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ORIGINAL ARTICLE

A novel LIPS assay for insulin autoantibodies

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Abstract

Aims Insulin autoantibodies (IAA) are often the first marker of autoimmunity detected in children in the preclinical phase of type 1 diabetes (T1D). Currently, the vast majority of laboratories adopt the radiobinding micro-assay (RBA) for measuring IAA. Our aim was to replace RBA with a novel non-radioactive IAA Luciferase Immuno Precipitation System (LIPS) assay with improved performance.

Methods We developed (pro)insulin antigens with alternative placements of a NanoLuc™ luciferase reporter (NLuc). Performance in LIPS was evaluated by testing sera from new onset T1D ($n = 80$), blood donors ($n = 123$), schoolchildren ($n = 186$), first-degree relatives (FDRs) from the Bart's Oxford family study ($n = 53$) and from the Belgian Diabetes Registry ($n = 136$), coded sera from the Islet Autoantibody Standardization Program (IASP) (T1D $n = 50$, blood donors $n = 90$).

Results IAA LIPS based on B chain-NLuc proinsulin or B chain-NLuc insulin, in which NLuc was fused at the C-terminus of the insulin B chain, required only 2 µL of serum and a short incubation time, showed high concordance with RBA (Spearman $r = 0.866$ and 0.833, respectively), high assay performance (B chain-NLuc proinsulin ROC-AUC = 0.894 and B chain-NLuc insulin ROC-AUC = 0.916), and an adjusted sensitivity at 95% specificity ranking on par with the best assays submitted to the two most recent IASP workshops. In FDRs, the IAA LIPS showed improved discrimination of progressors to T1D compared to RBA.

Conclusions We established a novel high-performance non-radioactive IAA LIPS that might replace the current gold standard RBA and find wide application in the study of the IAA response in T1D.

Keywords Insulin · Autoantibodies · T1D · Immunoassay · LIPS · Prediction

Abbreviations

IAA	Insulin autoantibodies
T1D	Type 1 diabetes
FDR	First-degree relative
RBA	Radiobinding micro-assay
LIPS	Luciferase Immuno Precipitation System

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BOX study	Bart's-Oxford study
BDR	Belgian Diabetes Registry
IASP	Islet Autoantibodies Standardization Program
ROC-AUC	Area under the receiver operator curve
AS95	Adjusted sensitivity at 95% specificity
NLuc	NanoLuc™ luciferase
sNLuc	Secretory NanoLuc™ luciferase
LU	Light units
AU	Arbitrary units

Introduction

Insulin autoantibodies (IAA) [1] are frequently the first autoantibodies detected in children in the preclinical phase of type 1 diabetes (T1D) [2]. Therefore, IAA measurement is a cornerstone of the study of the T1D natural history [3–5] and of all screening strategies aimed at identifying and enrolling children at increased T1D risk in prevention trials [6]. Currently, the vast majority of laboratories measuring IAA adopt the radiobinding micro-assay (RBA), based on the immunoprecipitation of radiolabeled ^{125}I-insulin with patient serum [7, 8]. While years of effort, including several international standardization workshops, have led to assay improvements [9–13], compared with assays measuring other T1D autoantibodies, the IAA RBA shows lower concordance and reproducibility across laboratories, requires more serum and a lengthy incubation, and is critically dependent on a limited number of suppliers for the provision of radiolabeled tracer. Furthermore, a significant fraction of subjects identified as single IAA positive by RBA during screening do not progress to T1D, suggesting an imperfect discrimination of IAA responses truly correlated with disease progression [14].

Currently, novel IAA assays that aim to replace RBA are under active development, of which the most mature example is an ECL method [15–17]. In particular, most efforts are aimed at substituting radiolabeled tracers with non-radioactive and more stable antigens, reducing serum consumption and assay duration, while at the same time improving assay sensitivity, specificity, and prediction of future disease development [16, 18]. In this study, we report the development of a novel IAA assay based on the Luciferase Immuno Precipitation System (LIPS) [19–24], a format in which luciferase-tagged antigens are immunoprecipitated with test sera, aimed at retaining most features of the classical IAA RBA while avoiding its shortcomings.

