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Immunogenicity of *Mycobacterium avium* subsp. *paratuberculosis* epitopes cross-reacting with human ZnT8 and proinsulin peptides in autoimmune diabetes

Supervisor

Prof. Leonardo A. Sechi

PhD student

Magdalena Niegowska Conforti

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Preface

The present thesis is based on my original research outcomes published during enrolment in the PhD programme in Biomolecular and Biotechnological Sciences. The work was carried out at the Department of Biomedical Sciences, Microbiology and Virology Unit, University of Sassari, Italy, under the supervision of Prof. Leonardo A. Sechi. Part of the project was performed at the Faculty of Pharmacy, Laboratory of Clinical and Experimental Immunology, Nicolaus Copernicus University Collegium Medicum in Bydgoszcz, Poland, within Erasmus+ Traineeship.

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Abbreviations

7-AAD – 7-aminoactinomycin D
Abs – antibodies
AITD – autoimmune thyroiditis
APC – allophycocyanin
ARS – at-risk subjects
BMI – body mass index
CD – Crohn's disease
CRP – C-reactive protein
EBV – Epstein-Barr virus
ELISA – enzyme-linked immunosorbent assay
GWAS – genome wide association study
HCs – healthy controls
HERV – human endogenous retrovirus
HLA – human leucocyte antigen
HP – *Helicobacter pylori*
HT – Hashimoto's thyroiditis
IL – interleukin
INFγ – interferon gamma
L5P – lipopentapeptide
LADA – latent autoimmune diabetes of adults
LPS – lipopolysaccharide
MAP – *Mycobacterium avium* subsp. *paratuberculosis*
MAS – multiple autoimmune syndrome
MS – multiple sclerosis
NOD – non-obese diabetic (mouse)
OD – optical density
PBMCs – peripheral blood mononuclear cells
PBS-T – 1x phosphate-buffered saline containing 0.05% of Tween20
PD – Parkinson's disease
PE – phycoerythrin
PHA – phytohaemagglutinin
PI – proinsulin
ROC – receiver operating characteristic
T1D – type 1 diabetes
T2D – type 2 diabetes
TGFβ – transforming growth factor beta
TNFα – tumor necrosis factor alpha
TPO – thyroperoxidase
UC – ulcerative colitis
WHO – World Health Organization
ZnT8 – zinc transporter 8 protein

Summary

Although numerous studies put in evidence the increasing incidence of type 1 diabetes (T1D), its cause remains unclear. The role of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) as a putative environmental agent triggering or accelerating the disease has been previously hypothesized in Sardinian and Italian T1D populations. The present thesis further sustains this association by reporting an elevated seroreactivity to MAP-derived epitopes and homologous human peptides corresponding to proinsulin and ZnT8 fragments in populations at different T1D stages and originating from distinct biogeographic backgrounds. Anti-MAP antibodies (Abs) resulted detectable in the first months of life before the appearance of classical autoantigens and, in most cases, were maintained in time making the selected peptides good candidates for early biomarkers. Likewise, Abs responses to the same antigens were observed among LADA patients and subjects affected by Hashimoto's thyroiditis which frequently complicates T1D. Validation with a MAP-specific lipopentapeptide confirmed these results in coincidence with a stable Abs status. In PBMC primary culture, ZnT8 peptide and its MAP homolog induced the expression of proinflammatory cytokines along with increased cell-mediated responses and apoptosis. Good correlation between values obtained for the homologous MAP/human peptide pairs point at cross-reactivity through which mechanisms of self tolerance may be disrupted leading to autoimmunity.

Contents

I. Introduction

Most common types of diabetes mellitus

Diabetes mellitus is a chronic condition associated with high blood sugar levels occurring when the production of insulin by the pancreatic β-cells is insufficient or when insulin-resistant cells fail to efficiently respond to the hormone. Insulin regulates the uptake of glucose by muscles and adipose tissue as a source of energy. Hyperglycemia, or raised fasting glucose, is a common effect of uncontrolled diabetes that over time leads to serious long-term complications such as neuropathy with subsequent muscle atrophy, damage of blood vessels resulting in retinopathy or nephropathy, and ketoacidosis due to enhanced lipolysis via the β-oxidation pathway. Life-long requirement for insulin therapy and medical supervision generate a significant economic burden on national healthcare services going hand in hand with the continuously increasing incidence of diabetes worldwide. The number of subjects affected by the disease are estimated to 422 million in 2014[1] while WHO predictions indicate diabetes as the 7th leading cause of death in 2030[2].

T1D

T1D is an autoimmune form of diabetes resulting from T-cell mediated destruction of pancreatic β-cells accompanied by a simultaneous production of Abs against islet cell targets. This process starts in early infancy and can occur over many years during childhood without developing clinical symptoms[3].

Epidemiology

T1D accounts for about 5-10% of all diabetes cases and shows uneven distribution worldwide with over a 350-fold difference in incidence between countries[4]. Globally, the incidence of T1D presents elevated trends at higher geographical latitudes and in parallel to economic development[5].

Notably, Sardinia registers the second incidence among children aged 0-14 years after Finland (44.8/100,000 and 64.2/100,000 cases each year, respectively)[6-7] contrasted by China, India and Venezuela where autoimmune diabetes is uncommon (0.1/100,000; Figure 1)[8]. Several studies describe wide variations between genetically related and neighbouring populations such as Finnish children with a triple and six-fold risk compared to, respectively, Estonians[9] and Russia Karelians[10], or ten-fold difference between Omanis and patients from Saudi Arabia[11].

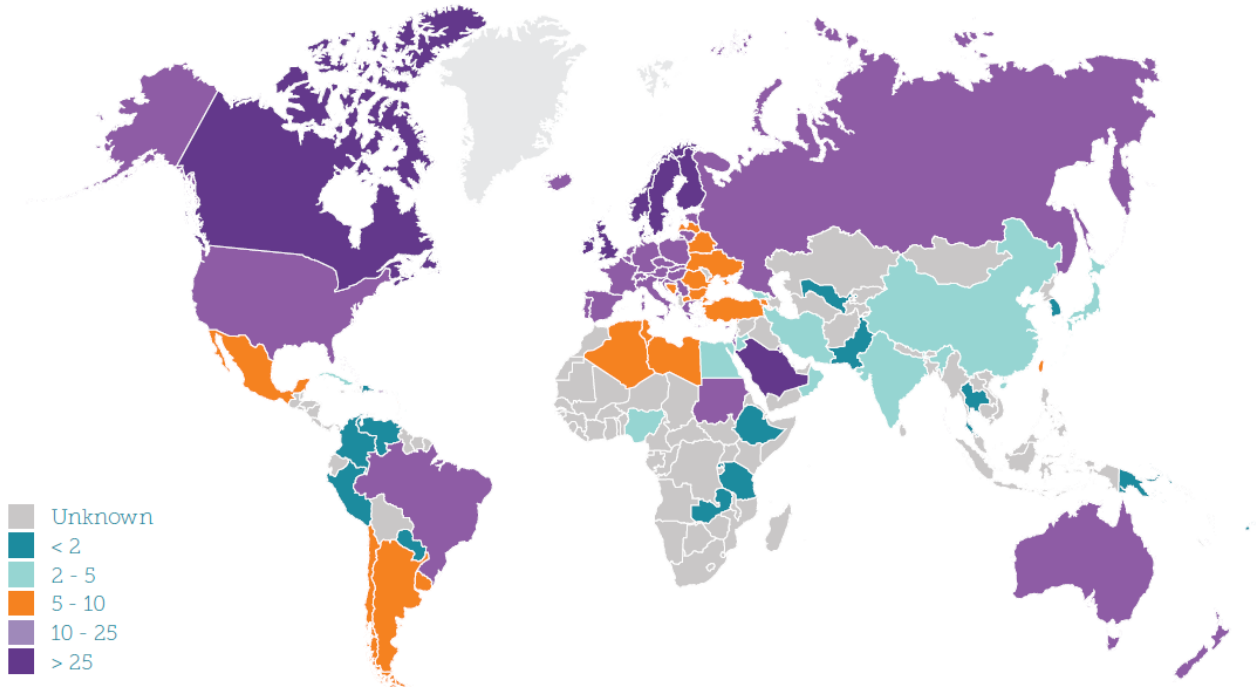


Figure 1. The global incidence of T1D among 0-14 year-old children. Values indicate the number of cases per 100,000 people each year (from the International Diabetes Federation, 2015)[12].

In contrast to other autoimmune diseases and studies in NOD models which show a strong female excess at onset[13], T1D affects males and females equally with a slight male excess in high-incidence populations[14]. This picture is inverted in low-incidence countries and ethnical groups of African and Asian origin[15]. Historically, Western populations had a low constant rate of the disease until the middle of the 20th century with a subsequent rise in incidence maintained until the present day[16]. However, marked regional differences reveal a levelling off in high-incidence countries such as Sweden[17] and a rapid increase in Eastern Europe[18].

Pathogenesis and diagnosis

Although a progressive loss of β-cell function and the requirement for daily insulin treatment for patient survival has been apparent for over a century, the precise cascade of immunologic, genetic and physiologic events that guide disease onset and progression appear thus far incomplete[19]. The β-cells are crucial for glucose sensing and release of insulin to maintain correct sugar levels within a physiologically narrow range. In the recent years, the studies in animal models spontaneously developing autoimmune diabetes such as NOD mice have demonstrated that T1D occurs as a consequence of a breakdown in immune regulation, resulting in the expansion of autoreactive CD4+ and CD8+ T cells[20-22], autoantibody-producing B lymphocytes[23-25], and activation of the innate immune system that collaborate to destroy the insulin-producing β-cells[26].

Consistent with studies in humans, the hallmark of preclinical T1D is the infiltration of antigen-specific T cells into the pancreatic islets[27]. Further β-cell damage leads to the release of self-antigens followed by epitope spreading. In the peripheral blood of T1D patients, autoantibodies against several islet targets can be detected long before disease onset and are commonly used for prediction of increased risk and to distinguish T1D from other forms of diabetes.

