



Article

Autoantibodies against Proinsulin, Human Endogenous Retrovirus W (HERV-W) and *Mycobacterium avium* Subspecies *Paratuberculosis* (MAP) Slowly Decrease Years after T1DM Diagnosis

Marta Noli ¹, Gianfranco Meloni ², Elena Rita Simula ¹, Maria Antonietta Manca ¹, Seyedesomaye Jasemi ¹, Stefano Ruberto ¹, Davide Cossu ¹, Mario Palermo ³ and Leonardo A. Sechi ^{1,4,*}

¹ Dipartimento di Scienze Biomediche, Università di Sassari, 07100 Sassari, Italy; martanoli@outlook.it (M.N.); simulaelena@gmail.com (E.R.S.); m.anto.manca@gmail.com (M.A.M.); s.jasemi@studenti.uniss.it (S.J.); ruberto.ste@gmail.com (S.R.); dcossu@uniss.it (D.C.)

² Dipartimento di Medicina Mediche, Chirurgiche e Sperimentali, Università di Sassari, 07100 Sassari, Italy; gfmeloni@uniss.it

³ Servizio di Endocrinologia, Azienda Ospedaliera Universitaria, 07100 Sassari, Italy; mario.palermo@aousassari.it

⁴ Struttura Complessa di Microbiologia e Virologia, Azienda Ospedaliera Universitaria, 07100 Sassari, Italy

* Correspondence: sechila@uniss.it



Citation: Noli, M.; Meloni, G.; Simula, E.R.; Manca, M.A.; Jasemi, S.; Ruberto, S.; Cossu, D.; Palermo, M.; Sechi, L.A. Autoantibodies against Proinsulin, Human Endogenous Retrovirus W (HERV-W) and *Mycobacterium avium* Subspecies *Paratuberculosis* (MAP) Slowly Decrease Years after T1DM Diagnosis. *Zoonotic Dis.* **2022**, *2*, 37–43. <https://doi.org/10.3390/zoonoticdis2010005>

Academic Editor: Seiya Yamayoshi

Received: 24 January 2022

Accepted: 16 March 2022

Published: 18 March 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Previous studies have highlighted the potential role of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and human endogenous retrovirus W (HERV-W) in the pathogenesis of type 1 diabetes (T1DM) among Sardinian subjects. To better understand how antibody responses evolve during disease progression, a serological evaluation of IgG antibodies was performed in Sardinian children with T1DM collected at different time-points following the onset of the disease. It is known that anti-PI and anti-insulin (IAA) autoantibodies are the first to appear before the clinical onset of T1DM. In order to investigate the humoral responses, 69 children with T1DM were enrolled in the study, including 25 with new onset, 25 with T1DM at 1–5 years since diagnosis and 19 with T1DM at 6–12 years since diagnosis. Serum samples were tested for the presence of antibodies (Abs) against PI_{46–61}, three MAP epitopes (including MAP 2404c, which has a homologous sequence with PI) and two HERV-W-derived epitopes via indirect enzyme-linked immunosorbent assay (ELISA). The data obtained from the analysis showed significantly higher IgG responses against all peptides detected in the new onset group compared to longer suffering (1–5 and 6–12 years) T1DM patients, also showing a robust correlation between the proinsulin autoantibody and anti-MAP/HERV antibodies, characterized by a progressive decline the first year after onset. Taken together, these findings support the hypothesis that MAP and HERV could act as risk factors for T1DM, suggesting that they may serve as potential biomarkers of disease progression in early-stage T1DM.

Keywords: HERV-W; *Mycobacterium paratuberculosis*; proinsulin; antibodies; molecular mimicry; children T1DM onset; follow up; peptides

1. Introduction

Type 1 diabetes (T1DM) is an autoimmune disorder characterized by the destruction of insulin-secreting pancreatic β -cells by autoreactive T cells [1].

Autoantibodies against β -cells are important markers for the early diagnosis of T1DM. Indeed, they tend to appear in individuals who developed T1DM several years before the disease onset [2].