Materials and methods

Study subjects

All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from all individual participants included in the study. Samples (ESM Table 1) included 80 new onset T1D patients and 123 blood donors from the San Raffaele Scientific Institute; 186 non-diabetic schoolchildren and 53 non-diabetic first-degree relatives (FDRs) of patients with T1D, from the Bart's Oxford (BOX) study [25]; 136 non-diabetic FDRs from the Belgian Diabetes Registry (BDR) family study [26]; 50 new onset T1D patients and 90 blood donor controls coded samples from the 2015 and 2016 Islet Autoantibody Standardization Program (IASP) workshops.

Recombinant luciferase-tagged antigens production

Custom synthetic genes (Eurofins Genomics GmbH, Munich, Germany) of recombinant proinsulin or insulin antigens tagged with a NanoLuc™ (NLuc) luciferase reporter (Promega, Madison WI, USA) were sub-cloned into a modified pCMVTNT™ vector (Promega). To express luciferase-tagged insulins, PC1/PC2 convertase recognition sites were replaced with those of furin convertases [27–29], allowing for cleavage of the C-peptide. Antigens were expressed by transfection into Expi293F™ cells (Expi293™ Expression System, Thermo Fisher Scientific Life Technologies, Carlsbad, CA, USA). After 48 h, cells were removed by centrifugation and the culture medium harvested stored frozen at − 80 °C as single-use aliquots. The recovered luciferase activity was quantified by seeding the wells of an OptiPlate™ 96-well plate (PerkinElmer, Waltham, MA, USA) with 25-µL aliquots of serial tenfold dilutions of the antigen in 50 mM tris buffer, 1% Tween-20, pH 8 (TBT) followed by the addition of 40 µL of Nano-Glo® substrate (Promega), mixing and measurement of light units (LU) bioluminescence for 2 s/well in a Berthold Centro XS3 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

IAA LIPS assay

Antigens were diluted with TBT to adjust the luciferase activity to a final concentration of 10^7 LU/25 µL, and then filtered with a Durapore™ PVDF 0.45-µm Millex-HV

syringe filter (Millipore, Billerica, MA, USA). Immunoprecipitation was performed with and without competition of IAA binding using ACTRAPID® insulin (Novo Nordisk, Bagsværd, Denmark) added at a final concentration of 4.5×10^{-5} mol/L.

Four 2-µL replicates of each serum were pipetted into 96-deep-well plates (Beckman Coulter Inc., Brea, CA, USA), 25 µL of antigen w/o competitor was added to half of the replicates, while the rest received antigen plus competitor. Plates were then mixed briefly on a rotary shaker, centrifuged for 2′ at 500*g* at 4 °C, and incubated for 24 h at 4 °C. Immunocomplexes were captured by incubation with 5 µL of blocked [12] rProtein A and 2.5 µL of G Sepharose 4Fast Flow in 50 µL TBT (GE Healthcare Europe GmbH, Freiburg, Germany), for 1 h at 4 °C, with shaking. Plates were washed 5 times by sequential dispensing of 750 µL of TBT per well, centrifugation at 500*g* for 2′ at 4 °C, and removal of supernatant using a micro-plate plate washer (BioTek Instruments Inc., Winooski, VT, USA). Resin pellets were then transferred to an OptiPlate™, and the luciferase activity was measured after addition of each well with 40 µL of Nano-Glo® substrate, followed by a 2″ readout in the luminometer.

