mainly hydrophobic). Microspheres have been precleaned using centrifugation and their size was determined visually by Scanning Electron Microscope and/or Beckman Coulter Multisizer.

2.4.1.2. Passive adsorption protocol

This protocol was performed using the manufacturer's instructions with minor modifications. Microspheres (4 µm, 36.4 µL of 5% w/V suspension) were mixed in 0.5 mL Eppendorf tubes with 28 µg TrxGAD65 purified preparation per 100 cm^2 of microspheres in a final volume of 220 µL in IBS, and incubated overnight at 4°C on an end-over-end shaker. The preparation was centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The pellet was suspended with 220 µL of IBS-BSA and incubated for 1 h at room temperature on an end-over-end shaker. The preparation was centrifuged again at 10,000 rpm for 10 min, the supernatant was discarded and the pellet was washed with 400 µL of PBS-SA. Finally, another 400 µL of PBS-SA were added (resulting in $1x10^5$ microspheres-TrxGAD65/µL) and the suspension was stored at 4°C.

2.4.1.3. Stability study of TrxGAD65-microspheres

In order to determine if conformational non-continuous epitopes were properly folded after adsorption onto the microspheres, the GAD enzymatic activity was carried out measuring the formation of $^{14}CO_2$ from decarboxylation of L-[^{14}C(U)]-glutamic acid to form gamma-aminobutyric acid (GABA)[14, 22]. Briefly, 2 µL L-[^{14}C(U)]-glutamic acid (New England Nuclear, Life Science Products, Inc., Boston, MA, USA) were assayed in glass tubes with 50 µL of samples (PBS as negative control, TrxGAD65 preparation as positive control, and microspheres-TrxGAD65 as samples) in 200 µL of activity buffer (50 mM K_3PO_4, 1 mM EDTA, 1 mM 2-aminoethylisothiouronium bromide, 0.2 mM pyridoxal 5-phosphate, 20 mM glutamic acid, pH 7.2). A small piece of 3MM Whatman paper, soaked with 50 µL of 1 M hyamine in methanol was

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introduced into the reaction tube. After 1 h incubation at 37°C, 0.25 mL of 2.5 M H_2SO_4 was injected into the solution to stop the reaction. The mixture was incubated for 1 h at 37°C to ensure the complete evolution of CO_2 and its absorption to hyamine. Papers were transferred to vials containing 2 mL of scintillation fluid and $^{14}CO_2$ was determined in a liquid scintillation counter. Results were expressed as a sample cpm/blank cpm ratio.

2.4.2. FloCMIA protocols

2.4.2.1. Reagents

PBS was used as compatible buffer for Flow Cytometric acquisition, the washing buffer was PBS-T and sample or reagent dilutions were prepared using 0.5% w/V BSA in 0.05% V/V PBS –Tween 20 (PBS-BSA-T). Goat anti-human IgG-Alexa Fluor 488 was purchased from Molecular probes; Inc. (Eugene, OR, USA) and rabbit anti-human IgG-biotin and streptavidin-dichlorotriazinyl aminofluorescein (DTAF) were purchased from Jackson ImmunoResearch Laboratories, Inc.

2.4.2.2. Protocol A

Except when otherwise indicated, all washing steps were performed with 200 µL of PBS-T on a Multiscreen$_{HTS}$ vacuum manifold (Millipore Corporation, Billerica, MA, USA). Fifty microliters of sample diluted $^1/_5$ in PBS-BSA-T were mixed with 20 µL of microsphere-TrxGAD65 preparation ($2.5x10^3$ microspheres/µL) in 0.2 mL tubes. Incubation was performed in 2 steps: i) 3 h at room temperature and ii) overnight at 4°C, both on an end-over-end shaker. After the incubation step, mixtures were transferred to a Multiscreen$_{HTS}$-HV 96-well filtration plate (Millipore Ireland BV) and washed 5 times. For detection of bound antibody, 50 µL of goat anti-human IgG-Alexa Fluor 488 diluted 1/500 was used and plates were incubated for 1 h at room temperature in the dark. After that, plates were washed 4 times plus 1 final washing step with 200 µL of PBS and microspheres were suspended with another 200 µL of PBS. The suspension was transferred to Röhren-tubes (3.5 mL, 55x12 mm, Sarstedt, Germany) and acquired on a PAS III PARTEC Flow Cytometer (PARTEC, Görlitz, Germany) equipped with a 488 nm Argon laser. Samples were analyzed using Cyflogic software (CyFlo Ltd., Turku, Finland), singlet population of microspheres was gated in forward scatter (FSC) *vs*. side scatter (SSC) dot plot, fluorescence signals were measured in FL1 channel and reported as median fluorescence intensities (MFI) (Electronic Supplementary Figure 1). Results were expressed as SDs = (MFI_s - MFI_c) /SD_c where MFI_s is the mean MFI of samples in duplicate, MFI_c is the mean MFI of control sera and SD_c its standard deviation. Twenty human control sera were included in each assay and samples were considered positive when SDs > 2. The schematic description of this protocol is shown in Fig. 1 and Electronic Supplementary Figure 2.

