




## RESEARCH ARTICLE

# Antibodies to oxidized insulin improve prediction of type 1 diabetes in children with positive standard islet autoantibodies

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## Abstract

**Background:** Antibodies to posttranslationally modified insulin (oxPTM-INS-Ab) are a novel biomarker of type 1 diabetes (T1D). Here, we evaluated whether oxPTM-INS-Ab can improve T1D prediction in children with positive standard islet autoantibodies (AAB).

**Methods:** We evaluated sensitivity, specificity, accuracy, and risk for progression to T1D associated with oxPTM-INS-Ab and the standard islet AAB that include insulin (IAA), GAD (GADA), and tyrosine phosphatase 2 (IA-2A) in a cohort of islet AAB-positive (AAB<sup>+</sup>) children from the general population (median follow-up 8.8 years).

**Results:** oxPTM-INS-Ab was the most sensitive and specific autoantibody biomarker (74% sensitivity, 91% specificity), followed by IA-2A (71% sensitivity, 91% specificity). GADA and IAA showed lower sensitivity (65% and 50%, respectively) and specificity (66% and 68%, respectively). Accuracy (AUC of ROC) of oxPTM-INS-Ab was higher than GADA and IAA ( $P = 0.003$  and  $P = 0.017$ , respectively), and similar to IA-2A ( $P = 0.896$ ). oxPTM-INS-Ab and IA-2A were more effective than IAA for detecting progr-T1D when used as second-line biomarker in GADA<sup>+</sup> children. Risk for diabetes was higher ( $P = 0.03$ ) among multiple AAB<sup>+</sup> who were also oxPTM-INS-Ab<sup>+</sup> compared with those who were oxPTM-INS-Ab<sup>-</sup>. Importantly, when replacing IAA with oxPTM-INS-Ab, diabetes risk increased to 100% in children with oxPTM-INS-Ab<sup>+</sup> in combination with GADA<sup>+</sup> and IA-2A<sup>+</sup>, compared with 84.37% in those with IAA<sup>+</sup>, GADA<sup>+</sup>, and IA-2A<sup>+</sup> ( $P = 0.04$ ).

**Conclusions:** Antibodies to oxidized insulin (oxPTM-INS-Ab), compared with IAA which measure autoantibodies to native insulin, improve T1D risk assessment and prediction accuracy in AAB<sup>+</sup> children.

## KEYWORDS

biomarker, insulin autoantibodies, posttranslational modifications, prediction, type 1 diabetes

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Rocky Strollo and Chiara Vinci contributed equally to the work. Paolo Pozzilli, Johnny Ludvigsson, and Ahuva Nissim share senior authorship.

## 1 | INTRODUCTION

Type 1 diabetes (T1D) is characterized by a prodromal phase of variable duration marked by islet cytoplasmic autoantibodies (ICA) and circulating islet autoantibodies (AAB) to insulin (IAA), GAD (GADA), tyrosine phosphatase 2 (IA-2A), and zinc transporter 8 (ZnT8A).

Several approaches have been tested for effective prediction of T1D, ranging from genetic risk assessment, number and levels of islet AAB, and appearance of dysglycaemia.<sup>1-3</sup> Islet AAB represent the most robust approach to predict progression to clinical T1D.<sup>4</sup> Number of AAB may incrementally increase the risk of progression.<sup>1,4</sup> When used in combination, islet AAB can identify up to 80% or more of patients at disease onset or at risk of developing disease,<sup>5</sup> but except for IA-2A, their predictive potential is extremely reduced when assessed in single test.<sup>6</sup> Based on this, staging of preclinical T1D has been recently updated to include multiple AAB, also termed islet autoimmunity, as the earliest stage of T1D.<sup>7</sup> Measurement of islet AAB has been instrumental for selecting patients entering clinical trials, but currently fall below the high sensitivity and specificity needed for detection of preclinical diabetes in the general population, where the prevalence of diabetes is of the order of 0.3%.<sup>8</sup> Furthermore, newly identified islet antibody specificities might redefine diabetes risk and disease staging.

