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## Method Article

# ELISA based on peptide antigens reproducing cross-reactive viral epitopes to detect antibodies in latent autoimmune diabetes in adults vs. type 1 diabetes



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## A B S T R A C T

Diagnosis of Latent Autoimmune Diabetes in Adults (LADA) is based on the adult-age, anti-islet autoantibodies, and temporary insulin-independence. As in Type-1-Diabetes (T1DM), autoimmunity may trigger LADA and enteroviruses-infections can play a role. Anti-human Glutamic-Acid-Decarboxylase (hGAD) autoantibodies are accepted clinical biomarkers, but do not discriminate LADA vs. T1DM. The hypothesis is that protein antigens detecting anti-hGAD antibodies do not expose epitopes specific for different disease forms.

We investigated the diagnostic value of autoantibodies in LADA vs. T1DM to peptides of hGAD65/67 isoforms, and Enterovirus-Coxsackie-B4 (CVB4), as antigens sharing the epitope PEVKXX (X: E/T) included in CD8 T-cell CVB4 epitope restricted by diabetes-associated HLA-A2.1. Statistically significant differences of IgM and/or IgG in LADA and T1DM vs. controls were identified. In LADA IgMs to GAD65/67 peptides are diagnostics, IgGs to GAD65/67 peptides correlate with anti-CVB4 peptide antibodies. IgM and/or IgG to all tested peptides can predict LADA, monitoring CVB4 infected patients, improving LADA vs. T1DM stratification.

DOI of original article: [10.1016/j.cca.2021.01.002](https://doi.org/10.1016/j.cca.2021.01.002)

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<https://doi.org/10.1016/j.mex.2021.101452>

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- A customized SP-ELISA based on synthetic peptides Ac-hGAD65(250-273)-NH<sub>2</sub> (**1**), Ac-hGAD67(258-281)-NH<sub>2</sub> (**2**), and Ac-CVB4P2C(28-50)-NH<sub>2</sub> (**3**) is described.
- The method was designed to detect specific IgM and/or IgG in LADA, T1DM, vs. controls
- Final aim is improvement of LADA vs. T1DM patient stratification.

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## ARTICLE INFO

*Method name:* Customized peptide-based solid-phase Enzyme Linked ImmunoSorbent Assay

*Keywords:* Glutamic acid decarboxylase, Enterovirus Coxsackie B4, LADA, T1DM

*Article history:* Received 22 March 2021; Accepted 10 July 2021; Available online 12 July 2021

## Specifications table

Subject area:	Immunology and Microbiology
More specific subject area:	Diagnostics of autoimmune diabetes
Method name:	Customized peptide-based solid-phase Enzyme Linked ImmunoSorbent Assay
Name and reference of original method:	Not applicable
Resource availability:	Not applicable

## Method details

One of the challenges in diagnosis of Latent Autoimmune Diabetes in Adults (LADA) vs. Type 1 Diabetes Mellitus (T1DM) is the development of a simple immunodiagnostic method helping clinicians in patient stratification. In fact, diagnosis and prognosis of LADA is, up to now, usually based on: 1) the adult age; 2) anti-islet autoantibody detection; 3) insulin independence [1].

Since years, we have extensively demonstrated that synthetic peptides including or mimicking epitopes for high affinity antibody recognition can be used as efficient antigenic probes to replace proteins in immunoenzymatic assays, increasing specificity of the assay [2,3]. Recently we have identified antibodies to post-translational modified mitochondrial peptide PDC-E2(167-184) in T1DM [4]. Few peptide-based ELISA are commercially available for different autoimmune diseases [5].