2.4.2.3. Protocol B

This design was similar to protocol A, except that 20 µL of sample diluted ¼ in PBS-BSA-T were mixed with 20 µL of microsphere-TrxGAD65 preparation ($5x10^3$ microspheres/µL) and directly incubated overnight at 4°C on an end-over-end shaker. In this case, detection of bound antibodies required a two-step incubation (1 h at room temperature each, with 5 washes in between): i) 50 µL of rabbit anti-human IgG-biotin diluted 1/500 was added and ii) 50 µL of streptavidin-DTAF

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diluted 1/150 were used for amplification of the antigen-antibody interaction. Samples were considered positive when SDs > 2. The schematic description of this protocol is shown in Fig. 1 and Electronic Supplementary Figure 2.

2.4.2.4. Protocol C

This was similar to protocol A, except that 20 µL of pure human serum were mixed with 20 µL of microsphere-TrxGAD65 preparation (3.125×10^3 microspheres/µL) and directly incubated overnight at 4°C on an end-over-end shaker. As described above, detection of bound antibodies required a two-step incubation (1 h at room temperature each, with 5 washes in between): i) 50 µL of TrxGAD65-biotin diluted 1/50 was added and ii) 50 µL of streptavidin-DTAF diluted 1/100 were used for detection of bound TrxGAD-biotin. Samples were considered positive when SDs > 3. The schematic description of this protocol is shown in Fig. 1 and Electronic Supplementary Figure 2.

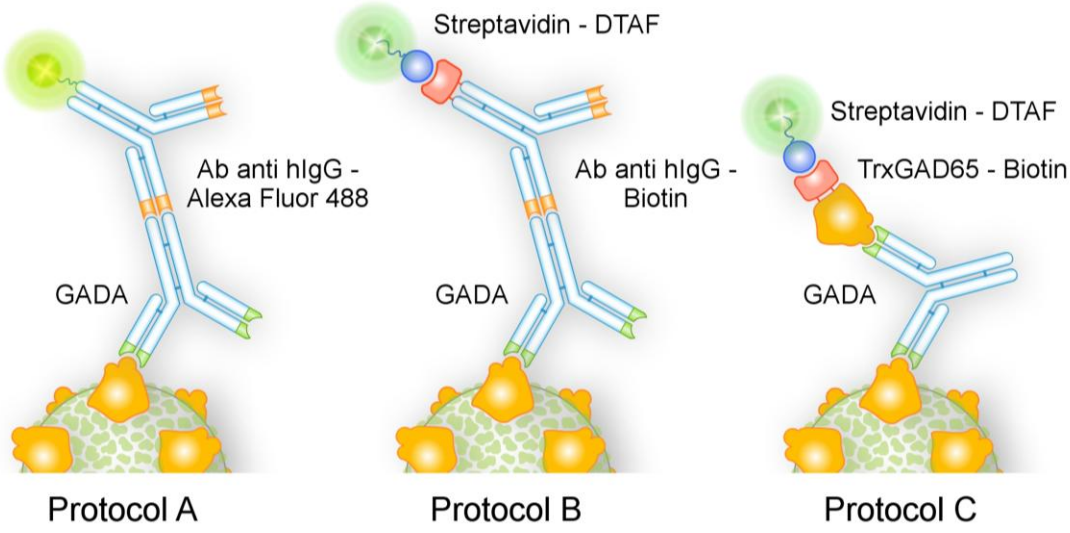

Fig. 1. **Schematic representations of alternative FloCMIA protocols for GADA detection.** Four µm microspheres-TrxGAD65 were used as the solid phase.