Recent evidence by our group and others have shown that chemical or enzymatic posttranslational modifications (PTM) of self-antigens, such as oxidation,<sup>9,10</sup> glycosylation,<sup>9</sup> citrullination,<sup>11,12</sup> and deamination,<sup>13</sup> provide a large and heterogeneous source of neoepitopes able to breach immune tolerance in T1D. This concept is well established in other autoimmune diseases such as rheumatoid arthritis (RA) where citrullination and oxidation are prevalent traits of the disease. Indeed, anticitrullinated peptide antibodies (ACPA) have been included in the classification criteria for RA,<sup>14</sup> while antibodies to oxidative PTM (oxPTM) collagen type II have been recently proposed as a highly accurate biomarker of the disease.<sup>15</sup>

We have demonstrated the proof of concept that oxPTM of insulin are neoantigens. Most of T1D subjects<sup>10</sup> or prediabetic children<sup>16</sup> have autoantibodies to oxPTM insulin (oxPTM-INS-Ab). We showed that oxPTM-INS-Ab is highly accurate for diabetes diagnosis<sup>10</sup> and may identify subjects who are negative to the other standard islet AAB, and its appearance can antedate diabetes diagnosis by several years.<sup>16</sup> However, it is not clear whether oxPTM-INS-Ab can improve early diagnosis and prediction in association with the standard islet AAB.

Therefore, the primary aim of this study was to evaluate the ability of oxPTM-INS-Ab to predict T1D in children with positive standard islet AAB; primary outcome was to evaluate its predictive accuracy compared with autoantibodies to native insulin, IAA. As secondary aim, we evaluated whether oxPTM-INS-Ab was able to refine diabetes risk in multiple AAB positive (AAB<sup>+</sup>) children. We therefore analysed data available<sup>16</sup> from AAB<sup>+</sup> children and prediabetic children from the All Babies in Southeast Sweden (ABIS) cohort, a large prospective study in the general population.

## 2 | MATERIALS AND METHODS

### 2.1 | Subjects

We used serum samples obtained from the ABIS study, a large prospective birth cohort study in which 17 055 unselected children from the general population born between 1 October 1997 to 1 October 1999 have been followed prospectively with regular evaluation of islet AAB before the development of T1D.<sup>17,18</sup> Of the screened children, 116 developed type 1 diabetes during the follow-up. In the present study, we analysed data available from 23 children progressing to type 1 diabetes (progr-T1D), collected longitudinally before diagnosis at three different time points (at the ages of 5, 7, and 11 years), and 32 children who did not progress to T1D despite being islet AAB positive (NP-AAB<sup>+</sup>), after a median follow-up of 10.8 years (IQR 7.7-12.8). NP-AAB<sup>+</sup> children were defined as positive to at least one standard islet AAB (IAA, GADA, and/or IA-2A). Samples were selected where sufficient serum and autoantibody data were available for this study. A comprehensive description of clinical and biochemical features, as well as prevalence of oxPTM-INS-Ab, in the studied population is extensively reported elsewhere.<sup>16</sup> Progr-T1D were selected irrespective of AAB status; 19 of 23 progr-T1D were positive to at least one standard islet AAB (GADA, IAA, and/or IA-2A), while two were negative to the standard islet AAB but positive to oxPTM-INS-Ab. Overall, progr-T1D and NP-AAB<sup>+</sup> were all positive to at least one of the following tested autoantibodies—GADA, IAA, IA-2A, and oxPTM-INS-Ab. Informed consent was obtained from parents prior to the collection of blood. The study was approved by the Research Ethics Committees of the Medical Faculties of Linköping University, Linköping, and Lund University, Lund, Sweden, and by the Ethics Committee of the Università Campus Bio-Medico di Roma.

### 2.2 | Detection of oxPTM-INS-Ab

Antibodies to insulin modified by hydroxyl radical (oxPTM-INS) were measured by our developed ELISA platform. Insulin was chemically modified as previously described to generate oxPTM-INS modified by hydroxyl radical (\*OH).<sup>10,16</sup> Hen egg lysozyme (HEL; Sigma) was similarly modified and used as control antigens. An ELISA was performed using native and oxPTM-INS or control native and oxPTM-HEL as targets. Development and calibration of the ELISA are described in our previous publications.<sup>10,16</sup> Briefly, ELISA plates (Nunc, London, UK) were coated with 10 µg/ml of modified or native protein in 0.05 mol/l carbonate/bicarbonate buffer (pH 9.6) at 4°C overnight. Plates were then washed three times with PBS. After blocking for 2 hours with 5% BSA in 0.5% Tween PBS, 100 µl of 1:200-diluted serum samples in 5% BSA in 0.5% Tween PBS were added to each well, followed by 2-hour incubation at room temperature. Plates were then washed with PBS plus 0.1% Tween, followed by three washes with PBS. Anti-human IgG-horseradish peroxidase-conjugated antibodies (Sigma-Aldrich) were then added at 1:1000 dilution in 5% BSA in 0.5% Tween PBS for another 2-hour incubation. The ELISA