Acquired LU were converted to arbitrary units (AU) using a standard curve constructed from nine doubling dilutions in normal human serum of either a IAA positive serum or the monoclonal antibody HUI018 (Dako, Glostrup, Denmark), whose epitope is centered against the A chain loop of human insulin [30]. AU were calculated based on the delta of measured LU between competed and non-competed wells, using a logarithmic curve fitting algorithm in Excel (Microsoft Corporation, Redmond, WA, USA). All samples in which LU of non-competed replicates was below those of competed replicates were given a fixed value of 0.01 AU. The anti C-peptide monoclonal antibody C-PEP-01 (Santa Cruz Biotechnology, Inc. Dallas, TX, USA), directed against an epitope within the central part of the C-peptide [31], was used to check presence or absence of the C-peptide in the labeled antigen.

Statistical analysis

Statistical analysis was performed using the Prism v6 software (GraphPad Software, Inc., La Jolla CA, USA) and the pROC package for the R software [32, 33]. The area under the receiver operating characteristic (ROC) curve was used to verify the ability of the IAA LIPS test to discriminate cases from controls. Concordance of quantitative results of assays was assessed by calculating the Spearman's r correlation coefficient. Survival curves following Kaplan–Meier analysis were compared using the Mantel–Haenszel Log-rank test.

Results

Expression and immunoprecipitation of (pro)insulin luciferases

We generated and expressed in eukaryotic cells as secreted recombinant proteins eight different (pro)insulin luciferase constructs in which the NLuc reporter was placed either at the NH_2 or COOH termini of the insulin A or B chains (Fig. 1). We evaluated, by immunoprecipitation, antibody binding to each of the expressed (pro)insulin luciferases using serial dilutions of the HUI018 mAb (against a conformational epitope in the insulin A chain), the C-PEP01 mAb (against an epitope in the C-peptide), and two human sera, IAA positive and negative, respectively. All immunoprecipitations were performed with and without competition of binding using unlabeled insulin, as per the classical IAA RBA protocol. Our results show that the HUI018 mAb specifically bounds to all constructs and that the C-peptide was correctly removed from the insulin antigens (ESM Fig. 1a, b). Prolonged storage of the antigens at − 80 °C did not affect significantly luciferase activity or antibody binding up to 10 months post-freezing (ESM Fig. 2). Five constructs were selected for further analysis in LIPS using human sera: sNLuc proinsulin, B chain-NLuc proinsulin, B chain-NLuc insulin, NLuc-A chain proinsulin, and NLuc-A chain insulin.

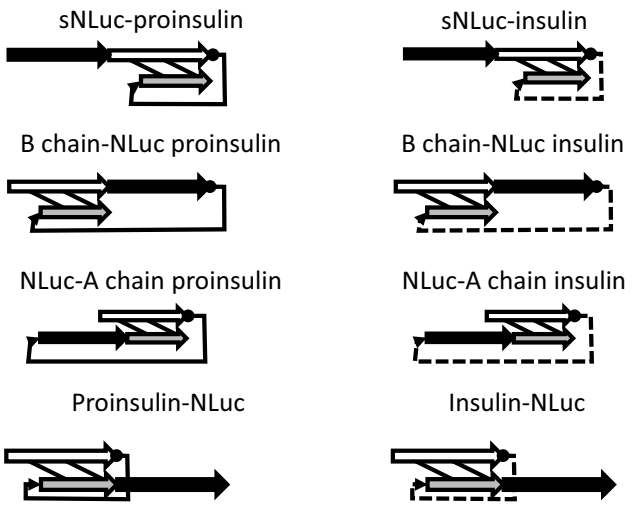

Fig. 1 Schematic representation of recombinant luciferase-tagged (pro)insulin antigens showing the placement of the NLuc reporter (black arrows) relative to the insulin B chain (white arrows) A chain (gray arrows) and C-peptide (black line). Dashed lines indicate the mutagenesis of recognition sites for native PC1/PC2 convertase to those of furin proteases

Performance in LIPS of alternative (pro)insulin luciferases

We conducted a preliminary evaluation of performance in LIPS of the selected (pro)insulin luciferases using 5 µL of serum per replicate, competition with unlabeled insulin, and 48 h incubation. All the antigens discriminated in LIPS newly diagnosed T1D ($n = 76$) from control ($n = 81$) sera, albeit with variable efficiency as measured by the analysis of the area under the ROC curve (ROC-AUC) (ESM Fig. 3a, b). Two constructs, in which the NLuc reporter was joined at the COOH terminus of the B chain and preceding the C-peptide (B chain-NLuc proinsulin and B chain-NLuc insulin), were selected for further study.