The likelihood of progression to T1D in autoantibody-positive individuals is influenced by factors linked to family history, age at Abs development, persistence and magnitude of the Abs response, Abs specificity (i.e. the presence or absence of certain epitopes known to be associated with high risk), and the number of detectable Abs types[28]. Abs against insulin (IAA) are usually the first to appear in young children and persist less among other classical autoantibodies[29]. Transient IAA titers in infants may result from maternal transfer but are not associated with progression to diabetes[30]. On the other hand, autoantibodies against the enzyme glutamic acid decarboxylase (GADA) are stable with age what makes them useful screening markers in young adults. The presence of Abs against zinc transporter type 8 protein (ZnT8A) is directly correlated with the age of T1D onset, whereas those directed against islet antigen-2 (IA-2A) are slightly more prevalent in younger cases and almost always detected in combination with other T1D autoantibodies. Both markers are supposed to indicate active β-cell destruction especially in late preclinical phase when they tend to cluster in association with a rapid progression to T1D and

increased diagnostic sensitivity[31]. Identification of at least two islet autoantibodies points at a significantly greater risk than a single autoantibody[32-33] and accounts for 61% of probability to develop diabetes within 10 years in relatives with T1D history[34]. Similarly, individuals with multiple islet autoantibodies but no T1D family history appear to be at high risk[35], however target specificity influenced by the strength of Abs binding determines the onset. For example, Abs directed towards the N-terminal GAD portion are associated with poor risk of T1D development, whereas Abs against the middle part and/or C-terminal of the antigen are indicative of disease progression[36].

Aetiology

Although T1D is among the most common autoimmune diseases, knowledge regarding the factors contributing to its development remains incomplete. The interplay between environmental determinants and multiple genes has been considered as a complex cascade of events leading to the loss of immune tolerance towards self antigens. The genetic background has been considered a major contributor until twin and migration studies along with in-depth association analyses involving human leukocyte antigen (HLA) genotypes showed only partial concordance that has given space to the hypothesis seeing environmental factors as key T1D contributors.

Genetic predisposition

Genes affecting immune systems are supposed to strongly influence the development of T1D. HLA is a family of more than 200 proteins involved in immune recognition of body's self components from exogenous antigens such as viruses or bacteria against which immune responses must be activated. While HLA class I are present on the surface of almost all cell types, HLA class II can be identified only on antigen presenting cells (APCs) including macrophages, dendritic cells or B cells and contribute to the activation of inflammatory responses or destruction of infected cells by displaying protein fragments to the immune system

that in turn recognizes their “self” or foreign origin. Fine mapping of a wide range of HLA variants confirmed that haplotypes strongly associated with T1D belong to HLA class II region[37-38] and classify from protective to those with extremely high risk, for example HLA DR3/DR4[39] impacting on disease development in approximately 50%.

Linkage and association studies permitted to identify single nucleotide polymorphisms (SNPs) in non-HLA genes shared within affected populations. A variable number tandem repeat (VNTR) in the insulin gene promoter contains three classes of antigens with different impact on transcription rates and T1D risk linked to the impaired negative selection of insulin reactive T cells[40]. Yet, T cell self-reactivity may be increased by inherited changes in the expression of cytotoxic T-lymphocyte antigen-4 gene (CTLA-4) gene that in normal conditions inhibits ligand interactions within T cell receptor signaling complex[41]. The increased frequency of the 1858T allele in protein tyrosine phosphatase non-receptor 22 (PTPN22) gene have been reported among T1D individuals suggesting an incorrect control of spontaneous T cell activation[42]. Certain SNPs in interleukin 2 receptor alpha (IL2RA) may affect the maintenance of tolerance to self-antigens by reduced capacity to bind IL-2 leading to uncontrolled proliferation of regulatory T cells (T_{reg})[43-44]. Recent technologies employed in genome-wide association studies (GWAS) permitted to identify over 40 new genetic loci associated to T1D[45] most of which involved in immune activation pathways such as toll-like receptor family[46], chemokine receptor CCR5[47], interferon-induced helices C domain (IFIH1)[48] or interactions between natural killer cells and HLA class I[49-52], each adding only a minimal risk to disease onset (Figure 2).

Despite more sensitive identification methods, high risk genotypes have been less frequently detected among new T1D cases over the last half century[53]. As this time frame is not sufficient for the human gene pool to change, environmental pressure is attracting more attention as a possible factor precipitating T1D in subjects with a lower genetic predisposition.

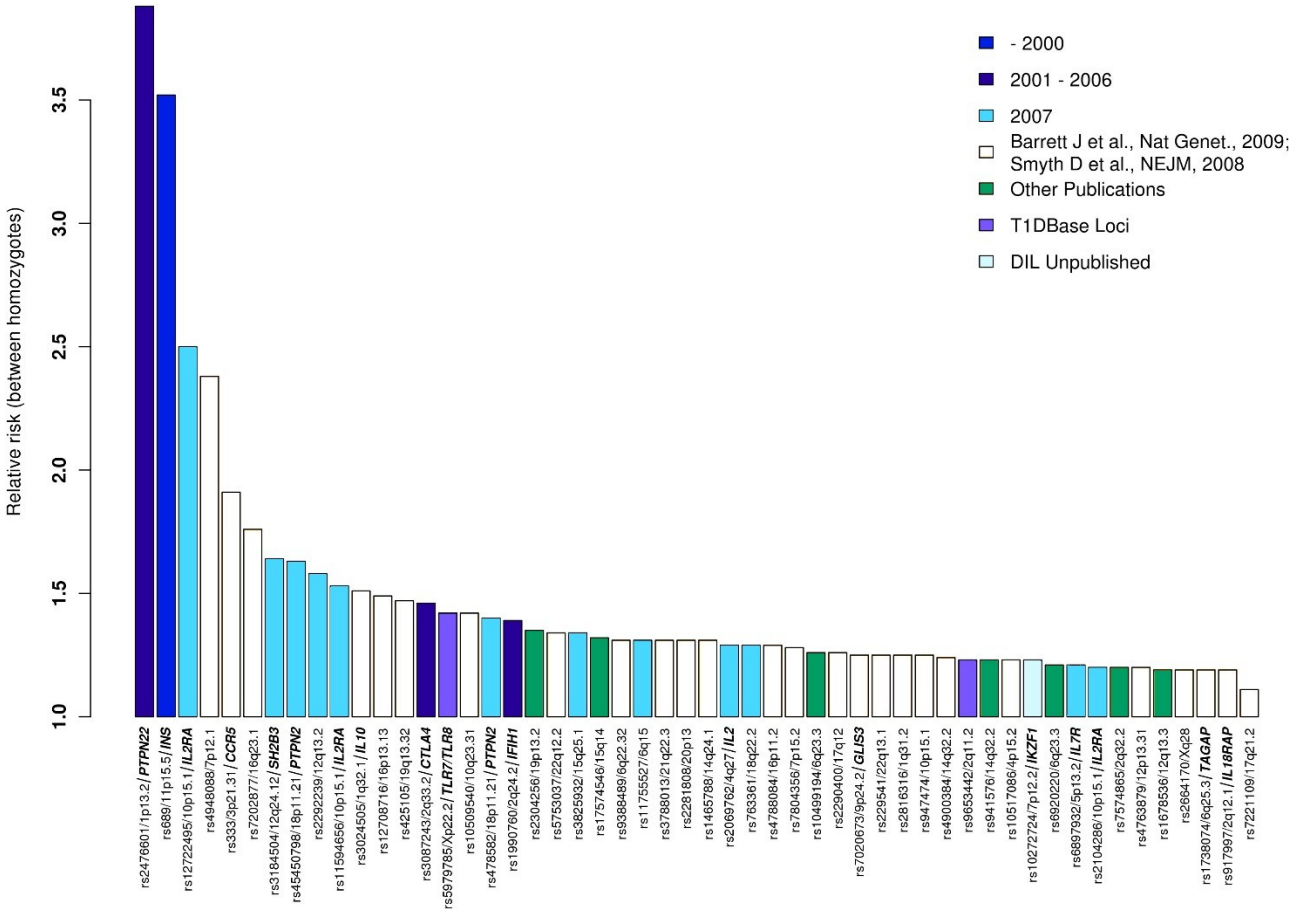


Figure 2. Currently mapped T1D non-HLA loci with respective chromosome locations and disease-associated SNPs (adapted from Todd JA, 2010)[54].

Environmental factors

Dietary components are the first source of exposure to exogenous substances in early life. While prolonged breastfeeding has generally been considered protective, the effect of cow's milk proteins on β-cell autoimmunity provided contradictory results[55-62] that, however, depend on geocultural preferences regarding milk formulas and types of complementary food to which infants are weaned. The recent Trial to Reduce Insulin-Dependent Diabetes Mellitus in the Genetically at Risk (TRIGR) evaluated the effects of weaning to an extensively hydrolyzed milk formula compared to a conventional milk based formula in Finnish babies with HLA-conferred T1D risk; after a 7-year follow-up period no differences in the incidence of T1D-associated autoantibodies was observed[63]. Likewise, a high consumption of cow's milk in childhood does

not present a linear contribution to T1D onset in Scandinavian populations[64-65] but the introduction of insulin-free formulas in the first 6 months of life reduces the cumulative incidence of standard T1D Abs by more than 60%[66] which may be favored by limiting a possible cross-reaction between bovine and human insulin that differ by only three amino acids. In a similar manner, gluten and other cereal-derived proteins were not confirmed as antigens driving the progression to T1D[67-69]. On the other hand, fatty acid status in the serum of breastfed children and the quantity of breast milk consumed per day negatively associated with islet autoimmunity[70].

In relation to the increased T1D prevalence along with the distance from the equator, supplementation with vitamin D in childhood has been considered protective against the disease. Even though several studies confirmed this association[71-73], low T1D incidence in some regions in Northern Europe[74] and high incidence in Sardinia argue with these outcomes[75]. The accelerator hypothesis linked the raised velocity of β-cell apoptosis to excess weight gain that causes insulin resistance and weakening of glucose control[76], therefore accelerated height may be a risk factor for progression to autoimmunity[77].

Seasonal T1D onset trends pointed at a probable involvement of enteroviruses[78]; persistent infections may lead to the loss of pancreatic cells through a cytolytic effect or by induction of autoimmune processes due to molecular mimicry that results from the structural similarity between viral and β-cell epitopes[79]. This concept was addressed in a recent study involving six newly diagnosed T1D patients whose pancreatic biopsy tissue presented enteroviral capsid protein 1 and specific RNA sequences indicating a low-grade infection[80]. However, impossibility to confirm whether the enteroviral particles are a cause or consequence of β-cell failure together with low number of assessed patients and previous reports excluding such an association[81-83] is far from explaining the role of viral factors. Yet, the transcription of non-protein coding HERV sequences integrated in the human genome as remnants of ancestral retroviral infections may be activated or stimulated by poorly defined exogenous agents[84-85].