plates were washed, and 100 µg/ml 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich) in 100 mmol/l sodium acetate (pH 6.0) was added. Subsequently, the reaction was stopped with 1 mol/l sulfuric acid. The absorbance was measured at 450 nm using a GENios plate reader and Magellan software (Tecan, Reading, UK). The ELISA absorbance values obtained for HEL and oxPTM-HEL were used as background controls that were subtracted from the absorbance values of NT-INS and oxPTM-INS, respectively. Levels of oxPTM-INS-Ab above the 99th percentile of 88 healthy individuals were defined as ELISA cut-off. Intra-assay CV was <8% (n = 10). Interassay CVs were <10% and <13% for NT-INS and oxPTM-INS-Ab (n = 12), respectively.

### 2.3 | Standard islet AAB determinations

The standard islet AAB were measured by radiobinding assay (RBA) as previously described.<sup>16,18,19</sup> In the 2005 Diabetes Autoantibody Standardization Programme (DASP), the RBA assays for GADA, IA-2A, and IAA achieved 76%, 72%, and 28% sensitivity with 96%, 100%, and 100% specificity, respectively.

### 2.4 | Sample size calculation

Sample size was calculated post hoc based on the results generated in our previous publication<sup>16</sup> to determine if the number of available sera would suffice for the hypothesis that oxPTM-INS-Ab would be more accurate than at least one standard islet AAB for identifying children progressing to diabetes. Using a significance level of 0.05 and 80% power, the minimum sample size required was of 23 subjects per group (progr-T1D and NP-AAB<sup>+</sup>). This was based on the area under curve (AUC) of ROC for oxPTM-INS-Ab and GADA of 0.86 and 0.56, respectively, and on the rank correlation coefficient between oxPTM-INS-Ab and GADA of -0.05 and -0.21 in progr-T1D and NP-AAB<sup>+</sup> groups, respectively.

### 2.5 | Statistical analysis

Statistical analyses were performed using Prism Software version 6.01 (GraphPad, San Diego, CA, USA) and MedCalc Statistical Software version 16.8.4 (MedCalc Software bvba, Ostend, Belgium). Diagnostic ability of oxPTM-INS-Ab vs the standard islet AAB was evaluated by receiver operating characteristic (ROC) curve analysis. ROC curves displaying sensitivity and specificity of a continuous marker (titre or absorbance) when comparing two markers, or a binary disease variable (positive vs negative) when comparing combinations of multiple markers, were tested. To evaluate sensitivity and specificity of continuous markers (titre or absorbance), cut-off points of positivity were specifically recalculated for the population studied based on the Youden's index *J* obtained by the ROC analysis. Area under the curve (AUC) of the ROC (ROC-AUC) of oxPTM-INS-Ab and the standard islet AAB is reported as absolute value ± standard error and was tested for equality according to DeLong et al.<sup>20</sup> The overall accuracy (%) was calculated as (number of progr-T1D identified as

positive + number of NP-AAB<sup>+</sup> identified as negative) / (total number of progr-T1D + total number of NP-AAB<sup>+</sup>). This calculation was based on predetermined cut-offs obtained from healthy individuals as reported in Section 2 and as described previously.<sup>18,19</sup> Categorical variables between groups were compared by the Fisher exact test. Survival analysis for diabetes onset used the Kaplan-Meier method. The log-rank test was used to compare cumulative incidence of diabetes between groups. The Wilcoxon test was used when proportional hazard validity was not respected. This study had only one primary aim. The primary outcome of this aim was the difference in the ROC-AUC between ox-PTM-INS-Ab and IAA, which was explored with one single test of equality (according to DeLong et al). Therefore, no correction for multiple testing was performed.