Several molecular targets of T1DM autoimmunity have been identified from studies on circulating autoantibodies in humans, and these have provided insights into the etiology, pathogenesis and natural history of this disease.

Instead of individual markers, the number of specific antibodies and positivity against two or more antigens are more reliable predictors of disease. Antibody determinations against these antigens may also be useful when assessing immunological therapeutic interventions targeting autoreactive T and B cells [3,4]. In these cases, antibody titers and epitope affinity and specificity are important markers of the immune response [5,6]. Humoral autoreactivity may precede clinical disease by months or years, and progression to clinical disease may take decades [7].

Proinsulin (PI) has been described as an important autoantigen [8] triggering the pathogenesis of T1DM in a non-obese diabetic (NOD) mouse model [9], while in humans anti-insulin antibodies (IAA), the processed form of PI, are the first to appear, especially in children, suggesting an early role for insulin in the development of T1DM [10]. In addition, PI shapes the autoreactive CD8 T-cell repertoire [11,12].

Insulin is a hormone that is initially synthesized from the precursor preproinsulin (PPI).

PPI contains a 24 aa leader sequence, which is then transformed into pro-insulin (PI) by the cleavage of the leader sequence. PI is composed of 86 amino acids and has three domains, namely the α -chain, β -chain and C-peptide. Through the proteolytic cleavage of the C-peptide, bioactive insulin is generated, which contains the alpha and beta chains.

Several epitopes within PPI and PI have been found to be recognized by CD8 or CD4 T cells isolated from patients with T1DM, suggesting that this antigen plays a role in the progression of the disease [13]. The results of previous studies indicate that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection might be associated with T1DM [14].

Our group previously identified different peptides of *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which share more than 50% residue identity with human PI (Table 1). Moreover, in previous reports, we portrayed the association of MAP and human endogenous retrovirus W (HERV-W) in T1DM etiopathogenesis in children from Sardinia [15], supporting for the first time the hypothesis that these two pathogens may both contribute to T1DM etiology. It has been suggested that HERV pathogenic elements could be reactivated by MAP in patients with a specific genetic background.

Table 1. Epitopes used in the study (aa: amino acids). Conserved amino acid residues are highlighted in yellow.

Peptides	Position	Sequence
PI	aa 46–61	RGFFYTPKTRREAEDL
MAP 2404c	aa 70–80	RGFVVLPVTRRDVTDV
MAP 3865c	aa 125–133	MIAVALAGL
MAP1,4- α -gpb	aa 157–173	GTVELLGGPLAHPFQPL
HERV-Wenv	aa 93–108	NPSCPGGLGVTVCWTY
HERV-Wenv	aa 248–262	NSQCIRWVTPPTQIV

The aim of this study was to investigate whether there is a correlation between anti-MAP, anti-HERV antibodies and PI autoantibodies in the sera of children with T1DM collected at different time points after onset, to assess whether MAP and HERV antibody titers may serve biomarkers for the early prediction of T1DM.

2. Materials and Methods

2.1. Patients

For this study, we recruited sixty-nine Sardinian children with T1DM (46 males and 23 females, median age 10.7, including twenty-five patients at onset of the disease (6 females and 19 males, median age 7.8), twenty-five with 1–5 years of T1DM (12 females and 13 males median age 10.4) and nineteen with 6–12 years of T1DM (5 females and 14 males, median age 14.2), attending the Department of Pediatrics of the AOU of Sassari and the Department of Experimental and Clinical Medicine of the University of Sassari.

The diagnosis of T1DM was based on criteria from the American Diabetes Association [16]. The main inclusion criteria were levels of glycated hemoglobin, ketoacidosis and

the presence of classical islet autoantibodies. In particular, regarding the onset population, we selected patients aged ≤ 12 years who were not yet on insulin therapy.

For the other groups we selected patients with T1D diagnosis from 1–12 years of age and up to 30 years, which were collected during regular diabetological visits.

The healthy controls (HCs) consisted of twenty-eight age-matched children (17 females, 11 males, median age 8.41) without inflammatory episodes in the last 2 months, with cancer and neurological diseases, recruited in the corresponding geographic areas during routine check-up visits from the Department of Endocrinology of the University Hospital (AOU) of Sassari. Subjects gave their written informed consent, which was obtained from their parents or legal caregivers, before participating in this study.