The B chain-NLuc proinsulin LIPS was then used to test FDRs from the BOX family study [25] that included high-risk subjects ($n = 15$), who either developed T1D or tested positive for multiple T1D autoantibodies, and low-risk subjects, who tested negative for other T1D autoantibodies and were either positive ($n = 18$) or negative ($n = 20$) for IAA in RBA (Fig. 2). The threshold for positivity was placed at the 97.5th percentile of AU measured by LIPS in serum samples from 186 healthy schoolchildren for both LIPS and RBA. The B chain-NLuc proinsulin LIPS showed a ROC-AUC of 0.877 (95% CI 0.762–0.993, $p < 0.0001$) compared to 0.774 for RBA (95% CI 0.648–0.899, $p = 0.002$). While the two ROC-AUCs were not statistically different ($p = 0.214$), a comparison of ROC-AUCs at a specificity greater than 90% showed a trend toward significance in this limited sample set ($p = 0.088$) (ESM Fig. 4).

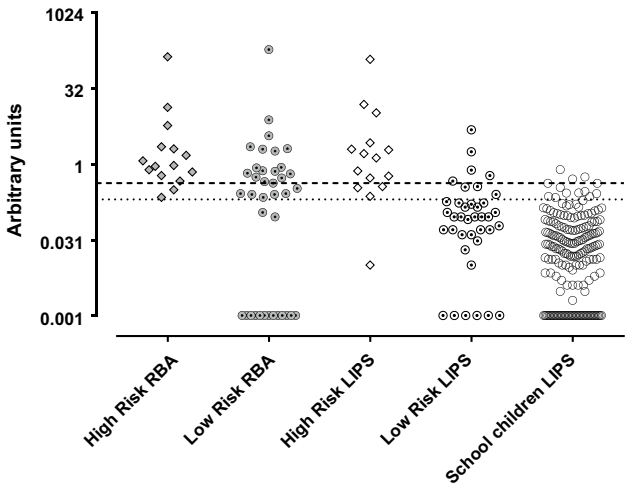

Fig. 2 B chain-NLuc proinsulin LIPS results (clear symbols) compared to RBA (gray symbols) in progressors/high-risk (diamonds $n = 15$) and non-progressors/low-risk (pointed circles $n = 38$) FDRs from the BOX study (dotted and dashed lines indicate the thresholds for positivity of RBA and LIPS placed at the 97.5th percentile of titers 186 non-diabetic schoolchild shown as clear circles)

In blind evaluation of IAA LIPS assays in IASP workshops

The B chain-NLuc proinsulin and insulin LIPS assay performance was verified by testing independently in two laboratories sets of coded human sera obtained from the IASP2015 and IASP2016 workshops. Both laboratories achieved for the B chain-NLuc proinsulin LIPS an adjusted sensitivity at 95% specificity (AS95) of 64%, ranking first of the 23 IAA assays that reported results for the 2015 workshop, and an AS95 of 50 and 47.9%, ranking third and fourth of the 25 assays reporting results in the IASP2016. The B chain-NLuc insulin LIPS showed an AS95 of 64% in IASP2015, again at the first rank of all assays in that workshop, and of 50 and 43.7% in IASP2016, at the third and sixth rank of all assays in the 2016 workshop.

The concordance of the B chain-NLuc proinsulin LIPS between the two laboratories was high, both as raw data in LU (Spearman $r = 0.967$, $p < 0.0001$ in T1D samples; Spearman $r = 0.618$, $p < 0.0001$ in control samples) and in AU (Spearman $r = 0.974$, $p < 0.0001$ in T1D samples; $r = 0.541$, $p < 0.0001$ in controls) (ESM Fig. 5). In addition, a variety of experimental conditions including reduced serum volume and assay incubation lengths were evaluated, showing that a comparable assay performance could be achieved by using only 2 µL of serum and shortening the assay incubation to 18 h (ESM Fig. 6).