## 3 | RESULTS

### 3.1 | Prevalence of oxPTM-INS-Ab and the standard AAB

Prevalence of oxPTM-INS-Ab and the standard AAB is summarized in Table 1. A detailed description of the distribution and overlapping prevalence of oxPTM-INS-Ab and the standard islet AAB in the tested population has been reported elsewhere.<sup>16</sup> Prevalence of oxPTM-INS-Ab was higher in progr-T1D compared with the NP-AAB<sup>+</sup> [82% (19/23) vs 19% (6/32), *P* < 0.0001]. In contrast, prevalence of GADA and IAA did not differ significantly between progr-T1D and NP-AAB<sup>+</sup> groups [GADA: 78% (18/23) vs 91% (29/32), *P* = 0.257; IAA: 56% (10/18) vs 33% (9/27), *P* = 0.218], while IA-2A were more prevalent in the progr-T1D group [70% (16/23) vs 13% (4/32), *P* < 0.0001].

### 3.2 | Sensitivity, specificity, and accuracy indices of oxPTM-INS-Ab and the standard AAB

ROC analyses were performed to obtain sensitivity, specificity, and accuracy (AUC) of oxPTM-INS-Ab in comparison with the standard autoantibodies for discriminating progr-T1D from NP-AAB<sup>+</sup> children. Best cut-off points for this high-risk population were derived from the Youden's index *J* of each biomarker. Among the four tested biomarkers, oxPTM-INS-Ab and IA-2A displayed the best sensitivity and specificity; oxPTM-INS-Ab had 74% sensitivity and 91% specificity, while IA-2A had 71% sensitivity and 91% specificity, respectively. Conversely, GADA and IAA showed lower sensitivity (65% and 50%, respectively) and specificity (66% and 68%, respectively) (Table 2). The ROC-derived accuracy (AUC) of oxPTM-INS-Ab for identification of progr-T1D was higher than GADA (AUC 0.862 ± 0.058 vs 0.566 ± 0.093; *P* = 0.003) and IAA (AUC 0.862 ± 0.058 vs 0.624 ± 0.082; *P* = 0.017) and similar to IA-2A (AUC 0.862 ± 0.058 vs 0.871 ± 0.049; *P* = ns). Similarly, the overall accuracy, based on predetermined cut-off points, was higher for oxPTM-INS (82%) and IA-2A (80%), followed by IAA (62%) and GADA (38%), respectively (Table 2).

**TABLE 1** Prevalence of oxPTM-INS-Ab and the standard islet autoantibodies

	AAB <sup>+</sup> Children	Progr-T1D	NP-AAB <sup>+</sup>
N	55	23	32
Follow-up time, years	8.75 (5.96, 10.78)	5.12 (3.22, 7.73)	10.78 (7.68, 12.85)
GADA	47 (85%)	18 (78%)	29 (91%)
IAA <sup>a</sup>	19 (42%)	10 (56%)	9 (33%)
IA-2A	20 (36%)	16 (70%)	4 (13%)
oxPTM-INS-Ab	25 (45%)	19 (82%)	6 (19%)
GADA only	23 (42%)	1 (4%)	22 (69%)
IAA only <sup>a</sup>	2 (4%)	1 (6%)	1 (4%)
IA-2A only	1 (2%)	0 (0%)	1 (3%)
oxPTM-INS-Ab only	3 (4%)	3 (9%)	0 (0%)
Multiple standard AAB	26 (47%)	18 (78%)	8 (25%)

Follow-up time is reported as median (interquartile range).

<sup>a</sup>IAA was available in 45 children (18 progr-T1D and 27 NP-AAB<sup>+</sup>).

### 3.3 | Evaluation of oxPTM-INS-Ab for prediction of T1D in GADA-positive children

To evaluate the efficiency of oxPTM-INS-Ab as a second-line Ab for the prediction of T1D, we compared the ROC-AUC of oxPTM-INS-Ab with those of IAA and IA-2A in subjects positive for GADA at the cut-off point used in the general population. In this group, both IA-2A and oxPTM-INS-Ab were highly accurate (AUC of IA-2A = 0.941 ± 0.04 vs AUC of oxPTM-INS-Ab = 0.911 ± 0.05;  $P = 0.620$ ) and more effective than IAA for detecting progr-T1D (AUC-IAA = 0.679 ± 0.09;  $P = 0.024$  vs oxPTM-INS-Ab and  $P = 0.009$  vs IA-2A). When two more autoantibodies were added to GADA, we found that IA-2A/oxPTM-INS-Ab provided the best combination with the highest accuracy (AUC = 0.960), which was superior to the IA-2A/IAA or oxPTM-INS-Ab/IAA ( $P = 0.002$ ).