2.2. Blood Samples

Peripheral blood samples were drawn in K+ EDTA tubes from each individual. Blood samples were processed with Ficoll-Paque[®] (Sigma-Aldrich, St. Louis, MO, USA) to collect the plasma. Plasma rates were obtained according to the protocol by gradient centrifugation and stored into 0.5 mL aliquots at -80°C .

2.3. Peptides

The peptide PI_{46–61} (RGFFYTPKTRREAEDL) is shown in Table 1, along with its respective derived homologs MAP 2404C_{70–85} (RGFVVLVPVTRRDVTDV) and MAP 3865C_{125–133} (MIAVALAGL).

MAP 1,4- α -gpb_{157–173} (GTVELLGGPLAHPFQPL), HERV-Wenv_{93–108} (NPSCPG-GLGVTVCWTY) and HERV-Wenv_{248–262} (NSQCIRWVTPPTQIV) were synthesized at $>95\%$ purity (LifeTein, South Plainfield, NJ, USA).

DMSO was used as a solvent to resuspend the peptides (Table 1 in single-use aliquots and stored at -80°C).

2.4. Serological Assays and Data Analysis

Serum samples were tested for the presence of Abs against PI, MAP and HERV-Wenv antigens using an indirect enzyme-linked immunosorbent assay (ELISA), as described previously [15]. A positive serum control, whose reactivity was set to 1.0 arbitrary units (AU)/mL, was used to normalize all results obtained in ELISA experiments. The precision of ELISA was determined by calculating both the inter- and intra-assay coefficients of variation (CV). Statistical analysis was performed with commercial software GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Kruskal–Wallis test and Dunn’s post hoc tests were used to analyze non-parametric data to compare differences among different groups. Statistical significance was defined as a p value < 0.05 .

3. Results

Prevalence and Titer of PI, MAP and HERV-W Antigens

The humoral responses against PI_{46–61}, its homologue MAP 2404C_{70–80} and four selected peptides, including two MAP derived and two from the envelope of HERV-W_{env}, were assessed in the plasma samples of the T1DM and HCs groups. In order to investigate a potential change in humoral response related to disease duration, the results of the four groups (onset T1DM, 1–5 years, 6–12 years and HCs) were analyzed by Kruskal–Wallis test and using Dunn’s post hoc analysis to compare quantitative values of the antibodies’ OD values among groups. The results exhibited statistically significant differences in the antibody responses against all peptides in the different groups, including for PI_{46–61} values (Figure 1A) between patients at onset, the 1–5 years T1DM group ($p = 0.03$) and HCs ($p = 0.04$). Significant differences were also found in the antibody responses directed against its homologue MAP 2404C_{70–80} (Figure 1B) between T1DM onset and T1DM and HCs ($p = 0.005$). For MAP 3865C_{125–133}, significant differences were detected between patients at the onset and the 6–12 years group ($p = 0.04$) and HCs ($p < 0.0001$) (Figure 1C). MAP1,4- α -gpb_{157–173} peptide

was also significantly recognized among the T1DM onset group, 1–5 years T1DM group ($p = 0.01$), 6–12 years T1DM ($p = 0.01$) and HCs ($p = 0.02$) (Figure 1D).