Optimized B chain-NLuc (pro)insulin LIPS performance

Optimized LIPS assays using 2 µL of serum per replicate and 24-h incubation were evaluated in new onset T1D ($n = 80$) and control ($n = 123$) samples. The proinsulin LIPS showed a ROC-AUC of 0.894 (95% CI 0.841–0.946, $p < 0.0001$), while the insulin LIPS showed a ROC-AUC of 0.916 (95% CI 0.872–0.960, $p < 0.0001$) (Fig. 3). The results in T1D sera were highly correlated between LIPS assays (Spearman $r = 0.978$, 95% CI 0.965–0.986, $p < 0.0001$) and between LIPS assays and RBA (B chain-NLuc proinsulin: Spearman $r = 0.866$, 95% CI 0.779–0.921, $p < 0.0001$; B chain-NLuc insulin: $r = 0.833$, 95% CI 0.726–0.901, $p < 0.0001$) (ESM Fig. 7).

B chain-NLuc proinsulin LIPS performance in FDRs

The optimized B chain-NLuc proinsulin LIPS assay was used to test in blind 136 FDRs from the BDR family study [26], and its results were compared to those of RBA. For both tests, the threshold for positivity was placed at the 99th percentile of control titers. A Kaplan–Meier analysis highlighted a different rate of progression to T1D in FDRs IAA positive in LIPS ($n = 43$) with a diabetes-free

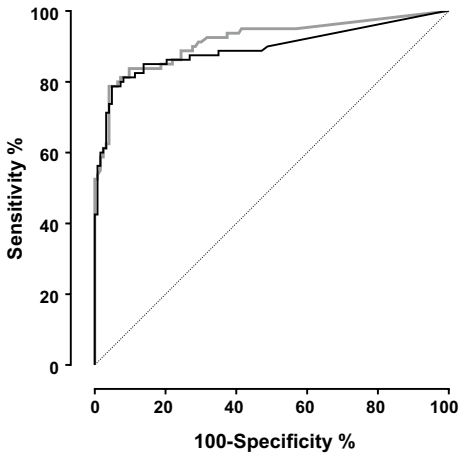

Fig. 3 ROC curve analysis of optimized B chain-NLuc proinsulin (black line) or B chain-NLuc insulin (gray line) LIPS performance in a cohort of new onset T1D ($n = 80$) and control ($n = 123$) samples (the dotted line represents the identity line)

survival fraction at 225 months of follow-up of 47%, compared to 79% in the IAA negatives ($n = 93$) (Mantel–Haenszel Log-rank test $p < 0.0001$) (Fig. 4a). Diabetes-free survival at 240 months was 59.6% in the IAA positives in RBA ($n = 44$), compared to 74% in IAA negatives ($n = 92$) (Mantel–Haenszel Log-rank test $p = 0.0804$) (Fig. 4b). After grouping according to the concordance of LIPS and RBA results, the diabetes-free survival fraction in FDRs at 181 months was 40% in subjects positive both in LIPS and in RBA, 48% in subjects positive only in LIPS, 90% in the subjects that tested IAA positive only in RBA, and 78% in subjects negative for both assays (Fig. 4c).