**TABLE 2** Comparison of the performance characteristics of oxPTM-INS-Ab and the standard islet autoantibodies

Antibody	% Sensitivity	% Specificity	AUC ± SE	% Accuracy	<i>P</i> vs oxPTM-INS-Ab
oxPTM-INS-Ab	74	91	0.862 ± 0.058	81.8	NA
GADA	65	66	0.566 ± 0.093	38.2	0.003
IAA	50	68	0.624 ± 0.082	62.2	0.017
IA-2A	71	91	0.871 ± 0.049	80.0	0.896

Sensitivity and specificity are derived from the ROC analysis based on the Youden's index *J* of each biomarker; % accuracy is calculated as (number of progr-T1D identified as positive + number of NP-AAB<sup>+</sup> identified as negative) / (total number of progr-T1D + total number of NP-AAB<sup>+</sup>), based on data reported in Table 1. *P* value refers to differences of AUC between oxPTM-INS-Ab and the standard islet autoantibodies.

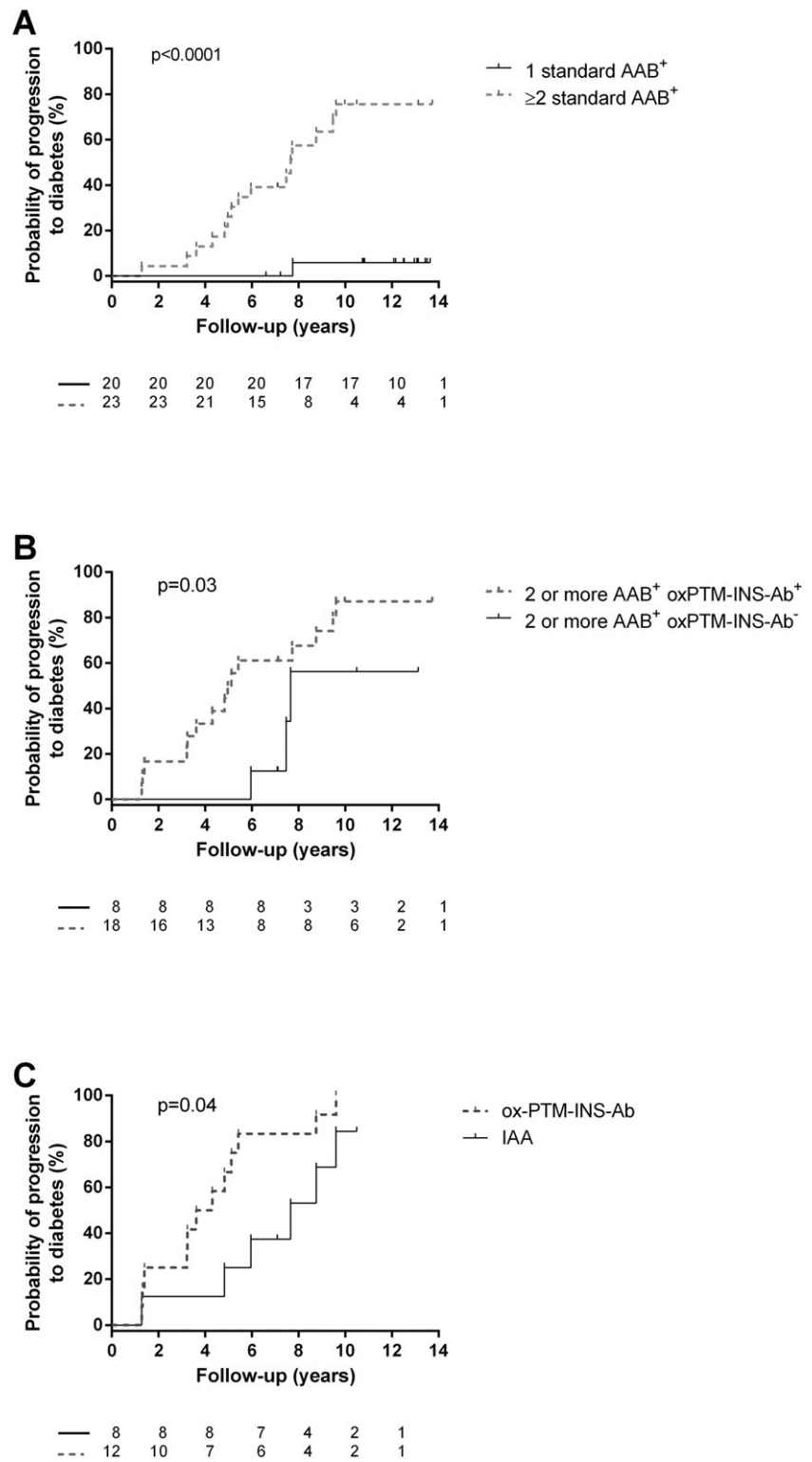
### 3.4 | Risk of progression to clinical diabetes in children positive to at least one standard autoantibody