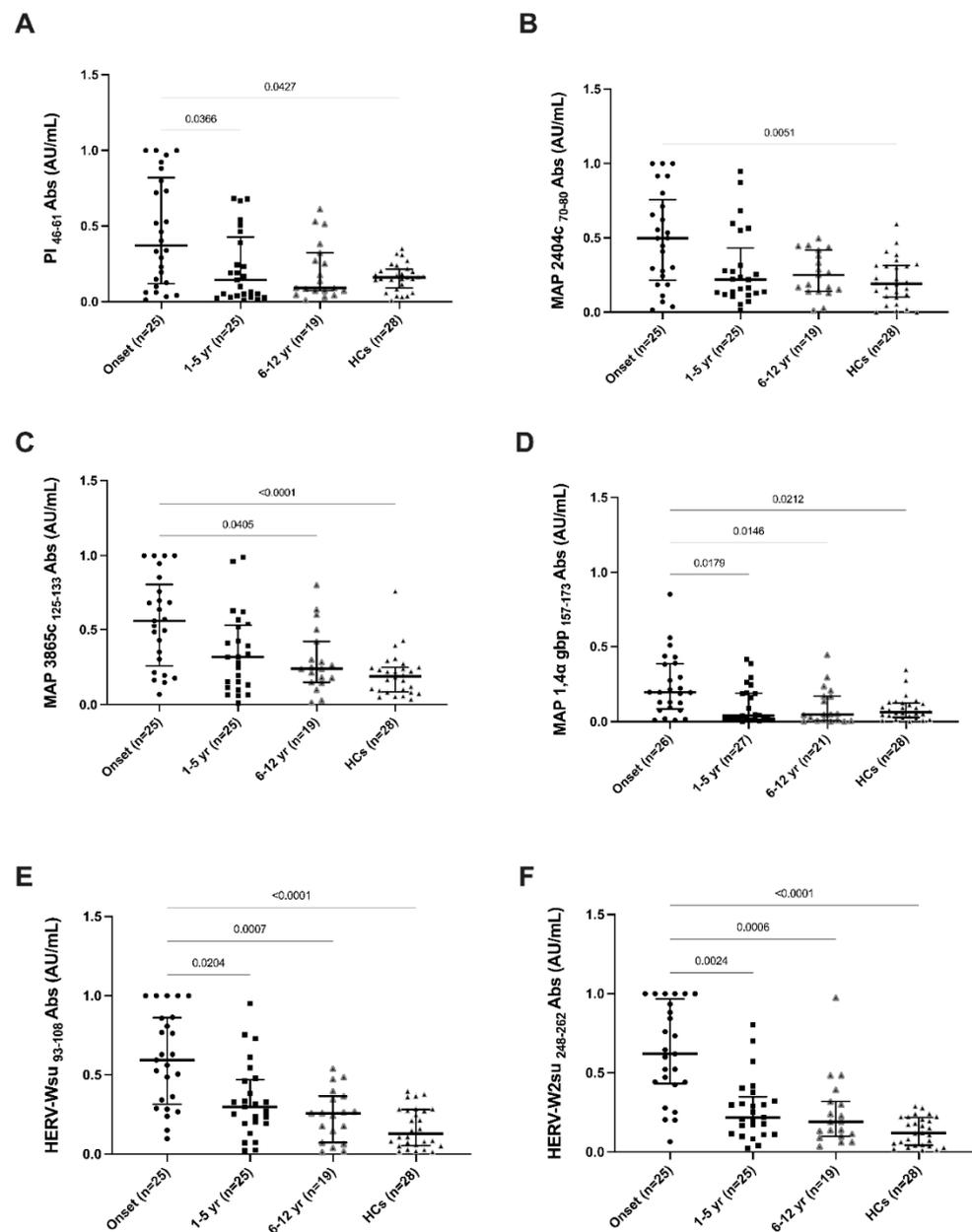


Figure 1. Prevalence of Abs against PI/MAP/HERV-Wenv antigens in Sardinian T1DM children. Plasma samples taken from different groups of T1DM patients (at onset/1–5 years/6–12 years) and HCs were tested against PI_{46–61} (A), MAP 2404C_{70–80} (B), MAP 3865C_{125–133} (C) MAP1,4- α -gbp_{157–173} (D), HERV-Wenv_{93–108} (E) and HERV-Wenv_{248–262} (F) peptides. Kruskal–Wallis p -values are indicated in the upper part of each graph.