T2D

In some persons, sedentary lifestyle and improper diet linked to overweight may lead to long-term metabolic alterations due to insulin resistance that result in increased release of glucose from the liver followed by hyperglycemia[86]. In contrast to T1D, the relative lack of insulin is mostly caused by reduced secretion of the hormone or by the inability of body tissues to respond to normal levels of insulin, without involving the autoimmune processes but including severe complications such as kidney failure, blindness, cardiovascular diseases and cognitive dysfunction. Chronic stress, epigenetic components and genetic risk contribute to the complexity of the disease[87-88]. Most predisposing genotypes are involved in β-cell functions and, among them, *TCF7L2* allele consistently associated to T2D in linkage studies conferring a relative risk of about 1.4[89]. To date, GWAS permitted to identify ~153 variants for T2D mapping with very few susceptibility loci shared with T1D that corroborate different mechanisms underlying the two forms of diabetes[90]. Genetic variants determine individual responses to the environment at the level of specific metabolic pathways; however, the inflammatory background of T2D-related obesity affects the studies on environmental associations.

LADA

Latent autoimmune diabetes in adults is a slowly developing subtype of immune-mediated diabetes characterized by a combination of clinical features typical to T2D and reactivity to islet cell antigens commonly detected in T1D. GADA and IA-2A are the most conclusive biomarkers[91] that, along with insulin independence period of at least 6 months following disease onset, permit to distinguish LADA from other forms of diabetes. Although general diagnostic criteria has been widely discussed[92-93], GADA titers remain a benchmark for discrimination between phenotypes recognized among LADA patients presenting clinical characteristics closer to classical T1D or T2D[94].

It is estimated that LADA accounts for almost 10% of newly diagnosed adult-onset diabetes cases, therefore its importance in terms of prevalence exceeds that of T1D. Nonetheless, LADA is far less investigated and its aetiology remains so far poorly understood. Even though some studies reported increased frequency of predisposing genotypes such as the T2D-associated variant in *TCF7L2* transcription factor[95] or HLA T1D-susceptibility haplotypes[96-97], major occurrence of less protective genotypes[98] and familial clustering linked to LADA pathogenesis[99], hereditary factors alone cannot explain progression towards β-cell failure in subjects at low genetic risk. Despite its

phenotypically heterogeneous nature, LADA is a distinct entity frequently associated with other endocrine disorders[100]. The scientific literature describes environmental risk factors related to dietary habits and lifestyle[101-105], however, unlike for T1D, scarce information is provided regarding infectious agents putatively contributing to LADA.

High-incidence comorbidities

Patients with diabetes show a greater prevalence for comorbidities compared to healthy population. T1D has been associated to other autoimmune diseases such as AIDT with up to 20% of T1D subjects bearing anti-thyroid autoantibodies that tend to increase with age and diabetes duration[106]. Additionally, GADA and ZnT8A are used as biomarkers of the risk to develop AITD[107-108]. Coeliac disease is observed in about 5% of T1D patients with the age at onset, female gender and concomitant AITD considered as predisposing factors[109-111]. These frequencies reflect the geographical incidence of T1D and, according to a Swedish study, the presence of coeliac-specific autoantibodies has been reported in 62% of T1D subjects diagnosed with coeliac disease within 24 months following onset of diabetes[112-113]. Addison's disease, polyglandular syndromes and collagenopathies co-occur with T1D to a lower extent[114].

In LADA, high GADA titers were associated with the presence of anti-TPO Abs and a more severe autoimmunity profile, which specifically predisposed for AIDT[115-116]. In contrast, metabolic complications are the most common comorbidities of T2D. Association studies indicated increased risk for 27 autoimmune diseases among T2D patients, however the impact of environmental confounders that might favour the imbalance in glucose metabolism or, vice versa, facilitate the progression to autoimmunity, was not taken into account[117].

Mycobacterium avium subsp. *paratuberculosis* (MAP)

MAP is widely known as the causative agent of Johne's disease affecting primarily ruminant livestock and defined by persistent gastroenteritis, progressive weight loss, reduced milk production and eventually death. Despite its worldwide distribution and over a century-long history of investigation, efficient diagnostic methods and prevention solutions are still unavailable due to the absence of symptoms in the first years after initial infection and to peculiar features of MAP with the very slow growth being the first drawback for strain characterization[118].

Features and transmission

Similar to other mycobacteria, MAP has a lipid-rich cell wall that confers limited permeability to biocides and increased tolerance to stress induced by antibiotics, disinfectants and high/low temperatures that permit a long-time survival in the environment[119-121]. As an obligate pathogen, it developed particular metabolic adaptations allowing massive proliferation in the host at late stages of infection with specific tropism to the intestine[122-124]. Its ability to reside intracellularly in macrophages is possible through the inhibition of phagosomal maturation, thereby modulating or evading host cell defence mechanisms[125-128]. Moreover, gene clusters involved in metal transport along with those encoding putative siderophore synthesis and uptake systems[129-130] help MAP overcome essential ions starvation induced by macrophages[131-135].

Young animals are most susceptible to MAP infections due to a large number of M cells that form the main way of entry due to the lack of hydrolytic enzymes and inability to form mature phagosomes[136]. After a latency period, the mycobacterium is shed in feaces and milk which may be the source of feed contamination and oral transmission within herd. Even though MAP is not considered a zoonotic agent, it has been detected in a wide range of foods, primarily meat, milk, dairy products and infant formula, creating a potential risk of daily exposure for humans[137-140]. Many reports confirm that pasteurization process is not sufficient for a complete elimination of viable MAP cells, especially when isolated from human PBMCs[141-143].

MAP and human disease

In humans, MAP has been associated with CD considering symptoms similar to those observed in animals affected by Johne's disease and a high frequency of MAP isolation from intestinal biopsies or PBMCs of CD patients[144]. Both diseases emerged in the same continents, today most affected by Johne's disease: North America, Europe and Australia[145], however the causal role of MAP has not been as yet ascertained. Some theories suggest that MAP invades and colonizes an already inflamed bowel[146] as previously hypothesized for *H. pylori* and duodenal ulcer disease. Numerous investigators pointed at the contribution of the mycobacterium to UC based on the identification of the specific *IS900* gene sequence present in several copies in the MAP genome, epidemiological evidences showing similar special and temporal distribution of UC and CD, as well as the development of both diseases in unrelated individuals living together[147-148].

Recently, MAP has been linked with other autoimmune diseases due to molecular mimicry between mycobacterial and human protein fragments. A transmembrane domain of ZnT8 expressed in endocrine cells including pancreatic islets and the thyroid shares amino acid sequence homology with a putative cation efflux MAP3865c which leads to the cross-reactivity and erroneous recognition of self and exogenous antigens. A few findings contribute to the strength of this association. ZnT8A are present in blood of patients affected by T1D and autoimmune thyroiditis but not in T2D subjects; in a similar fashion, high prevalence of Abs against MAP3865c has been reported in Italian children adults with established T1D or HT, while the same antigen elicited poor reactivity in T2D and healthy populations[149-150]. Likewise, Italian children at T1D onset showed increased levels of Abs against MAP-derived peptides homologous to ZnT8 and proinsulin[151]. Moreover, MAP DNA has been isolated from 63% individuals of a Sardinian T1D cohort and viable MAP cells have been successfully cultured from blood[152-153].

Several studies described the involvement of MAP in diseases affecting the central nervous system. Positivity for *IS900* detection tests and cross-recognition between EBV, MAP and myelin basic protein was reported in MS Sardinian patients with major prevalence when associated to polymorphisms in *SLC11A1* gene involved in host resistance to intracellular pathogens and iron metabolism[154-155]. In a Japanese MS population, Abs response directed against MAP crude extracts appeared with a major frequency[156]. The role of MAP in disease pathogenesis has also been hypothesized for PD, even though not confirmed by cross-reactivity with homologous fragments of human zinc transporters[157].

Hypothesis and purpose of the study

Numerous sequence homologies existing between MAP and human proteins may potentially be on the bases of cross-reactivity enhancing organ-specific autoimmune responses. Both ZnT8 and proinsulin show amino acid stretches overlapping with several MAP components are involved in T1D pathogenesis as self-antigens. The ubiquitous nature of MAP, its ability to survive heat sterilization and, in turn, presence in foods may facilitate the transmission to humans. Even though latent MAP infection could persist asymptomatically, exposure to mycobacterial antigens generates the production of Abs and, in some subjects, loss of tolerance towards homologous peptides.

The studies described in the present thesis aimed at evaluating seroreactivity against selected MAP-derived epitopes characterized by high sequence identity with human molecules in autoimmune diabetes and concomitant disorders. Time-related correlation with regard to the

appearance of the first classical autoantibodies, genetically-conferred risk and the phase of disease was performed with the purpose to characterize anti-MAP Abs as possible biomarkers for early prediction of T1D. Finally, selection of cohorts with distinct biogeographical and cultural background was performed in order to assess how different lifestyles and environmental conditions mirror humoral responses to MAP.

II. Seroreactivity of Sardinian newborns at risk for T1D

This chapter is based on the following study:

Niegowska M et al. Recognition of ZnT8, Proinsulin, and Homologous MAP Peptides in Sardinian Children at Risk of T1D Precedes Detection of Classical Islet Antibodies. J Diabetes Res. 2016;2016:5842701.

The study involved the analysis of plasma samples obtained from Sardinian children enrolled in the TRIGR study, for the presence of Abs against selected MAP peptides and homologous ZnT8 and PI fragments to identify possible biomarkers for early prediction of T1D development towards overt clinical disease. Moreover, peptides homologous to the human ZnT8 protein derived from *H. pylori* were assessed in order to achieve a picture of seroreactivity to a pathogen not associated with T1D and a possible cross-reaction with anti-MAP Abs.

Results

In the study population, 10 out of 23 T1D ARS (43.48%) resulted positive for anti-MAP1,4αgbp$_{157-173}$ (M1) Abs, compared to 9.09% of HCs. The same prevalence among ARS was detected for the homologous PI$_{64-80}$ (PI1) peptide, but only in 13.64% of HCs.

Abs against MAP2404c$_{70-85}$ (M2) were recognized by 7 ARS (30.43%) and 13.64% HCs, while 6 ARS (26.09%) and 9.09% of HCs were positive for its homolog PI$_{46-61}$ (PI2) (Figure 3).

Reactivity to MAP3865c$_{133–141}$ (M3) was registered for 6 ARS (26.09%) and 9.09% of HCs, whereas 5 ARS (21.74%) resulted positive for the homologous ZnT8$_{186-194}$ (Z1) peptide, comparing to 4.55% among HCs.