The risk for diabetes increased incrementally according to the number of positive tests for the standard AAB (GADA, IA-2A, and IAA). Thus, the 10-year risk (95% confidence limits) in children positive for one, two, and three autoantibodies were 6% (1%-67%), 70% (47%-84%), and 84% (75%-94%), respectively. A total of 18/23 children (78%) with multiple standard AAB progressed to diabetes (HR compared with single islet AAB, 23.8 [95% CI, 3.6-22.5]) after a median follow-up of 7.47 years (Figure 1A). oxPTM-INS-Ab was able to stratify multiple AAB<sup>+</sup> children in two subgroups with different risk. Figure 1B shows diabetes risks in children positive for at least two standard AAB, subdivided by oxPTM-INS-Ab. The risk for diabetes was significantly higher ( $P = 0.03$ ) among multiple (two or more) AAB<sup>+</sup> (IAA, IA-2A, GAD) who were also oxPTM-INS-Ab<sup>+</sup> compared with those who were oxPTM-INS-Ab<sup>-</sup>. In these two groups, the 10-year cumulative diabetes incidences (95% confidence limits) were 87% (77-93) and 56% (14-84), respectively. Importantly, by replacing IAA with oxPTM-INS-Ab, children that were GADA, IA-2A, and oxPTM-INS positive (oxPTM-INS-Ab<sup>+</sup>, GADA<sup>+</sup>, and IA-2A<sup>+</sup>) had twofold higher risk of progression to clinical diabetes within 5 years (66.67% [95% CI 43.17-85.25]) compared with children with the three standard AAB (25% [95% CI 1-70.82%]). At 10 years of follow-up, diabetes risk increased to 100% in children with oxPTM-INS-Ab, GADA, and IA-2A, compared with 84.37% (95% CI 64.18-93.69;  $P = 0.04$ ) in those with IAA, GADA, and IA-2A (Figure 1C).

## 4 | DISCUSSION

Our results suggest that the inclusion of oxPTM-INS-Ab in the panel of autoantibody assay was able to improve significantly the prediction of T1D. We found that oxPTM-INS-Ab positivity increased risk in multiple AAB<sup>+</sup> children. oxPTM-INS-Ab and IA-2A were more effective than IAA for detecting children progressing to T1D when used as second-line marker in GADA-positive children.

In this study, oxPTM-INS showed better accuracy (AUC of ROC) than IAA and GADA. The superiority of oxPTM-INS-Ab over IAA is consistent with a previous study carried out by our group. In newly diagnosed T1D, oxPTM-INS-Ab was more sensitive than IAA (84% vs 61%), detecting over one-third of subjects negative to IAA.<sup>10</sup> A similar result was obtained in the ABIS cohort, where oxPTM-INS-Ab identified 38% of prediabetic children who were negative to IAA.<sup>16</sup> More recently, Sidney et al showed that peptides derived from oxPTM-INS B-chain can bind HLA class I with better affinity than unmodified insulin peptides.<sup>21</sup> Taken together, such findings fit with the hypothesis that insulin autoimmunity is predominant in most T1D subjects and that oxidative modifications may amplify (and theoretically trigger) autoimmune reactions against insulin. Circulating markers of oxidative stress are increased not only in diabetes<sup>22</sup> but also in euglycaemic subjects at risk for developing the disease.<sup>23</sup> We speculate that such early changes in redox status may lead to insulin