Regarding HERV-W peptides, significant differences in Ab distribution were also detected between the different groups of patients and the control group. Indeed, the HERV-Wenv_{93–108} results were statistically significant between T1DM onset and 1–5 years ($p = 0.02$), 6–12 years ($p = 0.0007$) and HCs groups ($p < 0.0001$) (Figure 1E). Anti-HERV-Wenv_{248–262} Abs differences were also found between onset T1DM patients and the 1–5 years group ($p = 0.002$), 6–12 years ($p = 0.0006$) and HCs ($p < 0.0001$) (Figure 1F). An additional analysis was carried out to evaluate possible correlations between anti-PI, -MAP and -HERV-W humoral responses. The results showed correlations between the levels of antibodies

in T1DM patients; high, moderate and low correlations were observed based on their r values according to international recognized scores [17]. Indeed, moderate correlations were found between PI_{46-61} and its homologue $MAP\ 2404c_{70-80}$ ($r = 0.64$, $p < 0.0001$, Figure 2A), between PI_{46-61} and $MAP1,4-\alpha-gbp_{157-173}$ ($r = 0.63$, $p < 0.0001$, Figure 2C), between PI_{46-61} and $HERV-Wenv_{93-108}$ ($r = 0.63$, $p < 0.0001$, Figure 2D) and between PI_{46-61} and $HERV-Wenv_{248-262}$ ($r = 0.56$, $p < 0.0001$, Figure 2E).

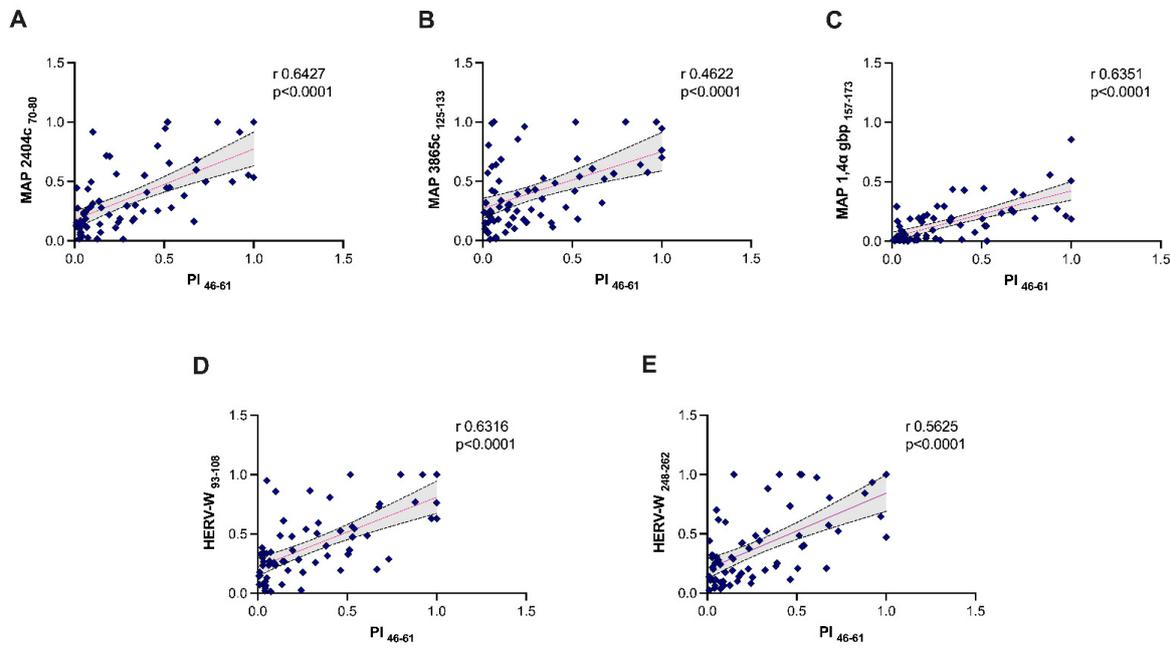


Figure 2. Scatter plot expressing correlations between PI and MAP/HERV-Wenv-derived peptides in all patients with T1DM. The graphs show the correlations among PI_{46-61} and $MAP2404c_{70-80}$ (A), PI_{46-61} and $MAP3865c_{125-133}$ (B), PI_{46-61} and $MAP1,4-\alpha-gbp_{157-173}$ (C), PI_{46-61} and $HERV-Wenv_{93-108}$ (D) and PI_{46-61} and $HERV-Wenv_{248-262}$ (E).