2.5. Statistical analysis

To assess if data from control subjects were normally distributed, the D'Agostino & Pearson omnibus normality test was applied. Inter-assay correlation was assessed by standard linear regression. Differences between results obtained with FloCMIA protocols *vs*. RBA or ELISA were evaluated by the Wilcoxon matched-pairs signed rank test. In order to remove outliers from normally distributed control sera the Grubbs' test was performed. Calculations were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego California, USA, www.graphpad.com).

3. Results and discussion

3.1. Selection and characterization of STAN-CONICET sera

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3.1.1. Detection of GADA by RBA and ELISA

Forty six sera from the STAN-CONICET were analyzed in parallel by RBA and ELISA. In the detection of GADA by RBA, SDs, used as precision units for inter-assay comparison purposes, ranged from 9.06 to 67.99, median SDs = 47.86 (Fig. 2. a and Table 1). The RBA for the determination of GADA in clinical samples was partially replaced in our laboratory by a non-radiometric method such as ELISA. The rationale for this replacement was to avoid the use of radioactive isotopes in routine methods because of their cost, their low stability and because they are environmentally harmful. Disadvantages, as the prior mentioned, resulted in difficulty of implementation in low complexity laboratories. Out of the 46 GADA positive sera by RBA, 32 scored positive by ELISA, whereas 14 patients were negative (Fig. 2. b). These results indicated that ELISA had a relative sensitivity of 69.6% for this sera collection (percentage of patients RBA positive that were ELISA positive) and 100% specificity when analysis of control human sera was performed (calculated as 100% minus the percentage of false positives). These data are similar to those reported previously [12]. The ELISA showed SDs ranging from 0.11 to 10.03, and a median SDs = 5.02 (Table 1). As previously described, the ELISA protocol relies on GADA crosslinking of immobilized TrxGAD65 and fluid phase TrxGAD65-biotin, resulting in a highly specific method but with lower sensitivity than RBA. For this reason it was considered as a first line method for screening purposes (for instance, negative results may be reexamined by RBA). Furthermore, it showed a narrow dynamic range and lower GADA titers than those obtained by RBA. Several ELISA procedures for the detection of GADA have previously been reported [10, 15, 23-27] but most of them have presented lower sensitivity than the reference RBA [28-30]. This finding appears to be due to a relative inability of these assays to distinguish true low levels of GADA in T1DM patients from unspecific signal levels in normal control sera. It could have been due to modifications of the epitopes created by interaction and a partial or complete denaturation of the antigen adsorbed to the solid phase plastic matrix. Epitopes would be modified since adsorption onto solid phases can expose regions of the protein that were hidden in the native form. Proteins adsorbed to solid surfaces may undergo some conformational change because of their relatively low structural stability and their tendency to unfold allowing the formation of additional interaction points with the surface. For this reason, the amount of GADA bound could be decreased since they are mainly directed against discontinuous conformational epitopes.

Assay	Median (SDs)	Range (SDs)	Sensitivity[a] (%)	Specificity[b] (%)
RBA	47.86	9.06-67.99	100	100
ELISA	5.02	0.11-10.03	69.6	100
FloCMIA				
A	2.32	(-1.69)-10.05	56.5	91.4
B	3.08	(-1.19)-18.07	63.0	94.9
C	23.60	1.00-134.40	97.4	97.8

[a] Percentage of patients RBA positive that were positive by each method

[b] 100% - percentage of false positive

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Table 1. **Analytical parameters from normal human sera and patients' sera from STAN-CONICET.** The results were obtained by RBA, ELISA and FloCMIA protocols A, B and C.

3.1.2. Detection of GADA by FloCMIA protocols

The aim of this study was to improve the analytical performance of methods employed in the determination of humoral markers, with considerably repercussion in terms of diagnostic support for autoimmune Diabetes and socioeconomic impact associated with this medical specialty. Our group has developed a new analytical method based on the use of microspheres as a solid phase and a Flow Cytometer as the platform for detection and data acquisition. In this work we introduce the development of three different protocols for the determination of GADA by FC, named FloCMIA. To the best of our knowledge, no studies evaluating the detection of GADA by FloCMIA-like methods have been published so far. The biggest challenge for this type of assays is the low concentration of autoantibodies frequently present in sera from patients with T1DM (~1.10^{-12} M for GADA). That is the reason for requiring a highly sensitive method, such as FC, which relies in fluorescence detection.