Herein, we propose a detailed Enzyme Linked ImmunoSorbent Assay (ELISA) that has been successfully used to measure specific antibodies in patient sera, using three synthetic peptides – Ac-hGAD65(250-273)-NH<sub>2</sub> (**1**), Ac-hGAD67(258-281)-NH<sub>2</sub> (**2**), and Ac-CVB4P2C(28-50)-NH<sub>2</sub> (**3**) – as antigens of human Glutamic Acid Decarboxylase hGAD65/67 isoforms and Enterovirus-Coxsackie-B4 (CVB4), respectively. The three peptides share the sequence PEVKXX (X: E/T) that is included in the CD8 T-cell CVB4 epitope and restricted by the diabetes-associated HLA-A2.1 molecule [6]. In particular, they were designed based on the homology between hGAD65 and CVB4. In fact, both sequences contain the "PEVKEK" fragment, which differs by a single residue in the hGAD67 protein containing a threonine (T) instead of a glutamic acid (E) residue. Following the hypothesis of a molecular mimicry effect in LADA patients, we hypothesize that anti-virus antibodies that are generated during the infection, may not be able to distinguish between a self- and a non-self antigen, attacking indiscriminately both of them. Therefore, we decided to evaluate antibodies to the three peptides. The method to detect serum IgM and/or IgG antibody profiles in LADA, T1DM, and controls, including statistical analysis, is proposed with the final aim to improve patient stratification.

## Patient selection

LADA patients (27) and T1DM patients (23) regularly attending the Diabetology and Dietetics outpatient Clinic of Padova University, and 24 controls were recruited for the study. The sample size was determined applying a non-parametric Mann-Whitney test, indicating that 80% power at 5% significance level is obtained selecting 22 subjects per group to detect a Cohen's  $d = 0.9$  effect size

**Table 1**  
Selected hGAD and CVB4P2C peptide sequences.

No	Peptides	Sequence
1	<b>Ac-hGAD65(250-273)-NH<sub>2</sub></b>	Ac- <sup>250</sup> AMMIARFKMFPEVKEKGMALPRL <sup>273</sup> -NH <sub>2</sub>
2	<b>Ac-hGAD67(258-281)-NH<sub>2</sub></b>	Ac- <sup>258</sup> SIMAARYKYFPEVKTKGMAAVPKL <sup>281</sup> -NH <sub>2</sub>
3	<b>Ac-CVB4P2C(28-50)-NH<sub>2</sub></b>	Ac- <sup>28</sup> FIEWLKVILPEVKEKHEFLSRL <sup>50</sup> -NH <sub>2</sub>

in antibodies against the selected peptides between LADA/T1DM vs. control. Recruitment considered a potential drop-out and the total number of subjects, was adjusted in the groups of interest.

LADA patients were selected only on the positivity to the clinical anti-GAD ELISA (IgG) test kit (EUROIMMUN) and enrolled at diagnosis. T1DM subjects were treated with insulin (4 injections/day). None of the patients had monogenic diabetes [7] and/or type 2 diabetes. Fasting plasma glucose (FPG), HbA1c, and autoantibody levels were detected in blood samples collected in the morning, after a 12 h overnight fast. After collection, blood was immediately centrifuged at 1700 g, 4 °C for 20 min. Glucose was measured immediately, and the rest of the sample was frozen to -20 °C until analysis.

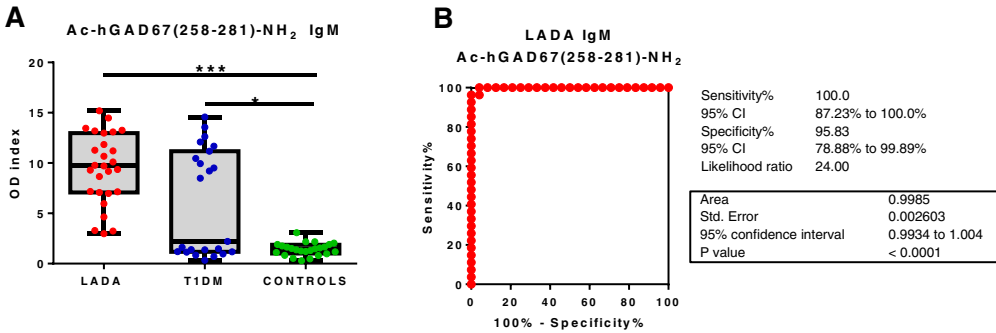
Mean age was significantly lower in T1DM with respect to LADA and control subjects ( $13.9 \pm 10.5$  vs.  $38.9 \pm 13.5$ , vs.  $35.0 \pm 5.0$  years,  $p < 0.001$ ). Diabetic patients have significantly higher values of fasting plasma glucose and HbA1c with respect to controls. No differences were found in terms of metabolic control (FPG and HbA1c), residual  $\beta$ -cell secretion (C-peptide levels), and amount of insulin requested to obtain an acceptable glycemic control in the two groups.