**FIGURE 1** Probability of progression to diabetes in AAB<sup>+</sup> children from the ABIS study. A, Probability of progression to diabetes according to the number of standard AAB<sup>+</sup> tests for GADA, IAA, and IA-2A. Risk of progression was higher in children with multiple vs single positive AAB ( $P < 0.0001$ ). B, Probability of progression in multiple standard AAB<sup>+</sup> children, according to oxPTM-INS-Ab status. oxPTM-INS-Ab<sup>+</sup> subjects had higher risk than those who were oxPTM-INS-Ab<sup>-</sup> ( $P = 0.03$ , Wilcoxon test). C, Probability of progression in triple AAB<sup>+</sup> increased when oxPTM-INS-Ab was used in place of IAA ( $P = 0.04$ ). Number of subjects at risk is showed below each graph

modifications and then autoimmunity in those who are immunologically susceptible to recognize such modifications as abnormal or unable to clear the modified antigen. We hypothesize two main mechanisms for the involvement of insulin modification in T1D immune pathogenesis: (1) generation of oxPTM by reactive oxidants released by leukocytes during insulinitis or (2) by altered redox state within

beta-cell under stress. The concept that posttranslational modifications of insulin may enhance autoimmune reactions in diabetes is not limited to oxPTM. Other modifications of (pro)insulin, such as deamidation of C-peptide<sup>13</sup> and covalent cross-linking of proinsulin peptides to other peptides (insulin hybrid peptides),<sup>24</sup> are targets of T cell responses. More recently, Wang et al showed that mutations

at the C-terminal end of the insulin epitope B9:23 dramatically improved presentation to pathogenic T cells, suggesting PTM-like transpeptidation as another potential mechanism to create pathogenic epitopes in T1D.<sup>25</sup>

In the present study, replacement of IAA with oxPTM-INS-Ab lead to improved prediction. This can be justified by the low prevalence and low specificity of IAA. This is consistent with results from two other studies carried out in the general populations. In the study by Knip and collaborator, IAA showed low sensitivity being detected only in 28% (5 of 18) subjects progressing to T1D (age range 2.9 to 18.9 years),<sup>26</sup> a percentage even lower than in our study (55%). The low IAA specificity in our study is similar to that from the Washington State Diabetes Prediction Study carried out on school children from the general population; in this study, 39% (40/102) of children with single positive islet AAB were positive to IAA but did not progress to T1D.<sup>27</sup> IAA prevalence may vary according to clinical and genetic features of the study populations, being higher in infants and cohorts selected for high genetic or familiar risk. For example, in the DAISY study carried out on young children with increased genetic susceptibility to T1D, 22 of 24 children developing diabetes were IAA positive (age at diagnosis 1 to 3 years).<sup>28</sup> Similar prevalence for IAA was found in progressor children from the DIPP<sup>29</sup> and the BABY-DIAB<sup>30</sup> studies. However, IAA disappear more often than other AAB,<sup>29</sup> and loosing IAA reactivity is associated with delayed progression to T1D in multiple AAB-positive children.<sup>31,32</sup> Therefore, we cannot exclude that children defined as IAA negative in our study have not been previously IAA positive.

An interesting finding of our study is the low accuracy of GADA. This is not completely unexpected, as GADA is less effective in predicting diabetes than other islet AAB. In a study on genetically at-risk subjects from three large prospective cohorts, the 10-year risk of progression conferred by GADA was about threefold lower than that provided by IA-2A (12.9% vs 40.5%).<sup>4</sup> This is also in line with the study by Yu et al performed in T1D relatives, where GADA failed to independently predict diabetes progression over a 5-year follow-up.<sup>33</sup> In the same study, the risk associated with GADA was up to fourfold lower than that conferred by IA-2A or IAA.<sup>33</sup> In a recent cluster analysis of the TEDDY study, stable single GADA positivity (with or without IAA) conferred 30% or lower diabetogenic effect than IA-2A clusters.<sup>31</sup> However, these results should be interpreted with caution. Features specific to our population, including the high prevalence of GADA (85%) and its tendency to be the most prevalent single autoantibody, may also explain our finding. This is consistent with studies in T1D relatives. GADA was highly prevalent (74%) in first-, second-, or third-degree T1D relatives from the TrialNet cohort<sup>33</sup> and the most prevalent autoantibody in initially single positive relatives from the Belgian Diabetes Registry.<sup>34</sup> However, we cannot exclude that subjects positive to GADA will develop diabetes at a later follow-up not yet assessed in our study. According to the report from the Belgian Diabetes Registry, relatives positive to GADA and IAA progressed more slowly than double autoantibody-positive individuals carrying IA-2A and/or ZnT8A.<sup>34</sup> In a study by Knip and collaborators on subjects from the general population, 6 of 18 subjects (33%) developed

diabetes after age of 20 (2 of 18 after age of 30), despite being islet autoantibody positive for up to 20 years.<sup>26</sup> It is also worth noting that GADA as single antibody is the most common antibody biomarker for adult onset autoimmune diabetes.<sup>35</sup>