A somewhat low correlation instead was observed between PI_{46-61} and $MAP\ 3865C_{125-133}$ ($r = 0.46$, $p < 0.0001$, Figure 2B).

A summary of these results are depicted in Table 2.

Table 2. Relationship between PI antigens and MAP/HERV-Wenv-derived epitopes, expressed as r . Values were obtained based on all seventy-four samples with T1DM.

Antigen	PI_{46-61}
$MAP2404c_{70-80}$	$r\ 0.64$ $p < 0.0001$
$MAP3865C_{125-133}$	$r\ 0.46$ $p = 0.03$
$MAP1,4-\alpha-gbp_{157-173}$	$r\ 0.63$ $p < 0.0001$
$HERV-Wenv_{93-108}$	$r\ 0.63$ $p < 0.0001$
$HERV-Wenv_{248-262}$	$r\ 0.56$ $p < 0.0001$

4. Discussion

T1DM is an autoimmune disorder characterized by T cell infiltration, resulting in the destruction of insulin-secreting pancreatic β -cells and marked by the production of Abs against islet cells.

The primary biomarkers of β -cell autoimmunity at the time of diagnosis involve the identification of Abs against islet cell antibodies (ICAs), zinc transporter 8 (ZnT8), glutamic acid decarboxylase (GAD), islet antigen-2 (IA2) and insulin (IAA), among which anti-proinsulin (PI) can be detected at a younger mean age [18,19] and is common in patients progressing rapidly to overt diabetes [20].

The major objective of this study was to evaluate how the prevalence of Abs against MAP and HERV-Wenv may change from the diagnosis of disease to subsequent years, and whether these changes follow a trend referable to measurable β -cell autoimmunity Abs, such as PI₄₆₋₆₁.

In light of our previous finding reporting an elevated sero-prevalence of MAP and HERV-Wenv in pediatric patients at T1DM onset from Sardinia, we demonstrate here that epitopes belonging to different MAP proteins (MAP2404c₇₀₋₈₀/MAP3865C₁₂₅₋₁₃₃/MAP1,4- α -gpb₁₅₇₋₁₇₃) and HERV-Wenv (HERV-Wenv₉₃₋₁₀₈/HERV-Wenv₂₄₈₋₂₆₂) are targets of an Abs-mediated response that produced data somehow comparable to those obtained for PI₄₆₋₆₁ during the following years.

In general, in no instances did we observe a higher Abs level against these epitopes after onset, but there is a progressive and marked decline in antibody level as early as from the first year of the disease. This pattern occurred with all peptides and was also mirrored by the selected autoantibody (Figure 1).

Importantly, the higher titers against the selected epitopes of different proteins of MAP and HERV-Wenv at onset of the disease declined over the years, with statistically significant differences, as observed for the T1DM 1–5 years group, in many cases reaching levels below the level of detection compared to healthy controls for MAP1,4- α -gpb₁₅₇₋₁₇₃ and HERV-Wenv₂₄₈₋₂₆₂.

The prevalence of Abs to these epitopes appears to reach a plateau 6–12 years after the diagnosis of T1DM.

This reflects the development of an active autoimmune process at the onset, while the progressive decrease in titers probably reflects a reduction in these processes over time or an impaired immune response to counteract MAP and HERV-W replication.

Our observations support the existence of an association between Abs positivity for MAP/HERV and PI₄₆₋₆₁ (Table 2)

The reported Abs patterns related to time of the disease indicated a possible reactivation of HERV-W following exposure to MAP, which through cross-reacting of homologous epitopes with PI₄₆₋₆₁ could act as a risk factor for loss of immune tolerance.

In conclusion, the high frequencies of Abs reacting against the different selected epitopes, their distribution over time, together with the correlation found with autoantibodies such as PI₄₆₋₆₁, also considering the sequence homology between MAP 2404c₇₀₋₈₀ and PI₄₆₋₆₁ (Table 2), suggest that the detection of Abs against these peptides, in conjunction with anti-GAD and anti-IA2, might make them useful predictor biomarkers for a complete and specific picture without clinical manifestation of symptoms at the onset of T1DM.