3.1.2.1. Stability study of TrxGAD65-microspheres

The GAD enzymatic activity was evaluated in TrxGAD65 preparation, both previous (ratio = 4.76) and after (ratio = 10.67) the adsorption protocol. This demonstrated that the structure of the molecule was correctly folded at least in the proximity of the catalytic site, even when the recombinant enzyme was immobilized on a polystyrene surface.

3.1.2.2. FloCMIA protocol A

Protocol A was based on a standard design for detection of antibodies, similar to an indirect ELISA. The reporter antibody was conjugated to Alexa fluor 488, a fluorochrome of comparable excitation and emission spectra to fluorescein. This protocol also used a limited volume of sera (equivalent to 10 µL of undiluted sample), higher than that used in RBA (2.5 µL of undiluted sera), but much lower than those habitually reported in ELISAs (≈ 50 µL). Control sera (n= 35) results showed two different statistical populations that needed outliers removal by the Grubbs' test. Remaining results were normally distributed. Outliers removed by the Grubbs' test were included in all FC plots and specificity calculations, as they are considered false positives. For this alternative, specificity was 91.4%. In order to achieve maximum analytical sensitivity for this protocol, SDs > 2 was chosen as the cut-off value for positive results. Twenty six GADA positive sera by RBA scored positive when evaluated by FloCMIA protocol A, whereas 20 patients were negative (Fig. 2. c i.). These results showed a sensitivity of 56.5%, SDs ranging from -1.69 to 10.05 and a median SDs = 2.32 (Table 1). Neither its analytical sensitivity nor its specificity was adequate for clinical applications. Besides, it showed a narrow dynamic range in comparison with RBA.

3.1.2.3. FloCMIA protocol B

Upon trying to improve the results obtained with protocol A, an alternative protocol B was developed using the same basic design but including an amplification step. This step consisted in the use of a xenogeneic antibody conjugated with biotin, while the fluorochrome (DTAF) was

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covalently bound to streptavidin. As biotin-streptavidin is one of the strongest non-covalent interactions usually employed in immunochemistry and each antibody is conjugated with several biotin residues, the detection of GADA was expected to be more distinguishable than signals from control sera. After outlier removal from control sera (n = 39) by the Grubbs' test, remaining results were normally distributed. Specificity for this protocol was 94.9% and its sensitivity was 63.0% when a cut-off for positive results of SDs > 2 was chosen. Thus 29 GADA positive sera by RBA scored positive when evaluated by FloCMIA protocol B, whereas 17 patients were negative (Fig. 2. c ii.). This assay exhibited signals ranging from -1.19 to 18.07 SDs and a median SDs = 3.08 (Table 1). Results showed that, in fact, sensitivity and specificity were increased, as well as the dynamic range, even though a smaller sample volume was used (20 µL of diluted sample, equivalent to 4 µL of sera). Although these parameters were better than the ones obtained with protocol A, they were still not as good as expected for replacement of RBA and/or ELISA.