The study used patient information available on the database of the Department of Medicine (DIMED), University of Padova (Italy). The study protocol was approved by the ethical committee for clinical study n. 149 DCCT/HbA1c and was conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from subjects before their participation in the study.

#### Customized SP-ELISA method for antibody profile determination

Antibody responses were determined in solid-phase ELISA (SP-ELISA). In previous studies, mimicry between the isoforms 65 and 67 of hGAD and the P2C protein of the CVB4 has been evidenced, hypothesizing that an early infection could be a possible environmental cause of the pathology [8]. Following this idea, the synthetic peptides Ac-hGAD65(250-273)-NH<sub>2</sub> (1), Ac-hGAD67(258-281)-NH<sub>2</sub> (2), and Ac-CVB4P2C(28-50)-NH<sub>2</sub> (3), covering the shared sequence, were synthesized and used as antigens (Table 1). All peptides were synthesized in the Centre of Competences of Molecular Diagnostics & Life Sciences PeptLab-MoD&LS of the University of Florence [6] and further used in SP-ELISA.

All buffers were daily prepared and maintained at room temperature prior to use, and filtered employing 0.22  $\mu$ m filters. 96-Well activated polystyrene ELISA microplates (NUNC Maxisorp) were coated with peptides **1-3** separately, using a solution 10  $\mu$ g/mL of diluted peptide in the coating buffer (carbonate buffer, 12 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, pH 9.8) and incubated overnight at 4 °C (100  $\mu$ L/well). The plates were then washed 3 times with washing buffer (0.9% NaCl, 0.05% Tween 20) using an automatic microplate washer (Tecan HydroFlex), and then blocked for 1h at room temperature using Fetal Bovine Serum (FBS) buffer (10% FBS in washing buffer, 100  $\mu$ L/well) to block uncovered sites on the microplate. After 3 washes, plates were incubated with patient sera and healthy control sera diluted 1:100 in FBS buffer (100  $\mu$ L/well) in triplicate at 4 °C overnight. After 3 washes, plates were then incubated with alkaline phosphatase conjugated anti-human IgG or anti-human IgM, opportunely diluted in FBS buffer (1:8000 and 1:200 respectively, 100  $\mu$ L/well). After 3 h incubation at room temperature and 3 washes, substrate solution (1 mg/mL p-nitrophenylphosphate (pNPP) in carbonate buffer with 10 mM MgCl<sub>2</sub>) was added (100  $\mu$ L/well). After 45 min approximately of incubation at room temperature (1 h in case of IgM detection), the absorbance was read in a multichannel ELISA reader at 405 nm. ELISA plates, coating conditions, reagent dilutions, buffers, and incubation times were previously tested. Each sample serum was tested in triplicates (3 rows/plate) and blanks were performed using FBS buffer instead of sample sera using identical conditions. The optical density (OD) index value for each serum was calculated as (mean



**Fig. 1. (A) Antibody reactivity among peptides.** Box plots of IgM and IgG antibody titers [expressed as optical density (OD) index] in LADA, T1DM, and control sera, identified by SP-ELISA, using peptide antigen Ac-hGAD67(258-281)-NH<sub>2</sub> (2). Data are presented as mean values with a significance level of \* $p < 0.05$  and \*\*\*  $p < 0.0001$  (Kruskal–Wallis test followed by *post hoc* Dunn's test vs. controls, with Bonferroni's adjustment for multiple comparisons).

**(B) Receiver Operating Characteristic (ROC) curve.** ROC curve analysis of antibodies to peptide antigen Ac-hGAD67(258-281)-NH<sub>2</sub> (2) in LADA or T1DM patient sera vs. controls for IgMs determined by SP-ELISA.

Abs of serum triplicate) – (mean Abs of blank triplicate). One positive and one negative serum, as references, were included in each plate for further normalization. Each experiment was performed at least twice in different days. Within-assays and between-assays coefficients of variations were below 10%.