Additional follow-up studies of Ab titers over longer time frames and with a larger T1DM population are necessary to determine the role of pathogens such as MAP and the human endogenous retrovirus HERV-W in T1DM, which will help verify whether multiple reactivity to the analyzed peptides, in particular complete Abs status, might more accurately indicate a prediabetes phase leading to overt T1DM.

Author Contributions: L.A.S. supervised, designed and conceived the study. L.A.S. and M.N. designed the experiments and statistically analyzed the data. M.N. drafted the manuscript and carried out the experiments. G.M. and M.P. recruited T1DM patients and healthy controls. L.A.S., M.N., G.M., M.A.M., S.J., D.C., E.R.S. and S.R. analyzed the data, discussed results and approved the manuscript. L.A.S. approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the UNISS Fondi Ricerca 2020 and Fondazione Sardegna 2017 to L.A.S.

Institutional Review Board Statement: The study was approved by the Ethical Committee of ASL N. 1 Sassari Prot. 2149/CE/2 on 16 June 2015.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Taplin, C.; Barker, J. Autoantibodies in Type 1 Diabetes. *Autoimmunity* **2008**, *41*, 11–18. [[CrossRef](#)] [[PubMed](#)]
2. Jaber-Douraki, M.; Liu, S.W.; Pietropaolo, M.; Khadra, A. Autoimmune Responses in T1DM: Quantitative Methods to Understand Onset, Progression, and Prevention of Disease. *Pediatric Diabetes* **2014**, *15*, 162–174. [[CrossRef](#)] [[PubMed](#)]
3. Keymeulen, B.; Vandemeulebroucke, E.; Ziegler, A.G.; Mathieu, C.; Kaufman, L.; Hale, G.; Gorus, F.; Goldman, M.; Walter, M.; Candon, S.; et al. Insulin Needs after CD3-Antibody Therapy in New-Onset Type 1 Diabetes. *N. Engl. J. Med.* **2005**, *23*, 2598–2608. [[CrossRef](#)] [[PubMed](#)]
4. Wenzlau, J.M.; Walter, M.; Gardner, T.J.; Frisch, L.M.; Yu, L.; Eisenbarth, G.S.; Ziegler, A.G.; Davidson, H.W.; Hutton, J.C. Kinetics of the Post-Onset Decline in Zinc Transporter 8 Autoantibodies in Type 1 Diabetic Human Subjects. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 4712–4719. [[CrossRef](#)] [[PubMed](#)]
5. Bingley, P.J. Clinical Applications of Diabetes Antibody Testing. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 25–33. [[CrossRef](#)] [[PubMed](#)]
6. Achenbach, P.; Schlosser, M.; Williams, A.J.K.; Yu, L.; Mueller, P.W.; Bingley, P.J.; Bonifacio, E. Combined Testing of Antibody Titer and Affinity Improves Insulin Autoantibody Measurement: Diabetes Antibody Standardization Program. *Clin. Immunol.* **2007**, *122*, 85–90. [[CrossRef](#)] [[PubMed](#)]
7. Regnell, S.E.; Lernmark, Å. Early Prediction of Autoimmune (Type 1) Diabetes. *Diabetologia* **2017**, *60*, 1370–1381. [[CrossRef](#)] [[PubMed](#)]
8. Narendran, P.; Mannering, S.I.; Harrison, L.C. Proinsulin—A Pathogenic Autoantigen in Type 1 Diabetes. *Autoimmun. Rev.* **2003**, *2*, 204–210. [[CrossRef](#)]