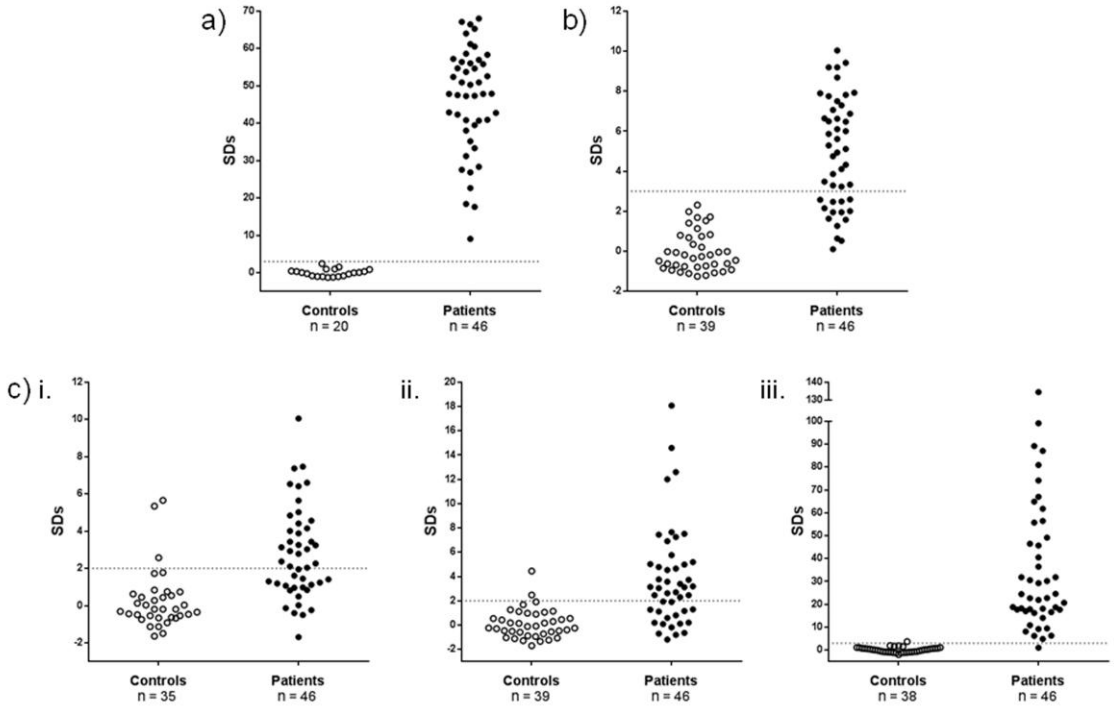

Fig. 2. **GADA results from normal human sera (controls) and diabetic patients' sera from STAN-CONICET.** The results, expressed as SDs, were obtained by RBA (a), ELISA (b) and FloCMIA protocols (c, i. protocol A, ii. Protocol B and iii. Protocol C). The cut-off value for each assay is indicated by a dotted line.

3.1.2.4. FloCMIA protocol C

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In order to improve FloCMIA's output, and considering the acceptable performance of ELISA for clinical applications in routinely GADA determination, FloCMIA's protocol C was developed. This design was similar to that of ELISA-based determination of GADA because it relies on GADA crosslinking of immobilized TrxGAD65 on the microsphere surface and fluid phase TrxGAD65-biotin. For this protocol, outlier removal from control sera (n = 38) by the Grubbs' test left a normally distributed population. This method exhibited a specificity of 97.8% and a sensitivity of 97.4% for a cut-off of SDs > 3. This meant that 45 GADA positive sera by RBA scored positive when evaluated by FloCMIA protocol C, whereas just one patient was negative (Fig. 2. c iii.). Furthermore, SDs values (median SDs = 23.60 and range = 1.00-134.4) were higher than any derived from the other FloCMIA protocols, or even RBA and ELISA, exhibiting a wide dynamic range (Table 1). This alternative protocol forced GADA to interact using its two paratopes, decreasing the unspecific signal when compared to other FloCMIA protocols. Such design, combined with Streptavidin-DTAF for fluorescence detection, resulted in the highest sensitivity, specificity and wider dynamic range observed for any of FloCMIA alternatives. Although this protocol required the use of a higher volume of patients' sera (20 µL of undiluted sample), it was still smaller than the volume normally used in ELISA.