Anti-MAP3865c$_{125-133}$ (M4) Abs were found in 5 ARS (21.74%) and in none of the HCs. Recognition of its homolog ZnT8$_{178-186}$ (Z2) among the analyzed subjects accounted for 6 ARS (26.09%) and 4.55% of positivity for HCs.

Overall, 47.82% of children (n=11) reacted to any of the analyzed peptides compared to 22.73% of age-matched HCs. A high degree of correlation (r^2>0.9) was found between anti-MAP and the respective anti-PI/ZnT8 Abs titers (Figure 4).

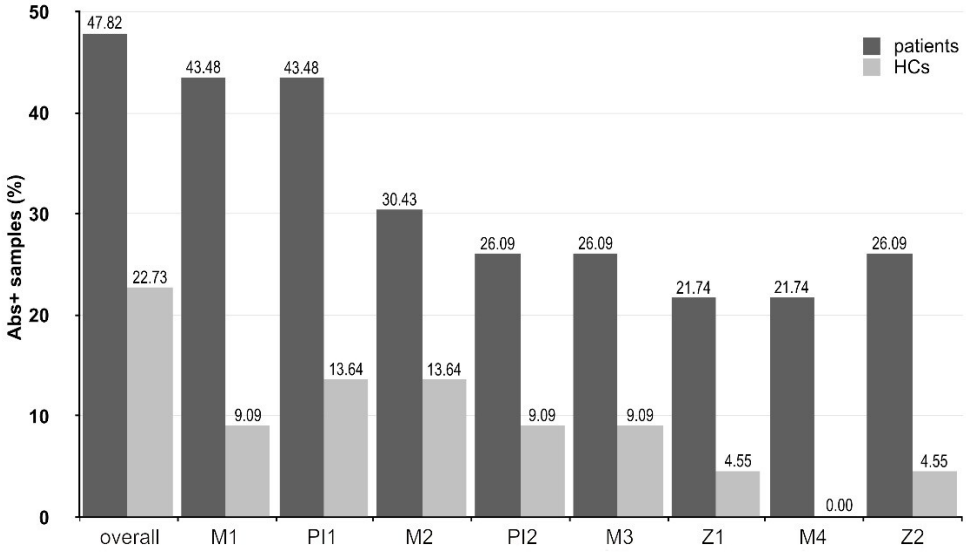


Figure 3. Prevalence of Abs against MAP, PI and ZnT8 homologous peptides in children at risk of T1D and healthy controls. Sera were tested in duplicate for their reactivity against plate-coated peptides. Percentage of children with Abs positivity to any of the analyzed peptides is indicated by the first column pair. Dark bars represent children at risk of T1D; light grey bars correspond to HCs.

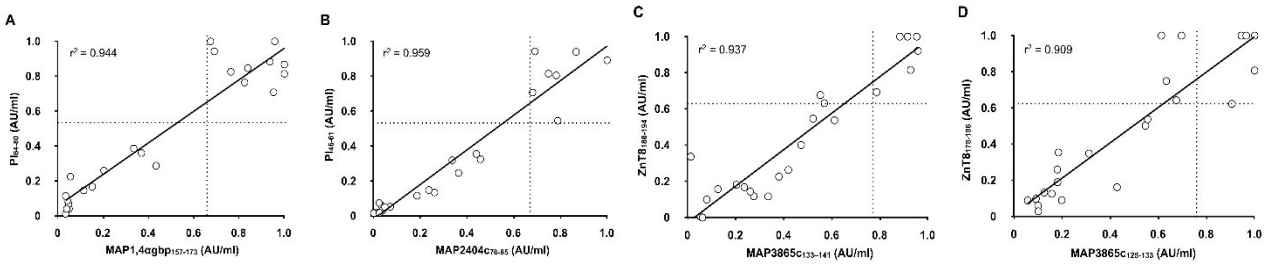


Figure 4. Correlation between Abs recognizing MAP and its homologous human epitopes in Sardinian children at risk for T1D. Distributions relative to peptide pairs MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ (A), MAP2404c$_{70-85}$/PI$_{46-61}$ (B), MAP3865c$_{133–141}$/ZnT8$_{186-194}$ (C) and MAP3865c$_{125-133}$/ZnT8$_{178-186}$ (D) in 23 at-risk children. Each circle represents Abs of one ARS. The dotted lines indicate the cut-off for positivity used in each assay, as calculated by ROC analysis.

Interestingly, of the 23 ARS followed in time, 5 resulted positive for all the peptides MAP3865c$_{133–141}$, MAP3865c$_{125-133}$, MAP2404c$_{70-85}$ and MAP1,4αgbp$_{157-173}$, ZnT8$_{186-194}$, ZnT8$_{178-186}$, PI$_{46-61}$ and PI$_{64-80}$ (Table 6); in other 5 ARS, response to at least three peptides was registered. The highest reactivity among subjects positive for the assessed epitopes was observed for MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ peptide pair, registered for 10 at-risk children (91%); two of them additionally recognized Abs against MAP2404c$_{70-85}$/PI$_{46-61}$ homologs and anti-MAP3865c$_{125-133}$/ZnT8$_{178-186}$ Abs were found in one child. Four children presented Abs positivity against non-homologous peptides MAP3865c$_{133–141}$, MAP3865c$_{125-133}$, MAP2404c$_{70-85}$, ZnT8$_{186-194}$ and PI$_{46-61}$. The overlap in reactivity against homologous antigens among positive HCs (n=5) accounted for 40% and regarded only two subjects with Abs directed against at least 4 epitopes: one HC recognized

MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ and MAP2404c$_{70-85}$/PI$_{46-61}$ peptide pair while the other one showed positivity to MAP3865c$_{133–141}$/ZnT8$_{186-194}$ and MAP2404c$_{70-85}$/PI$_{46-61}$ homologs. Non-homologous reactivity was detected for MAP1,4αgbp$_{157-173}$, PI$_{64-80}$, MAP3865c$_{133–141}$, ZnT8$_{178-186}$ and MAP2404c$_{70-85}$.

Five of the positive children presented autoimmunity against either the studied peptides or classical islet antibodies. Furthermore, positivity to our peptides not only appeared before detection of classical autoantibodies within 6 months of age (Table 1), but was also maintained in time. Eventually, 3 ARS developed diabetes few years later demonstrating the importance of the studied peptides as potential preclinical biomarkers for diagnosis of T1D in subjects at risk for T1D. However, another child developed T1D even though antibody values were not significantly high, emphasizing the fact that T1D is determined by multiple factors contributing to disease manifestation.

Anti-HP Abs, either directed against J0I929_HELPX$_{1-11}$ or T2T4W3_HELPX$_{99-105}$, were detected in one ARS (4.35%) and the results were not statistically significant (p=0.82 and 0.18, respectively). No positive cases were observed among controls (Figure 5) and no correlation with progression to T1D or response to the analyzed peptides was found.

Table 1. Age at detection of islet autoantibodies and immune reactivity to MAP, ZnT8 and PI homologous peptides in children at risk of T1D.

ID[a]	1 month	3 months	4 months	6 months	10 months	13 months	17 months	18 months	2 years	3 years	4 years	5 years	6 years	7 years	8 years	9 years
2			**M1**, PI1, Z4			Z3			**M2, M3**	PI2	**M4**					
4				PI2						**M1**, PI1				**M2**	**M4**	Z3
5							ICA, IAA, GADA, IA2A									
9								**M1**, **M2**, **M3**, Z3, **M4**,		PI2, Z4	ICA	PI1				
10		PI1, **M2**, PI2, **M3**, Z3, **M4**, Z4								**M1**					ICA	
12										**M1**, PI1, **M4**, Z2, **M2**, PI2, ICA, IAA, IA2A						
14						**M4**		ICA, IAA, GADA, IA2A								
16									PI1, **M2**	Z3			**M3**			
20									**M3**		PI1	**M1**, PI2, **M4**		ICA		
22						PI1, **M2**, PI2, **M3**, Z3, **M4**			**M1**, Z4							
24					**M2**, **M4**, Z4	**M1**, PI1, PI2, **M3**, Z3										
38	Z3											**M1**, PI1				

[a]: Identity of children positive to the studied peptides or/and islet autoantibodies (n=12); highlighted patients progressed to T1D at 5, 4 and 1,5 years of age, respectively; patient indicated in italics was lost during the follow-up.

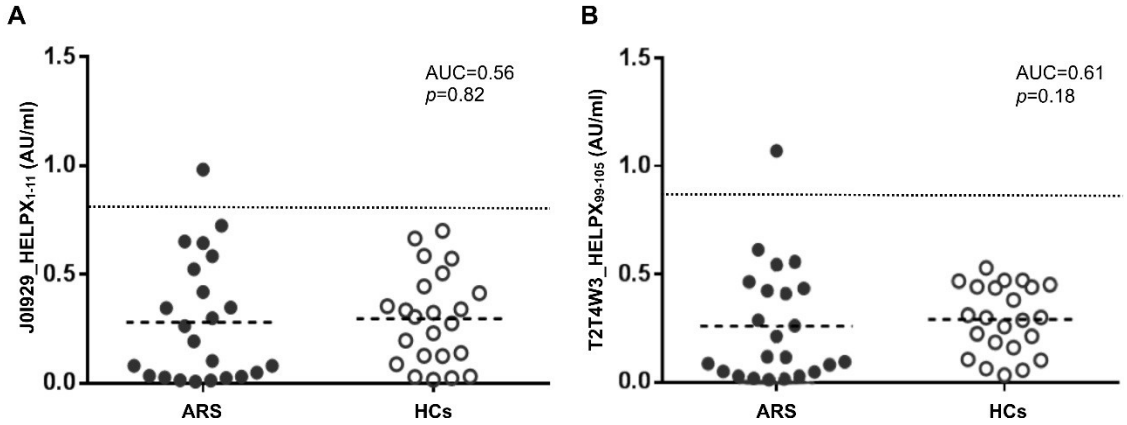


Figure 5. Distribution of anti-HP Abs levels measured by ELISA in T1D at-risk subjects and healthy controls. Sera of 23 ARS and 22 HCs were screened for Abs against J0I929_HELPX$_{1-11}$ (A) and T2T4W3_HELPX$_{99-105}$ (B). The dotted lines represent cut-off values calculated by ROC analysis and used to discriminate between positive and negative samples. Dashed lines indicate the respective mean OD values. Area under the curve (AUC) and *p* values are reported in the top-right corners.