As reported by others, we also found an association between number of positive standard autoantibodies and risk of progression to diabetes.<sup>1,4,26,33,34</sup> In the group of multiple autoantibody positive, oxPTM-INS-Ab testing refined risk by identifying a subgroup with a faster and greater risk of progression. This is similar to what has been shown for ZnT8A in a study on T1D relatives from the TrialNet cohort.<sup>33</sup> Therefore, these data may have implications for disease prediction and staging. Islet autoimmunity (as defined by the presence of two or more islet AAB) represents the earliest stage of T1D and identifies a target population for prevention trials and future preventive strategies.<sup>7</sup> Therefore, improved identification of subjects progressing to clinical disease has important clinical implications. If confirmed in larger studies, oxPTM-INS-Ab-based assay may be implemented as additional biomarker to further redefine disease taxonomy, allowing better prediction and therefore better stratification into T1D prevention trials.

The main strength of our study is the evaluation of oxPTM-INS-Ab in a well-characterized cohort of children from the general population, tested for the standard diabetes AAB and the fact that replacing IAA with oxPTM-INS-Ab in the equation resulted in a better prediction of risk to develop T1D. This discovery needs to be confirmed using independent and larger cohorts. Our study has limitations. First, the number of children progressing to T1D with a single standard autoantibody was small, and this did not allow us to find out whether oxPTM-INS-Ab would significantly redefine the risk in this category of subjects. Second, it was not possible to measure oxPTM-INS-Ab before seroconversion of the other islet AAB; thus, we cannot infer about timing of oxPTM-INS-Ab appearance compared with the other biomarkers. Third, although oxPTM-INS-Ab were not present in children with negative standard islet AAB, this should be further confirmed in larger cohorts of low-risk individuals. Finally, the post hoc sample size calculation is a limitation of the study. Although our study was powered to address the general question of the performance of oxPTM-INS-Ab against any other islet AAB, such data are based on minimal differences in the absolute number of subjects.

In conclusion, oxPTM-INS-Ab may identify children progressing to T1D with better accuracy than IAA and in combination with the current standard autoantibody markers such as GADA and IA-2A. Thus, oxPTM-INS-Ab may refine risk in multiple autoantibody-positive children and may maximize diabetes risk progression in children positive to the other islet AAB biomarkers. Additional studies with larger cohorts are required to confirm the predictive potential of oxPTM-INS-Ab in T1D.

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## DISCLOSURE STATEMENT

The authors have nothing to disclose.

## CONTRIBUTION STATEMENT

RS, PP, JL, and AN conceived the study. JL is the project leader of the ABIS study. CV performed experiments and provided the data. NN, EF, and EM contributed to data acquisition and analysis. LA and RC contributed to the islet autoantibody determination. RS analysed the data and wrote the first draft. All authors critically revised the manuscript for intellectual content. All authors have seen and approved the final draft. RS is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

## ABBREVIATIONS

AAB	autoantibody
AAB <sup>+</sup>	autoantibody positive
ABIS	All Babies in Southeast Sweden
ACPA	anticitrullinated peptides antibody
AUC	area under the curve
BSA	bovine serum albumin
ELISA	enzyme-linked immunosorbent assay
GADA	GAD autoantibodies
HEL	hen egg lysozyme
IA-2A	tyrosin phosphatase autoantibody
IAA	insulin autoantibodies
NP-AAB <sup>+</sup>	nonprogressors with positive autoantibodies
oxPTM	oxidative PTM
oxPTM-INS	oxidatively posttranslationally modified insulin
oxPTM-INS-Ab	antibody to oxPTM-INS
PTM	posttranslational modifications
RA	rheumatoid arthritis
RBA	radiobinding assay
ROC	receiver operating characteristic
T1D	type 1 diabetes
ZnT8A	zinc transporter 8 autoantibodies

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