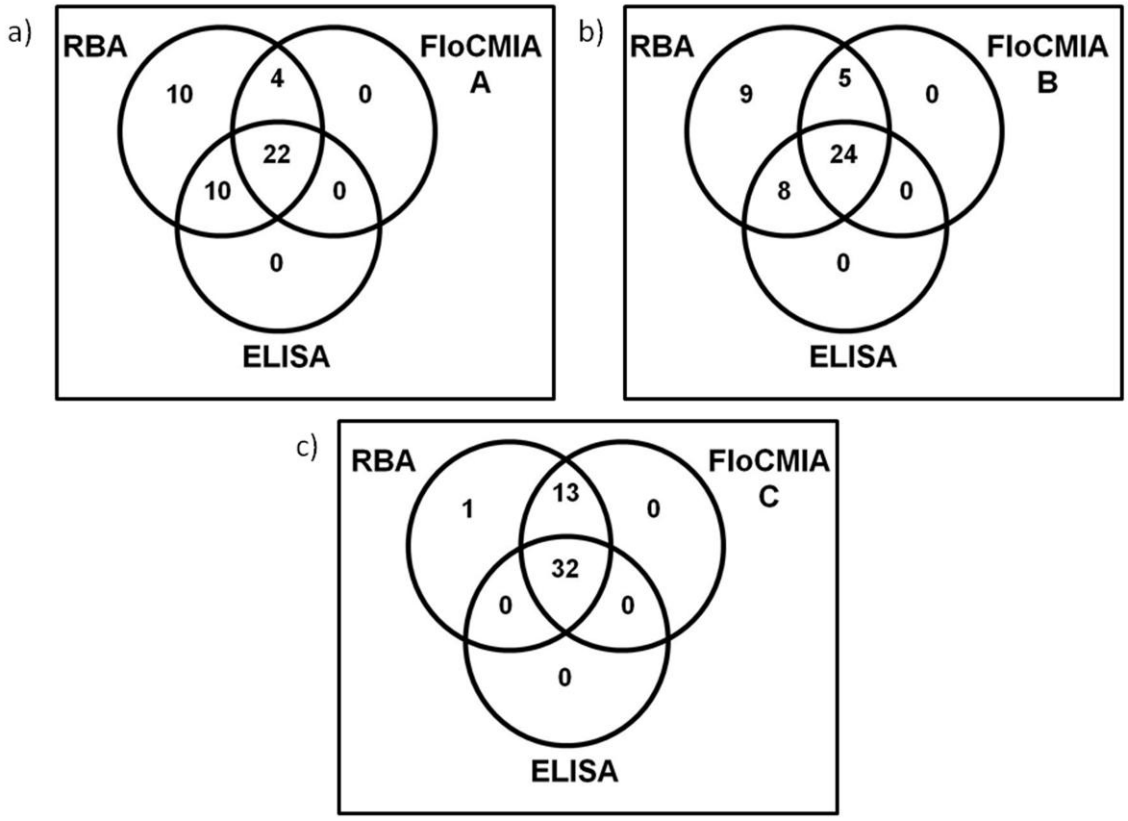

Fig. 3. **Integrated GADA results for diabetic patients' sera from STAN-CONICET.** The results obtained by RBA, ELISA, and FloCMIA protocol A (a), protocol B (b) and protocol C (c) are presented as Venn's diagrams.

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3.1.3. Integrated results

Venn's diagrams (Fig. 3) depict the integrated results of RBA, reference ELISA, and FloCMIA protocols A, B and C. As shown in panel (a), 22 out of 46 sera were positive by RBA, ELISA and FloCMIA protocol A. Ten sera were only positive by both RBA and ELISA, while 4 were only positive by both RBA and FloCMIA protocol A. As shown in panel (b), 24 out of 46 sera were positive by all RBA, ELISA and FloCMIA protocol B. Here, 8 sera were only positive by both RBA and ELISA, while 5 were only positive by both RBA and FloCMIA protocol B. Lastly, panel (c) shows that 32 out of 46 sera were positive by all RBA, ELISA and FloCMIA protocol C. Moreover, the latter was able to detect 13 patients positive by RBA, while ELISA could not. All of these results made protocol C the best candidate so far for replacement of RBA and/or ELISA for GADA assessment.

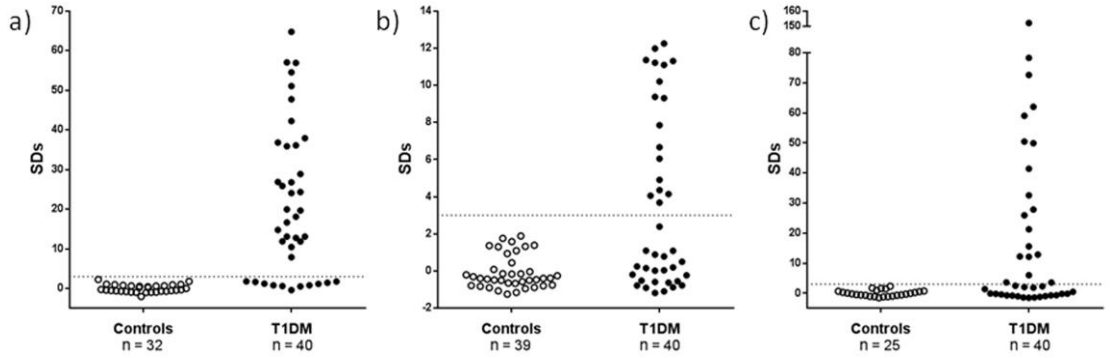

Fig. 4. **GADA results from normal human sera (controls) and children and adolescents with newly diagnosed T1DM.** The results, expressed as SDs, were obtained by RBA (a), ELISA (b) and FloCMIA protocol C (c). The cut-off value for each assay is indicated by a dotted line.