Discussion

As numerous studies put in evidence the raising incidence of diabetes in children, an early diagnosis is of great importance to define a correct method of restraining disease development and to establish ulterior treatment. Even though genetic predisposition has been considered a crucial factor for T1D onset, less newly registered cases present genotypes of high or moderate risk[158-163]. Environmental factors seem to be critical in the pathogenesis of diabetes and include diet, infectious agents, perinatal and psycho-social conditions that vary among countries[164]. Several intracellular pathogens have been studied as possible T1D triggers, but none of them was proved to cause the disease.

Cow's milk is the major mean of MAP transmission from infected cattle to humans. In developed countries, cow's milk-based formulas are certainly a frequent source of exposure to exogenous complex proteins in postnatal life[3]. Nonetheless, after 7 years of the TRIGR follow-up no significant difference in progression towards β-cell autoimmunity was detected between children with HLA-conferred T1D susceptibility weaned to a conventional or an extensively hydrolyzed formula, the latter containing 99.7% of peptides with a molecular weight >2 kDa[63]. If initially present, survival of MAP cells exposed to enzymatic treatment is plausible; nevertheless MAP-peptides whose molecular weight ranges from 0.86 kDa for MAP3865c$_{125-133}$ to 1.83 kDa for MAP3865c$_{125-133}$, could persist in the hydrolyzed formula following enzymatic digestion and act as antigens stimulating T1D autoimmunity. Furthermore, breastfeeding continued during the study period could contribute to the

transfer of MAP, MAP-derived peptides or anti-MAP antibodies from mother to child. Macrophages are the most abundant cells in the breast milk counting 40-80% of cell fraction[165] and the primary target of MAP. Studies on cattle with subclinical and overt Johne's disease confirmed that MAP is shed into colostrum and milk during lactation upon its isolation in culture and by real-time PCR targeting IS900 MAP-specific gene[166]. Similarly, MAP has been isolated from human breast milk of few Crohn's patients[167], however research is required to assess the coincidence of T1D onset with the presence of MAP in mother's breast milk and its transmission pathway to offspring.

MAP colonization of the mucosa lining the small intestine and the following uptake by M cells and enterocytes may play a triggering role in antibody production[168]. A contribution of MAP in inducing a latent infection in humans could result from molecular mimicry with ZnT8 and PI epitopes, leading to autoimmune responses. Cross-reactivity to the common target sequences and specificity of the homologous anti-MAP/ZnT8 Abs demonstrated recognition of a transmembrane domain of ZnT8 protein that cannot be evaluated by standard anti-ZnT8A tests which employ a fusion protein combining extra-luminal domains. The same ZnT8 region has a high homology with peptides derived from *H. pylori* already evaluated in association with autoimmune thyroiditis[169]; in contrast to MAP, anti-HP Abs were detected at very low levels with a similar prevalence in children at risk for T1D and the control group providing an additional support for the association of MAP with autoimmune diabetes. A high degree of correlation between the homologous peptides further points at their cross-reactivity and segregation within the same sera.

Although anti-IAA are considered the first circulating Abs to activate islet cell autoimmunity[170] and the single β-cell-specific autoantigen in postnatal period, birth cohort studies revealed that IAA can be detected only from 6 months of age in children genetically predisposed for T1D[171]. In the present study, all human peptides and their MAP homologs resulted detectable during the first 6 months after birth in 2 out of 6 positive children for which samples collected right after delivery were available. Response against insulin in three ARS partially followed positivity to the proinsulin peptides. One subject developed an early immunity against both PI peptides and their MAP homologs; regardless of missing anti-PI Abs in the other two children, although anti-MAP Abs were present in one of them, progression to diabetes was observed exclusively in these three cases (13,04%) during sample collection period and was accompanied by multiple islet autoimmunity. Two subjects positive to all the assessed islet autoantibodies were younger at T1D onset (17-18 months) compared to the subject with IAA, IA2A and ICA but negative for GADA (4 years). Children positive to ICA as a single autoantibody recognized at least five anti-MAP and the homologous peptides.

In the majority of cases positive to the eight analyzed peptides seroreactivity to most of the epitopes appeared at the same time-point. In contrast, children displaying incomplete responses to

MAP, ZnT8 and PI peptides presented an Abs pattern with gradual detection in the space of years. From the three cases of T1D onset, two were positive to at least one anti-MAP Abs detectable from 6 months to one year prior to β-cell autoimmunity. In other children this gap was even longer, ranging from 2 to 8 years. The lack of response to any of the investigated peptides in the child who progressed to diabetes may be explained by a high-risk HLA genotype or other risk factors such as concomitant diseases or high frequency of T1D familiarity, however these data were not available owing to the double-blind design of the TRIGR study.

Unfortunately, individual formula treatment assignments cannot be disclosed due to the ongoing follow-up. Individual clinical data relative to the progression of diabetes-related autoimmunity following the TRIGR's 7-year follow-up period are not revealed. Furthermore, samples covering the entire age range were not available for every participant, leading to the loss of an interesting subject with early response to all the analyzed peptides; lack of samples collected in the first months after birth for some children, including those who developed T1D, made the early Abs pattern impossible to evaluate ultimately.

III. Detection of anti-MAP Abs in Italian children and youths at T1D onset

This chapter is based on the following studies:

Niegowska M et al. Type 1 Diabetes at-risk children highly recognize Mycobacterium avium subspecies paratuberculosis epitopes homologous to human Znt8 and Proinsulin. Sci Rep. 2016 Feb 29;6:22266.

Niegowska M et al. Seroreactivity against Specific L5P Antigen from Mycobacterium avium subsp. paratuberculosis in Children at Risk for T1D. PLoS One. 2016 Jun 23;11(6):e0157962.

Sardinian populations display a high genetic homogeneity stemming from the shared ancestry coupled with evolutionary forces[172] and resulting in susceptibility to autoimmune diabetes, Considering the estimates that MAP infections among cattle herds in Sardinia are of particularly high frequency reaching 60%, exposure to MAP of an external population would occur with minor intensity providing an important ground for comparison of the two cohorts. In the this study, the Abs pattern involving seroreactivity to MAP-derived peptides and their human ZnT8/PI homologs was investigated in children and youth at risk for T1D from mainland Italy in order to verify whether the prevalence observed among Sardinian pediatric patients follows similar trends in a different biogeographical background. A correlation with classical islet autoantibodies was performed to evaluate a possible role of anti-MAP Abs as early predictive biomarkers. Moreover, time-point variations in Abs positivity were analyzed with reference to the further onset of T1D and risk factors including HLA genotype, concomitant diseases and familiarity. Occurrence and duration of cross-reactivity due to epitope homology with human T1D autoantigens was further evaluated upon validation with synthetic L5P antigen identified as a MAP distinctive feature among subspecies.

Results

Among 54 subjects at risk of T1D, 70.37% (n=38) resulted positive to at least one of the eight assessed peptides compared to 16.67% (n=7) of HCs. 78.95% (n=30) of the positive at-risk children had Abs targeting not less than four epitopes, 11.11% of whom were fully responsive to all peptide pairs. Considering healthy controls, multiple serum reactivity to at least three peptides was observed in four volunteers, whereas the remaining three positive subjects responded only to PI_{46-61}, PI_{64-80} or $MAP3865c_{133–141}$.

Upon single-peptide analysis of Abs reactivity, MAP3865c$_{133–141}$ was recognized by 61.11% of at-risk subjects and by only 7.14% of HCs (AUC=0.72, p<0.0001). The highest immunoreactivity among at-risk children was detected for ZnT8$_{186-194}$ and reached 62.96% in comparison to 7.14% among HCs (AUC=0.74, p<0.0001).

Serum Abs reactivity to MAP3865c$_{125-133}$ accounted for 62.96% as well (AUC=0.80, p<0.0001), while recognition of the homologous ZnT8$_{178-186}$ was observed in 51.85% (AUC=0.75, p<0.0001) of at-risk subjects, comparing to 4.76% of HCs for both peptides.

Concerning MAP homologs of proinsulin-derived peptides, anti-MAP1,4αgbp$_{157-173}$ Abs were found in 22.22% of at-risk children and in 7.14% of HCs (AUC=0.58, p=0.17). Abs positivity against PI$_{64-80}$ accounted for 16.67% among at-risk individuals, however prevalence among HCs maintained levels of 7.14% detected for the homologous peptide (AUC=0.57, p=0.19).

20.37% of at-risk subjects reacted to both MAP2404c$_{70-85}$ and PI$_{46-61}$ compared to 7.14% (AUC=0.54, p=0.53) and 9.52% (AUC=0.56, p=0.25) of positivity to the respective epitopes found among HCs. Figure 6 depicts percentages of each peptide pair. Interestingly, a slightly higher prevalence of positivity to MAP/PI peptides compared to MAP/ZnT8 was observed among HCs, in contrast to at-risk subjects presenting an inverse picture.

When time-point samples collected within further four years were analyzed for the presence of Abs against the homologous peptides, seroreactivity appeared maintained in 2 out of 3 at-risk subjects initially positive for the full set of epitope pairs. Similarly, Abs-negative status was constant in children (n=3) not demonstrating any response at the first blood collection. Fluctuations involving responses to the selected peptides were registered in subjects whose Abs positivity was at first incomplete (n=8), with the exception of two youth whose Abs status remained unvaried. Eight individuals lost their immune reactivity in the course of 1-3 years but not before the age of 5. These observations are reflected by changes in responsiveness over the time-points registering decreasing trends in prevalence of Abs against MAP/ZnT8 homologs and increased reactivity to MAP/PI, especially MAP2404c$_{70-85}$ (Figure 7A).