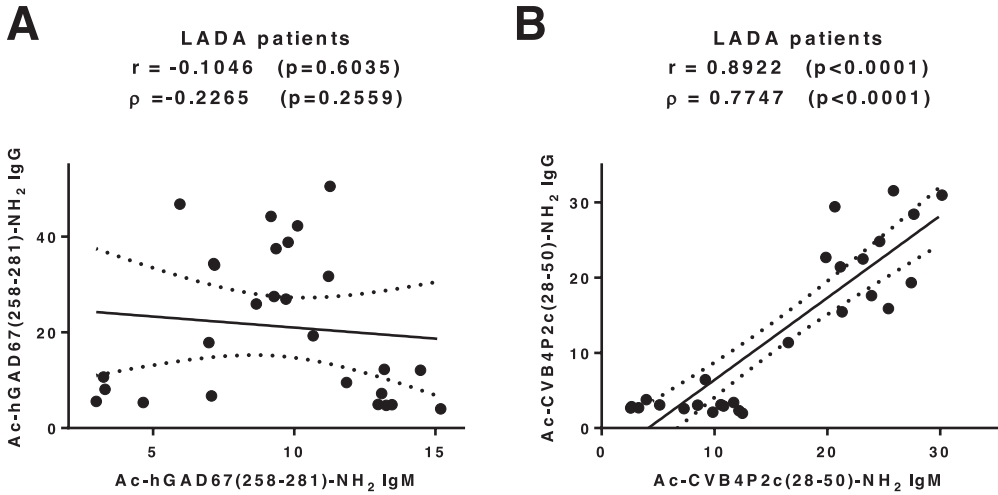
#### Statistical analysis: antibody profile evaluation and diagnostic power

Data were expressed as optical density (OD) index calculated as the mean absorbance values of replicates  $\pm$  SD and normalized to internal controls. An example of plot reporting values including the median and interquartile range (IQR) calculated as first quartile subtracted from the third quartile is shown in Fig. 1A. The statistical evaluation of normally distributed continuous variables was obtained applying ANOVA followed by Tukey's *post hoc* test. Moreover, we observed that a normal distribution cannot be always granted by using Shapiro-Wilk test. Therefore, data from the 3 groups of subjects were evaluated with nonparametric Kruskal–Wallis test followed by appropriate *post hoc* analysis (Dunn's test with Bonferroni's adjustment for multiple comparisons).

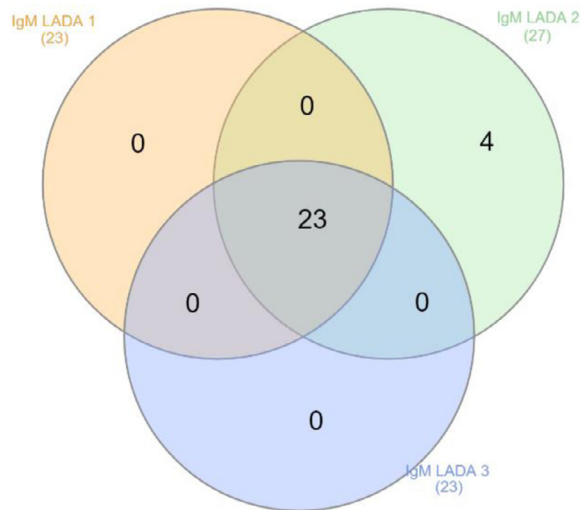
The statistical analysis continued applying the receiver operating characteristic (ROC) curve to evaluate the discrimination power of the peptides, calculating the corresponding cut-off values, sensitivities, and the rest of the statistical parameters following the previously described protocol [9]. We set-up a specificity value higher than 95%, taking into account the optimal likelihood ratio and then applying the corresponding cut-off value. A representative ROC-curve analysis for peptide Ac-hGAD67(258-281)-NH<sub>2</sub> (2) is reported in Fig. 2B. The characteristics of the curve, the shape and steepness, and the underlying area are useful to evidence the ability of the three synthetic peptides 1-3 as diagnostics in LADA patients in comparison with controls, with a high degree of confidence at the selected cut-off values ( $p$  values  $< 0.0001$ ).