3.2. GADA detection in new-onset Type 1 diabetic patients

3.2.1. Detection of GADA by RBA, ELISA and FloCMIA protocol C

Taking into account that results from tests performed on STAN-CONICET samples showed an adequate approach for the determination of GADA, protocol C was selected to evaluate new-onset diabetic patients. Forty sera from children and adolescents with T1DM were tested in parallel by RBA, ELISA and FloCMIA protocol C. These sera showed a wide range of reactivity expressed as SDs, when RBA was performed (ranging from -0.41 to 64.76, median SDs = 17.37) (Table 2). For this population, 29 sera scored positive by RBA (Fig.4. a), observing a considerable number of borderline sera. Thus, the reference method demonstrated a representative prevalence of 72.5% in this population (percentage of Type 1 diabetic patients GADA positive from the total population studied), in agreement with previous reports (70-80%) [5, 33-36]. On the other hand, 17 GADA positive sera by RBA scored positive when evaluated by ELISA, whereas 23 patients were negative (Fig. 4. b). These results showed that ELISA had a sensitivity of 58.6%; and a prevalence of 42.5% (SDs from -1.18 to 12.25, median SDs = 0.99) (Table 2), not near to those for RBA, but similar to data previously reported for this method [10, 12]. When evaluated by FloCMIA protocol C, the prevalence

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of positive results for this population was 47.5% and the relative sensitivity obtained was 58.6% (median SDs = 2.375), a value that was lower than that for RBA. The lower sensitivity showed by FloCMIA could be a consequence of partial denaturation of TrxGAD when adsorbed on the surface of polystyrene microspheres, as discussed for ELISA. Nevertheless, we presented evidence that the structure of the molecule was correctly folded since the immobilized enzyme maintained its activity. However, when a comparison was made with ELISA, FloCMIA achieved a slightly higher prevalence. Seventeen GADA positive sera by RBA scored also positive by FloCMIA protocol C, meanwhile 2 sera resulted positive only by FloCMIA protocol C. Again, SDs for sera were higher than RBA and ELISA (SDs from -1.49 to 152.1), showing a significantly wide dynamic range (Table 2).

Assay	Median (SDs)	Range (SDs)	Sensitivity[a] (%)	Prevalence[b] (%)
RBA	17.37	(-0.41)-64.76	100	72.5
ELISA	0.99	(-1.18)-12.25	58.6	42.5
FloCMIA C	2.38	(-1.49)-152.10	58.6	47.5

[a] Percentage of patients RBA positive that were positive by each method

[b] Percentage of GADA positive patients from the total population studied

Table 2. **Analytical parameters from normal human sera and sera from children and adolescents with newly diagnosed T1DM**. The results were obtained by RBA, ELISA and FloCMIA protocol C.

3.2.2. Integrated results and correlation analysis

Venn's diagrams (Fig. 5) illustrate integrated results of RBA, ELISA and FloCMIA protocol C. Twenty-nine sera were positive by RBA, while 11, resulted negative (27.5%). However, 2 of these RBA negative sera showed GADA positivity by FloCMIA protocol C, not being considered false positives since the sera had been obtained from newly diagnosed patients with T1DM. In this sense, it is currently accepted that RBA alone is not enough to rule out the existence of GADA in childhood diabetic patients [37]. Fifteen sera were positive by all RBA, ELISA and FloCMIA methods, whereas 10 were only positive by RBA and 9 were negative by all methods. Furthermore, FloCMIA was able to detect RBA positive sera that ELISA was not.