9. Culina, S.; Brezar, V.; Mallone, R. Insulin and Type 1 Diabetes: Immune Connections. *Eur. J. Endocrinol. Eur. Fed. Endocr. Soc.* **2013**, *168*, R19–R31. [[CrossRef](#)] [[PubMed](#)]
10. Ziegler, A.G.; Bonifacio, E. Age-Related Islet Autoantibody Incidence in Offspring of Patients with Type 1 Diabetes. *Diabetologia* **2012**, *55*, 1937–1943. [[CrossRef](#)] [[PubMed](#)]
11. Thayer, T.C.; Pearson, J.A.; de Leenheer, E.; Hanna, S.J.; Boldison, J.; Davies, J.; Tsui, A.; Ahmed, S.; Easton, P.; Siew, L.K.; et al. Peripheral Proinsulin Expression Controls Low-Avidity Proinsulin-Reactive Cd8 t Cells in Type 1 Diabetes. *Diabetes* **2016**, *65*, 3429–3439. [[CrossRef](#)] [[PubMed](#)]
12. Pearson, J.A.; Thayer, T.C.; McLaren, J.E.; Ladell, K.; de Leenheer, E.; Phillips, A.; Davies, J.; Kakabadse, D.; Miners, K.; Morgan, P.; et al. Proinsulin Expression Shapes the TCR Repertoire but Fails to Control the Development of Low-Avidity Insulin-Reactive CD8⁺ T Cells. *Diabetes* **2016**, *65*, 1679–1689. [[CrossRef](#)] [[PubMed](#)]
13. Babon, J.A.B.; Denicola, M.E.; Blodgett, D.M.; Crèvecoeur, I.; Buttrick, T.S.; Maehr, R.; Bottino, R.; Naji, A.; Kaddis, J.; Elyaman, W.; et al. Analysis of Self-Antigen Specificity of Islet-Infiltrating T Cells from Human Donors with Type 1 Diabetes. *Nat. Med.* **2016**, *22*, 1482–1487. [[CrossRef](#)] [[PubMed](#)]
14. Sechi, L.A.; Paccagnini, D.; Salza, S.; Pacifico, A.; Ahmed, N.; Zanetti, S. Mycobacterium Avium Subspecies Paratuberculosis Bacteremia in Type 1 Diabetes Mellitus: An Infectious Trigger? *Clin. Infect. Dis.* **2008**, *46*, 148–149. [[CrossRef](#)] [[PubMed](#)]
15. Noli, M.; Meloni, G.; Manca, P.; Cossu, D.; Palermo, M.; Sechi, L.A. HERV-W and Mycobacterium Avium Subspecies Paratuberculosis Are at Play in Pediatric Patients at Onset of Type 1 Diabetes. *Pathogens* **2021**, *10*, 1135. [[CrossRef](#)] [[PubMed](#)]
16. Diabetes, D.O.F. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* **2011**, *34*, S62–S69. [[CrossRef](#)]
17. Asuero, A.G.; Sayago, A.; González, A.G. The Correlation Coefficient: An Overview. *Crit. Rev. Anal. Chem.* **2006**, *36*, 41–59. [[CrossRef](#)]
18. Yu, L.; Dong, F.; Miao, D.; Fouts, A.R.; Wenzlau, J.M.; Steck, A.K. Proinsulin/Insulin Autoantibodies Measured with Electrochemiluminescent Assay Are the Earliest Indicator of Prediabetic Islet Autoimmunity. *Diabetes Care* **2013**, *36*, 2266–2270. [[CrossRef](#)] [[PubMed](#)]
19. Kikkas, I.; Mallone, R.; Tubiana-Rufi, N.; Chevenne, D.; Carel, J.C.; Créminon, C.; Volland, H.; Boitard, C.; Morel, N. A Simple and Fast Non-Radioactive Bridging Immunoassay for Insulin Autoantibodies. *PLoS ONE* **2013**, *8*, e0069021. [[CrossRef](#)] [[PubMed](#)]
20. Parikka, V.; Nantö-Salonen, K.; Saarinen, M.; Simell, T.; Ilonen, J.; Hyöty, H.; Veijola, R.; Knip, M.; Simell, O. Early Seroconversion and Rapidly Increasing Autoantibody Concentrations Predict Prepubertal Manifestation of Type 1 Diabetes in Children at Genetic Risk. *Diabetologia* **2012**, *55*, 1926–1936. [[CrossRef](#)] [[PubMed](#)]