All FDRs who tested IAA positive exclusively in LIPS ($n = 9$) also had additional T1D associated autoantibodies (5 subjects with single additional GADA and 4 with multiple auto-Abs). During follow-up, progression to T1D was observed in 5 subjects of the 9 IAA positive only in LIPS (1 with a single GADA and in all 4 with multiple auto-Abs). Conversely, among the 10 FDRs who tested IAA positive exclusively in RBA, additional antibodies were found in only 4 subjects (2 single GADA, 1 single IA-2A, and 1 single ZnT8A positive) of which only one progressed to T1D at a

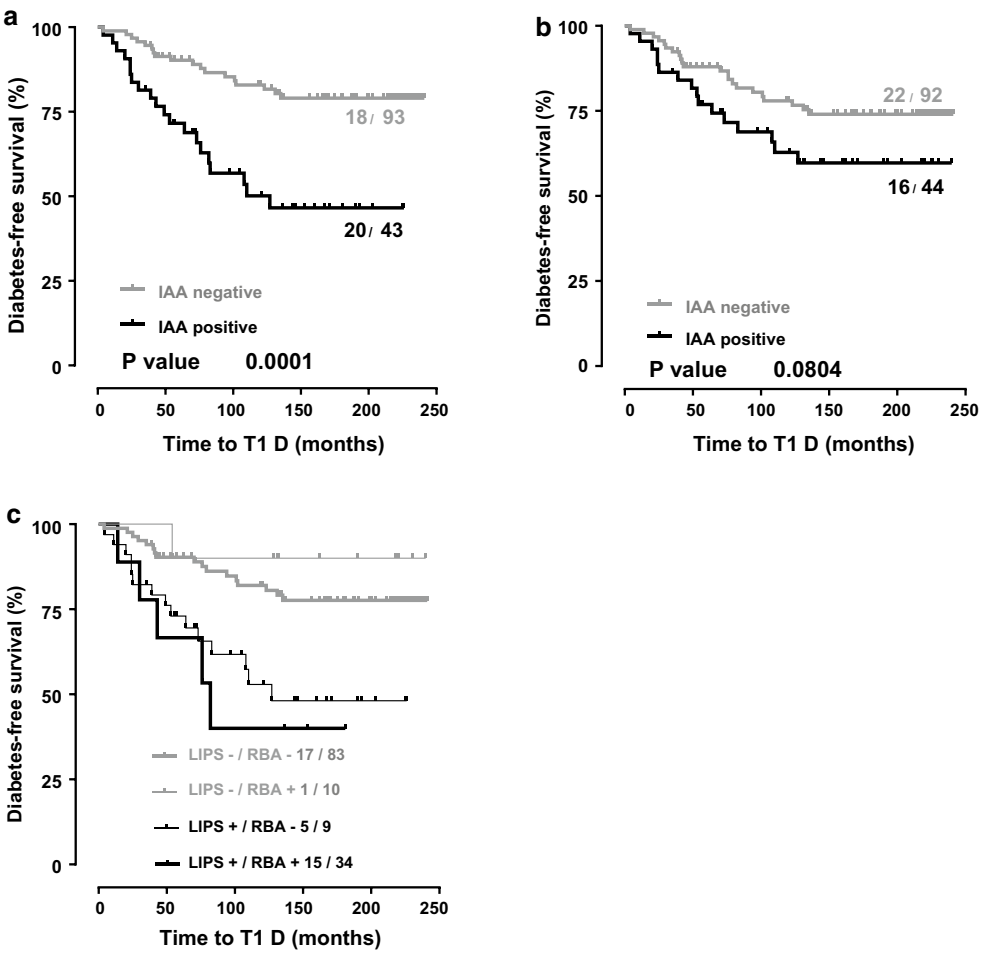

Fig. 4 Diabetes-free survival in 136 FDRs from the BDR study tested in the B chain-NLuc proinsulin LIPS (**a**) or RBA (**b**) or stratified according to presence or absence of IAA in both assays, (**c**) on the graphs are shown the number of progressors and the total number of subjects in each group and the *p* value of the Mantel–Haenszel Log-rank test

later stage. After stratification of the FDRs according to the presence of other T1D autoantibodies (0–3 additional autoAbs), the impact on T1D-free survival of IAA positivity in either LIPS or RBA did not reach statistical significance in any of the four grouping categories (ESM Fig. 8).

Discussion

In this study, we developed novel recombinant (pro)insulins tagged with a luciferase reporter and evaluated their performance as antigens for measuring IAA in LIPS, an immunoassay already successfully applied to the measurement of other T1D-associated autoantibodies [19–24].