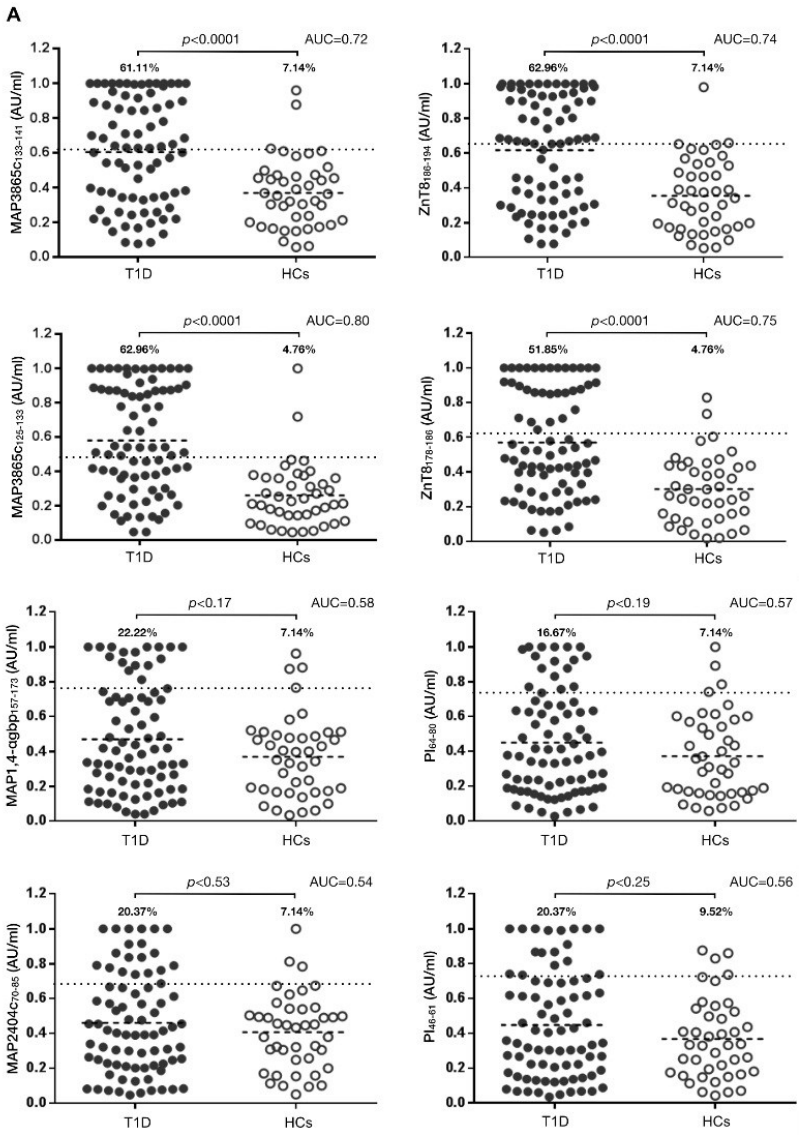
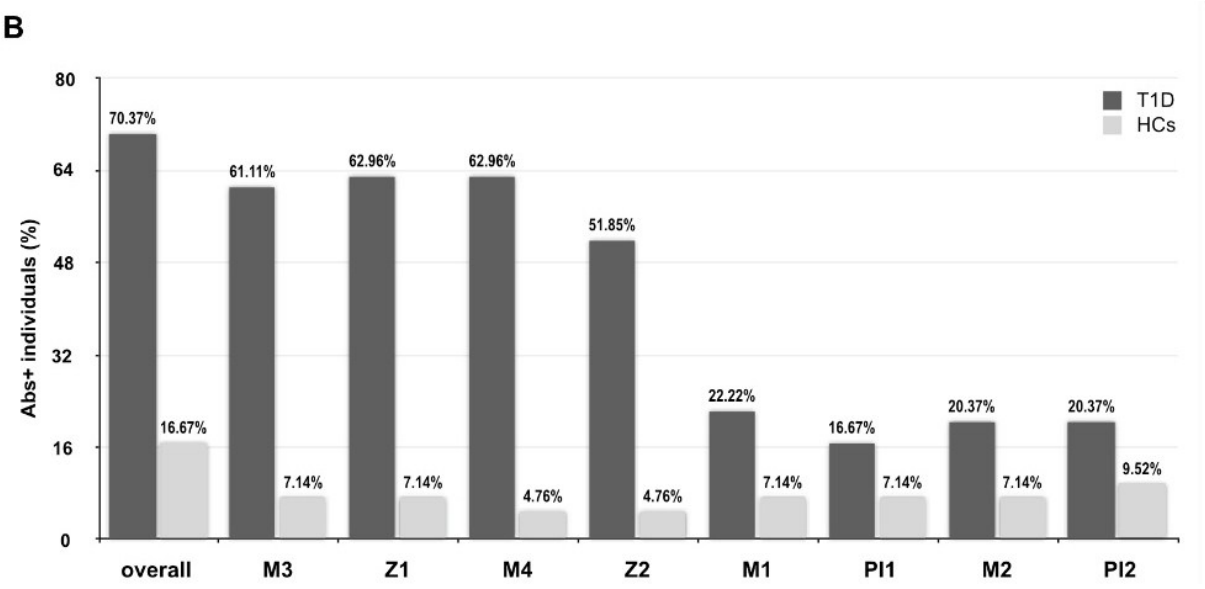


Figure 6. Prevalence of Abs against MAP, proinsulin and ZnT8 homologous epitopes in T1D at-risk subjects and HCs

(A) Distribution of Abs values based on the statistical analyses performed for all peptides separately. The dotted lines indicate thresholds of positivity relative to each assay calculated by ROC analysis. The percentage of Abs-positive at-risk subjects is reported on top of each distribution; horizontal bars specific for T1D and HCs groups correspond to means. AUC and p values (CI 95%) are indicated above the graphs.

(B) Percentage of children with Abs positivity to selected epitopes upon single-peptide analysis. The first column pair summarizes reactivity to any of the analyzed peptides. Dark bars represent subjects at risk for T1D; light grey bars correspond to HCs.



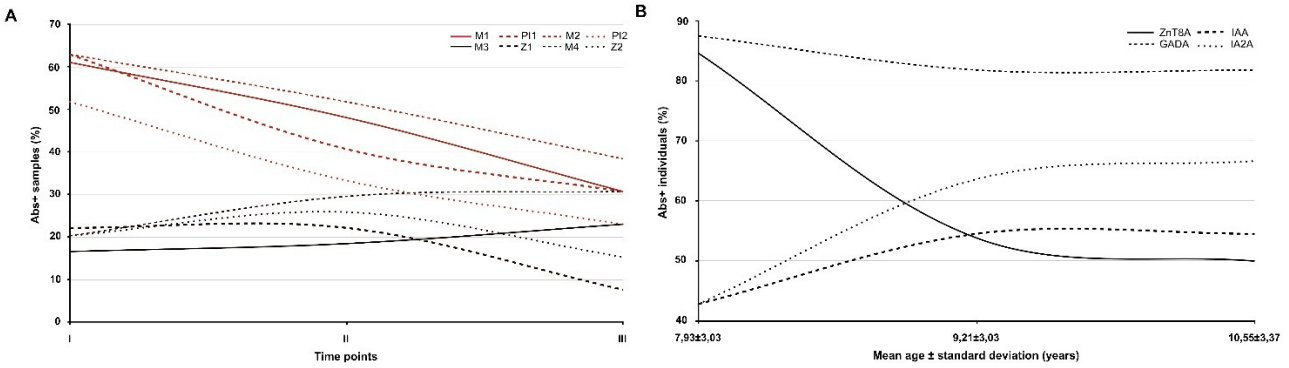


Figure 7. Time-related prevalence of Abs directed against the homologous epitopes and classical islet autoantibodies in children at risk for T1D. (A) Three time-point variations in Abs reactivity to the four peptide pairs. MAP/ZnT8 homologs are indicated by black lines, while grey lines indicate MAP/PI homologous epitopes. (B) Time-dependent classical Abs status evaluated including ZnT8, GADA, IAA and IA-2A in 13 at-risk subjects.

The overlap between the homologous peptides in all samples of T1D at-risk subjects equaled 83.87% for MAP3865c$_{133–141}$/ZnT8$_{186-194}$, 73.33% for MAP3865c$_{125-133}$/ZnT8$_{178-186}$, 41.38% for MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ and 55.88% for MAP2404c$_{70-85}$/PI$_{46-61}$. Among HCs, this pairwise reactivity was complete for MAP3865c$_{125-133}$/ZnT8$_{178-186}$ followed by 50% for MAP3865c$_{133–141}$/ZnT8$_{186-194}$ and MAP1,4αgbp$_{157-173}$/PI$_{64-80}$, and reaching 40% of overlap for MAP2404c$_{70-85}$/PI$_{46-61}$. A high degree of correlation (r^2>0.8) was found between anti-MAP and the respective anti-ZnT8/PI Abs titers with an even greater coefficient value (r^2>0.9) for the MAP3865c$_{125-133}$/ZnT8$_{178-186}$ pair (Figure 8).

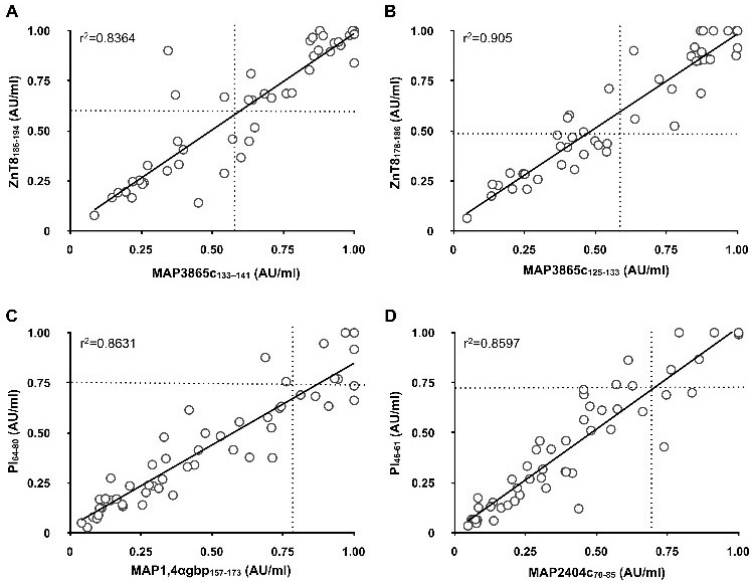


Figure 8. Correlation between Abs recognizing MAP and its homologous human epitopes in Italian children at risk for T1D. Correlations are shown between Abs against (A) MAP3865c$_{133–141}$/ZnT8$_{186-194}$, (B) MAP3865c$_{125-133}$/ZnT8$_{178-186}$, (C) MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ and (D) MAP2404c$_{70-85}$/PI$_{46-61}$. Each circle represents Abs detected in one sample. The dotted lines indicate cut-off points for positivity used in each assay, as calculated by ROC analysis.

Upon sex-related analysis of at-risk children, females presented a higher prevalence of Abs directed against MAP/ZnT8 (59.26-74.07%, $p<0.05$), with a greater positivity obtained for $ZnT8_{186-194}$ and $MAP3865c_{125-133}$ epitopes (Figure 9A). Prevalence to the same peptide pairs in males (44.44-59.26%) reached the highest values for both MAP-derived peptides homologous to ZnT8. These trends, even though with lower percentages (14.81-18.25% vs. 14.81-25.93%), were inverse for MAP/PI homologs in both genders with the exception of PI_{64-80} for which females displayed a slightly higher prevalence.