#### Correlation studies

Correlation among peptides was studied applying the Pearson's  $r$  correlation coefficient, as well as the non-parametric Spearman's  $\rho$  rank correlation coefficient. A value of  $p < 0.05$  (two-tailed) was considered as statistically significant. Examples of no correlation and correlation are shown in Fig. 2 (Panel A and B, respectively). A correlation study between IgM and IgG-isotype antibodies is shown in Fig. 2A, evidencing that IgG and IgM-type antibodies are not characteristic of the same patient population. This is relevant to be considered when evaluating antibody profiles for patient stratification.



**Fig. 2. IgM and IgG correlation analysis.** Correlation between IgM and IgG-isotype antibodies [expressed as optical density (OD) index] to the peptides Ac-hGAD67(258-281)-NH<sub>2</sub> (2) and Ac-CVB4P2c(28-50)-NH<sub>2</sub> (3) in LADA patients. Pearson's  $r$  correlation coefficient, as well as the Spearman's  $\rho$  rank correlation coefficient, with their respective  $p$  values, are reported in each plot. Linear regression lines are shown as continuous lines; dashed lines indicate the 95% confidence interval of the best-fit line.



**Fig. 3. Venn diagram.** Venn diagram of serum cross-reactivity for IgMs positive to peptides Ac-hGAD65(250-273)-NH<sub>2</sub> (1), Ac-hGAD67(258-281)-NH<sub>2</sub> (2), and Ac-CVB4P2c(28-50)-NH<sub>2</sub> (3) in LADA patients

The Spearman correlation study does not show any correlation between antibodies against commercial GAD65 ELISA kit and the studied peptides Ac-hGAD65(250-273)-NH<sub>2</sub> (1), Ac-hGAD67(258-281)-NH<sub>2</sub> (2), and Ac-CVB4P2c(28-50)-NH<sub>2</sub> (3). These results have been observed both for IgGs and IgMs. Our results are in agreement with previous observations by other authors [10] and our group [2,11], demonstrating that peptide sequences mimicking discontinuous protein antigenic determinant can disclose an antibody response hidden in recombinant and/or isolated protein antigens from a natural source used in most of the commercially available immunoassays. Therefore, the synthetic peptide antigens are identifying a family of antibodies possibly directed to a particular epitope inside the whole protein GAD65.

A deep study on IgM cross-reactivity in LADA screened sera has been performed by using the Venn diagram [12]. The use of overlapping circles to illustrate the logical relationships between IgMs among the three peptide antigens proposed, helps to graphically organize positivities among peptides, highlighting how the peptides identify similar or different patient populations. An example of IgMs positive to peptides Ac-hGAD65(250-273)-NH<sub>2</sub> (**1**), Ac-hGAD67(258-281)-NH<sub>2</sub> (**2**), and Ac-CVB4P2C(28-50)-NH<sub>2</sub> (**3**) is shown in Fig. 3. The diagram helps to recognize an increased % of positivity for peptide **2**, able to recognize up to 4 patients more than peptides **1** and **3**. Positive patients were identified applying the corresponding cut-off values previously calculated with a ROC-curve analysis. The thresholds were independently calculated for each peptide and antibody type.

This methodology is useful to understand the ability of anti-peptide antibodies to react with their cognate sequence in intact folded proteins. Additionally, the quantification of IgMs (particularly if reminiscent of a viral epitope) might improve the stratification of patients in an early stage of the disease, and can be promising diagnostic tools for overcoming missing or incorrect diagnosis of LADA, particularly if triggered by an immune response starting years before the onset of the disease.

In conclusion, the customized peptide-based SP-ELISA developed to identify IgM and/or IgG-isotype serum antibodies, allows the identification of statistically significant differences between LADA and T1DM vs. controls.

In particular, in LADA, IgMs to GAD65/67 peptides **1** and **2** are diagnostics and IgGs to GAD65/67 peptides **1** and **2** correlate with anti-CVB4 peptide **3**. Therefore, IgM and/or IgG to peptides **1**, **2**, and **3** can predict LADA, monitoring CVB4 infected patients, improving LADA vs. T1DM patient stratification.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Acknowledgements

We agree to publish a standard "thank you" note in the article to acknowledge the efforts made by the respective reviewers.

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