Past studies led to the production of numerous variants of recombinant (pro)insulin proteins; however, we are aware of only two instances in which large GFP or firefly luciferase reporters were fused either within the C-peptide or at the carboxyl terminus of the proinsulin sequence [34, 35]. In both cases, the secretion of mature insulin from cells transfected with these tagged proinsulins could be confirmed, but was nevertheless associated with substantial misfolding and retention in the endoplasmic reticulum.

Initially, our work focused on the generation of (pro)insulins joined to NLuc [36], the smallest commercially available luciferase, and on the systematic analysis of parameters that could potentially affect IAA binding to NLuc-(pro)insulins, including the placement of the reporter relative to the antigen sequence. Our results indicate that, despite the NLuc relatively large size compared to proinsulin, NLuc-(pro) insulins are constitutively secreted by transfected eukaryotic cells and undergo post-translational modifications, including the removal of the C-peptide. More importantly, the engineered (pro)insulins appear to be at least partly correctly folded, an essential requirement for their use in assays measuring IAA [9], as implicitly demonstrated by their recognition, albeit with variable efficiency and background levels, by both monoclonal and human serum antibodies directed against conformational epitopes.

We selected two recombinant proteins in which NLuc was joined at the carboxyl terminus of the insulin B chain (B chain-NLuc proinsulin and B chain-NLuc insulin, respectively) as the most promising antigens for IAA measurement in LIPS. We further confirmed the ability of our IAA LIPS to discriminate cases from controls by testing *in blind* new onset T1D and blood donor sera distributed in IASP2015 and IASP2016, the most recent NIDDK sponsored workshops for the standardization of islet autoantibodies [13], and achieve a performance on a par with the best IAA assays that participated in the two workshops.

Overall, the comparison of LIPS and RBA results showed a high degree of correlation between the two tests, suggesting that the IAA responses measured by LIPS in new onset T1D patients are mostly consistent with those in obtained by RBA. Furthermore, our preliminary results in a selection of FDRs from the BOX study [25] and from the BDR [26] suggested that LIPS might be more sensitive and specific when used to discriminate future progressors to T1D from subjects with lower risk. However, while in isolation the IAA LIPS showed an advantage over RBA, our study was not sufficiently powered to clarify whether there might be an added benefit of IAA measurement by LIPS in discriminating progressors from non-progressors in the context of strategies based on multiple autoantibody testing. Similar to previous validation studies [18], only the testing of very large cohorts of subjects at risk of T1D will allow the superiority of the IAA LIPS assay over RBA for prediction of T1D, particularly in young children, to be confirmed.

Compared to RBA, LIPS offers several practical advantages, primarily in the avoidance of radiolabeled tracers, a major source of RBA variability and complexity, because of the short life of ^{125}I-insulin and the cumbersome and expensive requirements linked to the use of radioactive substances. In addition to using a non-radioactive tracer with a potentially very long half-life, LIPS offers a greatly shortened assay duration and requires less than half the amount of serum (2 vs 5–30 µL per replicate), a noteworthy advantage when IAA testing is performed on precious and essentially irreplaceable archival samples collected for T1D natural history studies [37–40], or from capillary blood samples increasingly used for general population screening [6, 41].

Compared to ECL, another novel non-radioactive immunoassay and currently the only other successfully validated IAA assay format [15, 18], the IAA LIPS offers the benefit of reduced serum consumption, relies on relatively inexpensive and widely available equipment and commercial reagents, and is amenable to future implementation on more advanced micro-fluidic platforms [42, 43].

In conclusion, we have established a novel non-radioactive IAA test that offers high performance while preserving an assay procedure overall similar to the gold standard IAA RBA. This suggests that the IAA LIPS might become an easily transferable replacement for tests based on radiolabeled antigen that might find wide application in the study of the IAA response in T1D.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of Human and Animal Rights All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

Informed consent Informed consent was obtained from all patients for being included in the study.

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