Samples were further analyzed after grouping in two age ranges (0-9 and 10-18 years) showing 75% of prevalence to any of the analyzed epitopes in the younger group when compared to children older than 9 years for whom 61,9% of positivity was registered (AUC=0.56, $p<0.04$; Figure 9B). However, a complete serum reactivity to the 8 peptides was found throughout different ages in both groups. Upon single-peptide analysis, the highest positivity was obtained at equal levels for $MAP3865c_{133–141}$/$ZnT8_{186-194}$ homologs (71.88%) within 0-9 years, while the lowest response in the same group corresponded to $MAP2404c_{70-85}$ (15.63%). The 10-18 year-old children displayed the highest prevalence for $MAP3865c_{125-133}$ (52.38%), whereas Abs against both proinsulin epitopes were less recognized (14.29%).

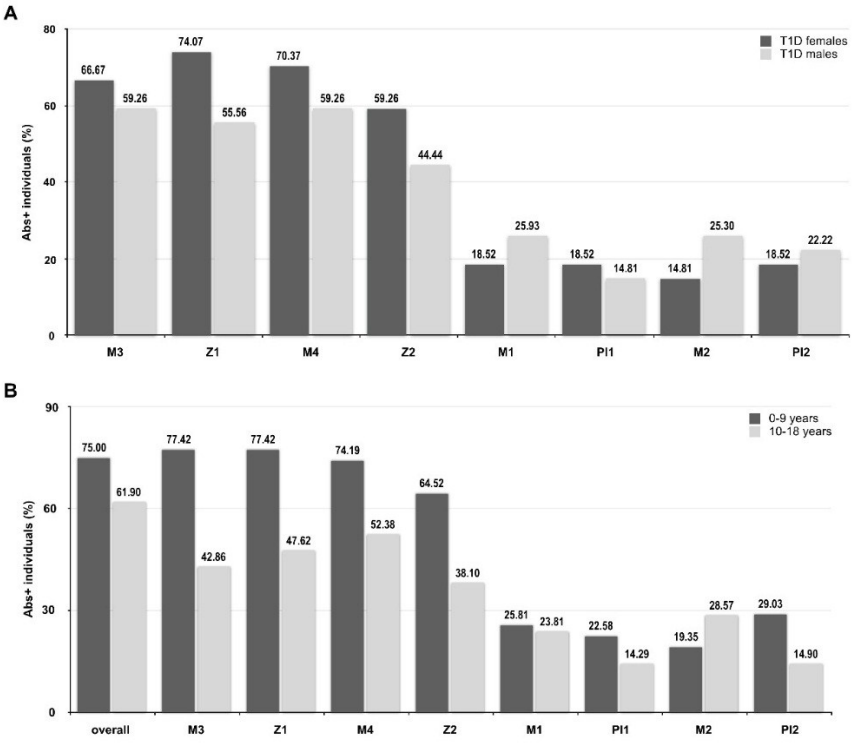


Figure 9. Age/sex-related prevalence of Abs against MAP, proinsulin and ZnT8 homologous epitopes in T1D at-risk subjects based on a single-peptide analysis. (A) Sex-related Abs status of positive individuals is indicated by dark grey bars for females and light grey bars for males. (B) Analysis performed for 0-9 (dark grey bars) and 10-18 (light grey bars) year-old groups. The first column pair indicates reactivity to any of the analyzed peptides.

Screening for classical islet autoantibodies in the first time-point samples revealed that ZnT8 were the most frequent Abs detected in 58.82% of at-risk subjects for which Abs status was determined, followed by IA-2A (42.10%), GADA (40%) and IAA (28.95%; Table 7). This picture changed following to further time-related analyses available for 13 subjects (Figure 7B). Among at-risk children with all measurements available, three (8.57%) resulted negative to both classical islet autoantibodies and the homologous peptides; one of them was affected by coeliac disease with impaired glucose tolerance and developed high IAA levels at the second blood collection.

Four at-risk children (13.79%) developed diabetes at the age of 6-11 years old with variable Abs status. One child was positive to IAA, IA-2A and ZnT8, the latter exceeding 1000 U/ml at T1D onset (9 years) and a complete reactivity to the 8 analyzed peptides. In contrast, a case with only ZnT8 measurements performed became Abs-negative when diagnosed one year later (11 years); likewise no Abs targeting MAP or the homologous epitopes were detected. Only one child developed a full set of classical islet autoantibodies at T1D onset (6 years) but not responded to the homologous peptides. A complete Abs response detected in another case at the second blood collection reduced to GADA and IA2A two years later at T1D diagnosis (11 years); this trend had a similar final for the investigated epitopes as the Abs pattern initially positive to anti-MAP/ZnT8 Abs (9 years) switched to MAP2404c$_{70\text{-}85}$ and PI$_{46\text{-}61}$ at the second blood collection one year later and reduced to no response at T1D onset.

Out of five subjects considered at high T1D risk due to their classical Abs status, one displayed initially a complete response to islet autoantibodies; this ratio increased to four upon further time-point analyses following fluctuations of Abs levels. Considering children at low or moderate risk for T1D, 42.85% were at first positive to multiple classical islet autoantibodies, while 17.86% subjects presented single Abs reactivity; however, data relative to the measured Abs levels were not available in some cases (Table 7). Further analysis of time-point samples revealed an almost unvaried prevalence of positivity to at least two classical islet autoantibodies (42.31%) while reactivity to single Abs increased twice (34.61%); individuals who lost or maintained their Abs negativity accounted for 23.08%.

Among subjects with a complete Abs identification, 55.56% of children positive to at least two classical autoantibodies reacted to non less than 4 homologous peptides, while no response was registered in 22.22%. On the other hand, individuals negative to classical islet autoantibodies presented the same levels of positivity to the analyzed epitopes. Both percentages equaled 33% in children positive to only one classical autoantibody. Of the six cases with a complete response to the homologous MAP and ZnT8/PI peptides, 16.67% showed a full set of classical autoantibodies, 33% had at least a double positivity and in 50% no response was detected.

37.78% (n=17) of at-risk subjects had high-risk HLA genotypes, however only 17.64% (n=3) of them developed T1D and 58.83% were positive to at least half of the analyzed MAP-derived and ZnT8/PI homologous peptides. Another child who progressed to diabetes had a low risk HLA genotype and reacted to MAP3865c$_{133–141}$/ZnT8$_{186-194}$, MAP3865c$_{125-133}$ and MAP2404c$_{70-85}$/PI$_{46-61}$. Males accounted for 58.82% of at-risk subjects with a high-risk HLA genotype, however the ratio of T1D onset between genders equaled 1:1. 54.05% of subjects with low-risk genotypes responded to at least four epitopes, whereas 50% of individuals with HLA DQA1*0501/DQB1*0201 and DQA1*0201/DQB1*0202 genotypes resulted negative.

When Abs status against the selected epitopes was analyzed in correlation with concomitant diseases in subjects at risk for T1D, 82.35% of children affected by coeliac disease or with coeliac familiarity resulted positive to at least two peptides. All individuals with autoimmune thyroiditis occurring alone or combined with coeliac disease reacted to at least four peptides including both MAP/ZnT8 homologous pairs. Among children with T1D familiarity, positivity to at least two epitopes was detected in 53.84% of cases. The lowest ratio (42.86%) was obtained for subjects suffering from occasional hyperglycemia, impaired glucose tolerance and/or obesity. Progression to T1D was associated with the disease familiarity coupled to a high-risk HLA genotype in two cases and a complete Abs positivity to the homologous peptides in one of them, whereas no Abs response was registered in the other one. Occasional hyperglycemia characterized the other two children at T1D onset, one of which without high genetic risk.

A full set of Abs directed against MAP and the homologous epitopes was observed in three cases with T1D familiarity and three cases with coeliac disease or familiarity, whereas immune responses to the peptides were absent in 38.46% of children with T1D familiarity, 57.14% suffering from occasional hyperglycemia and 11.76% with coeliac disease or familiarity. No correlation with age, high-risk HLA genotype or concomitant diseases was found for any of the classical autoantibodies.

Further analysis of Abs responsiveness to L5P was performed in order to specifically validate seroreactivity in relation to the four homologous peptide pairs. 81.25% of children at risk for T1D and 16.67% of HCs were positive to at least one of the eight homologous peptides. Reactivity to L5P antigen was lower among at-risk subjects and accounted for 54.12%, whereas responses in Hs reached 19.05%. Nevertheless, the difference between means relative to L5P-positive cases and HCs was not markedly pronounced and the results did not attain statistical significance (Figure 10A). MAP-derived epitopes homologous to human ZnT8 showed the highest and equal correspondence with levels of anti-L5P Abs, thus MAP3865c$_{133–141}$ has been chosen as a representative peptide for further analysis. Similarly, our previous results demonstrated remarkable prevalence (>60%) of Abs

directed against both MAP/ZnT8 homologs with high degree of statistical significance (p<0.0001) in comparison to values obtained for MAP/PI homologs (prevalence <23%). In this study, the initial seroreactivity to MAP3865c$_{133–141}$ among T1D at-risk subjects was higher compared to that of L5P and reached 65% but followed a decrease to 50% when samples relative to the last blood collection were included. Prevalence among HCs accounted for only 7% and the results were highly significant (p<0.0001, Figure 10B). Both epitopes presented a 1:1 ratio of Abs positivity between males and females. When genders were assessed separately, prevalence among girls was higher than in boys (92.3% vs. 63.7% for the homologous peptides and 69.2% vs. 42.1% for L5P).