When correlation plots for RBA and ELISA *vs*. the corresponding parallel determinations performed by FloCMIA protocol C were analyzed, the statistic parameters obtained were: r^2 = 0.2815 for RBA and FloCMIA, and r^2 = 0.6712 for ELISA and FloCMIA. To further assess the correlation between FloCMIA protocol C and the two conventional methods, paired data were subjected to a Wilcoxon matched-pairs signed rank test: the Spearman's correlation coefficient (*rs* = 0.6650 for RBA and FloCMIA, and *rs* = 0.7766 for ELISA and FloCMIA) and *P* value ($P < 0.0001$ for both analysis) showed that the pairing was not significantly effective and there was not an acceptable concordance between methods. FloCMIA protocol C joined solid phase and fluid phase-like interactions due to polystyrene microspheres, probably enabling antigen-GADA linkage

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(though it did not significantly correlate with a solid phase method or with a fluid phase one). Despite the fact that FloCMIA could not detect every low titer GADA sera (determined by RBA), as seen also for ELISA, it revealed high signals for many sera with low/medium GADA titers as shown in the correlation analysis between RBA and FlocMIA. Since GADA was successfully semi-quantified by FloCMIA in most sera that were sent to our laboratory from STAN-CONICET, this can be the method of choice even though it lacked enough sensitivity to detect this marker in all newly onset T1DM patients.

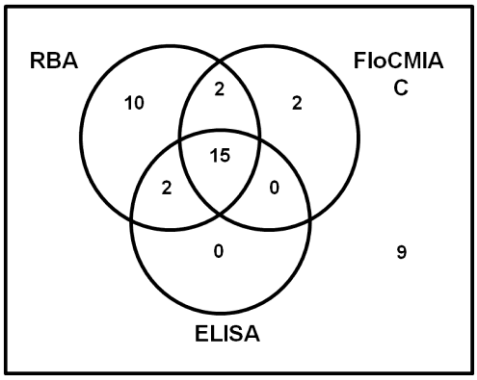

Fig. 5. **Integrated GADA results for children and adolescents with newly diagnosed T1DM.** The results obtained by RBA, ELISA, and FloCMIA protocol C are presented as Venn's diagrams.

4. Conclusion

From the experimental evidence presented herein, it may be concluded that microsphere-based immunoassays performed on Flow Cytometers constitute an innovative and cost-effective alternative to traditional determination of GADA by RBA and/or ELISA. Its advantages can be resumed as follows: i) highly sensitive detection due to fluorescent-labeling, ii) the use of easy-to-produce recombinant proteins from prokaryotic cells, iii) intact immunoreactivity of antigens after adsorption on microspheres, iv) the combination of solid and fluid phase interactions, v) a good specificity, vi) a wide dynamic range, vii) a small sample volume required, viii) low cost and environmentally harmless and ix) applicable in most medium-complexity laboratories.

In addition, the methods based on FloCMIA have also the potential to determine various humoral markers simultaneously. This is possible by using microspheres of variable diameters distinguishable by Flow Cytometers. Measuring simultaneously a number of related analytes (multiplexing) overcomes some of the limitations of conventional single and combined methods and appears to be advantageous to assess markers exhibiting differential profiles in the multiple variants and presentations of DM.

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Disclosures

This article has been written, reviewed, and approved by all contributing authors. The authors declare that they have no competing interests as defined by the Journal of Autoimmunity, or other interests that might be perceived to influence the results and discussion in this manuscript.

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Figure captions

Fig. 1. **Schematic representations of alternative FloCMIA protocols for GADA detection.** Four µm microspheres-TrxGAD65 were used as the solid phase.

Fig. 2. **GADA results from normal human sera (controls) and diabetic patients' sera from STAN-CONICET.** The results, expressed as SDs, were obtained by RBA (a), ELISA (b) and FloCMIA protocols (c, i. protocol A, ii. Protocol B and iii. Protocol C). The cut-off value for each assay is indicated by a dotted line.

Fig. 3. **Integrated GADA results for diabetic patients' sera from STAN-CONICET.** The results obtained by RBA, ELISA, and FloCMIA protocol A (a), protocol B (b) and protocol C (c) are presented as Venn's diagrams.

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Table 1. **Analytical parameters from normal human sera and patients' sera from STAN-CONICET.** The results were obtained by RBA, ELISA and FloCMIA protocols A, B and C.

Table 2. **Analytical parameters from normal human sera and sera from children and adolescents with newly diagnosed T1DM**. The results were obtained by RBA, ELISA and FloCMIA protocol C.

Electronic Supplementary Figure 1. **Four µm microspheres-TrxGAD65 set**. Population of microspheres seen in FSC vs. SSC dot plot for gating purposes (a), and FL1 vs. number of events histogram (b), for measuring of fluorescence (representative signals from normal human serum and diabetic patient's serum are shown).

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Electronic Supplementary Figure 2. **Flowchart of experimental protocols for FloCMIA A, B and C**.

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