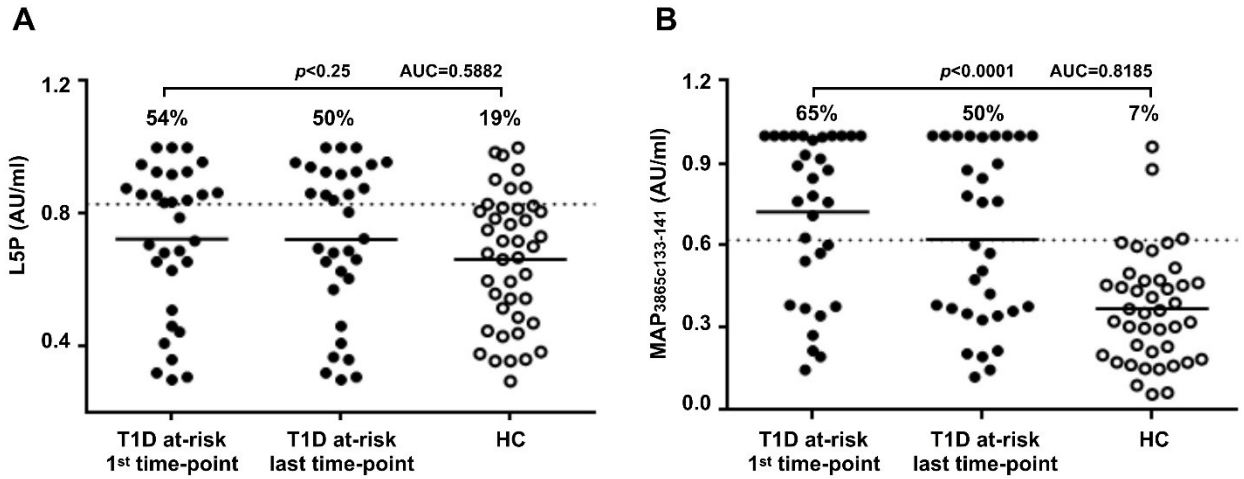


Figure 10. Prevalence of Abs against L5P and MAP-derived ZnT8 homolog in T1D at-risk subjects and HC. Plasma samples were analyzed in duplicate for Abs directed against L5P (A) and MAP3865c$_{133-141}$ (B) epitopes. Distributions are relative to the sample sets including only the first or the last time-point collection in comparison to HCs. Horizontal bars indicate means. The dotted line corresponds to the Abs positivity threshold. AUC and p-values relative to T1D at-risk subjects vs. Specific percentage of reactivity is indicated above each distribution.

Upon analysis of time-point samples, a stronger correlation between titers of Abs directed against MAP3865c$_{133–141}$ and L5P was observed among HCs and T1D at-risk subjects including values relative to the last blood collection compared to T1D at-risk children considering the first time-point samples (Figure 11). Children who maintained in time anti-MAP Abs positivity displayed a continuous response to L5P antigen, whereas loss of immunity against MAP epitopes was mirrored by reduced anti-L5P reactivity leading to a decrease of Abs levels below the established threshold. In these terms, coincidence in positivity to both MAP homologs and L5P antigen reached 90.63% in T1D at-risk subjects and 90.48% in HCs, pointing at the presence of immune responses independent from cross-reactivity observed for the homologous epitopes. 7 out of 11 children for whom time-point samples were available lost their positivity to MAP-derived homologous peptides. There were no cases of time-related acquisition of either anti-MAP or anti-L5P reactivity among the selected samples (Table 2).

Principal component analysis confirmed the association of Abs levels against MAP/human homologs with L5P but revealed a weak relationship between HLA genotype and reactivity to either homologous peptides or L5P antigen (Figure 12). Among L5P-positive subjects, 46.67% had a low-risk genotype, 55.55% moderate and 25% high genetic predisposition for T1D. Interestingly, in the study population, most of the high- and moderate-risk genotypes were found among males indicating an increased probability for the development of clinical symptoms by the combination of sex/genetics-related factors[173]. Age and the presence of islet cell autoantibodies were not associated with anti-L5P Abs levels or general positivity to L5P.

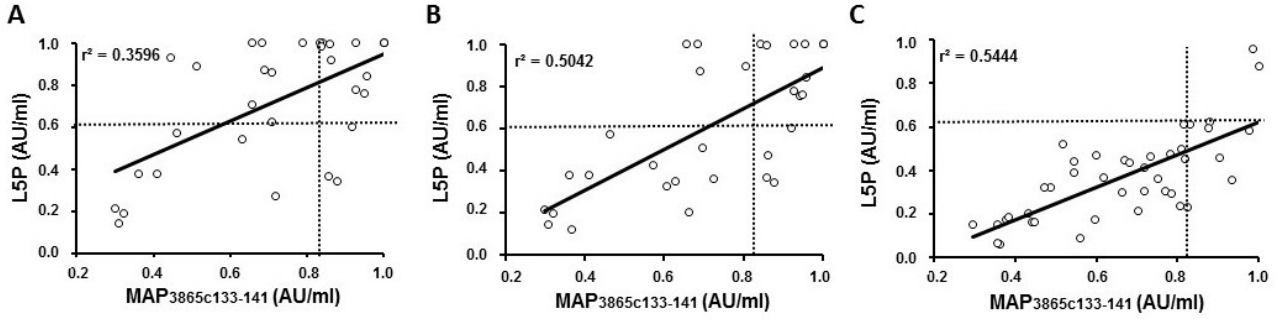


Figure 11. Correlation between Abs recognizing MAP-derived epitope and L5P antigen in children at risk for T1D and HC. The distributions represent correlations between Abs against MAP3865c$_{133–141}$ and L5P in T1D at-risk subjects including samples of the first time-point (A) or the last time-point (B) and HCs (C). Each circle corresponds to Abs detected in one sample. The dotted lines indicate cut-off values used in each assay to discriminate between positive and negative samples. R^2 coefficients are given for each distribution.

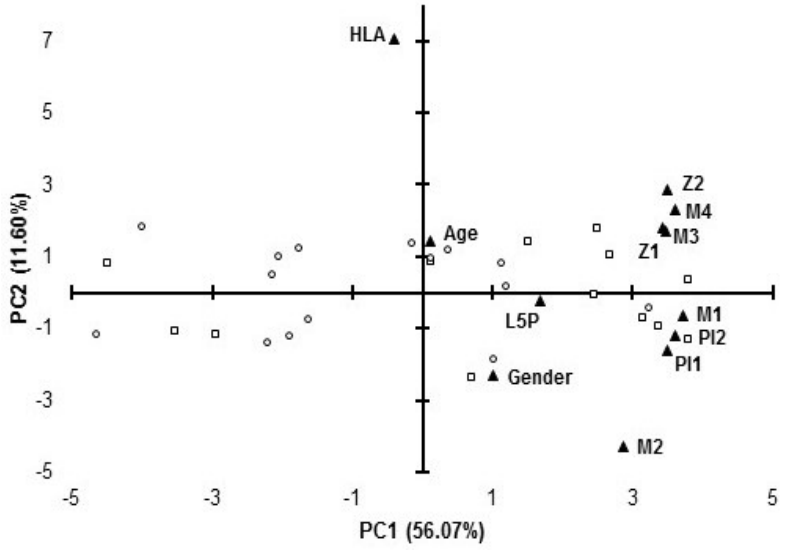


Figure 12. Principal component analysis of variables describing relationship with positivity to L5P in samples of children at risk for T1D. Bi-plot illustrates correlation between levels of anti-L5P Abs and variables relative to T1D genetic predisposition, available demographics data and titers of Abs against MAP-derived and human homologous peptides. Children reactive to L5P antigen are indicated by squares whereas circles correspond to negative samples. All variables are described by labels and their position on the plot is indicated by triangles. The distribution shows relationship between PC1 and PC2 explaining 67.67% of the total variation. Only samples of children with known HLA genotype are included.

Table 2. Clinical characteristics and Abs status in T1D at-risk subjects. Plus signs and hyphens indicate, respectively, the presence or absence of available time-point samples, Abs directed against MAP homologous peptides and L5P antigen with their maintenance in time, as well as coincidence of Abs status for both types of epitopes.

N.	Patient ID	Gender[a]	Age[b]	Time-point samples	Abs positivity		Abs maintenance[c]		Coincidence[d]
					MAP	L5P	MAP	L5P	
1	1	M	12.65	-	+	+			+
2	2	F	9.22	-	+	+			+
3	3	F	4.08	-	+	+			+
4	4	M	5.61	-	-	-			+
5	5	M	18.89	-	-	-			+
6	8	F	8.61	+	+	-	-	-	+
7	9	M	4.91	+	+	+	+	+	+
8	10	F	4.76	-	-	-			+
9	11	F	9.71	-	+	+			+
10	12	M	8.15	-	-	-			+
11	13	M	5.31	-	+	+			+
12	14	M	6.43	-	+	+			+
13	16	F	7.16	-	+	-			-
14	22	M	7.97	+	+	+	-	-	+
15	23	M	12.87	+	+	-	-	-	+
16	24	M	5.74	+	+	-	-	-	+
17	26	F	6.32	+	+	+	+	+	+
18	27	M	7.18	+	+	-	-	-	+
19	31	M	12.92	-	-	-			+
20	33	M	15.12	+	+	+	+	+	+
21	34	F	5.09	+	+	+	+	+	+
22	35	M	8.12	-	+	-			-
23	36	M	3.97	-	-	-			+
24	43	M	10.4	-	+	-			-
25	48	F	11.86	-	+	+			+
26	52	M	9.12	-	+	+			+
27	53	F	10.02	+	+	-	-	-	+
28	54	F	14.06	-	+	+			+
29	55	F	11.41	-	+	+			+
30	56	M	9.84	-	+	+			+
31	57	M	9.99	+	+	-	-	-	+
32	58	F	7.36	-	+	+			+

[a] F: females, M: males. [b] Age at blood collection; relative to the first sample for subjects with multiple time-point collections. [c] Given only for subjects for whom time-point samples were available. [d] Calculated based on the Abs status against MAP and L5P epitopes for single samples or its maintenance upon time-related analysis.

Discussion

The present results are in line with the earlier outcomes involving T1D at-risk Sardinian subjects enrolled in the TRIGR project. Both studies registered a high serum reactivity to MAP-derived epitopes in comparison with healthy individuals; while Sardinian children responded better to MAP/PI homologs, in the present work a particularly high prevalence was obtained for MAP/ZnT8.

The difference may be due to a younger mean age of the Sardinian participants with follow-up not exceeding 10 years old in most cases. This picture changed further upon the analysis of time-point plasma samples portraying downward trends of positivity to MAP/ZnT8 homologs and proinsulin peptides, paralleled by an increased response to MAP1,4αgbp$_{157-173}$ and MAP2404c$_{70-85}$.

In contrast to evidences claiming IAA as the first circulating autoantibody produced by subjects genetically susceptible to or affected by T1D and being their levels inversely proportional to age[170], IAA prevalence in the present cohort increased during follow-up. An opposite trend was followed by Abs against classical ZnT8 epitopes that diminished by almost 35%. Interestingly, levels of anti-MAP/ZnT8 Abs declined as well with further time-related analyses. Prevalence of GADA decreased slightly in consonance with reports describing its appearance pattern in distinct age periods[174].

A cross-reactivity of the analyzed peptides is emphasized by the overlap between the homologous peptides that exceeds 80% for MAP/ZnT8. The pairwise positivity was lower in case of MAP/PI homologs, probably caused by a gradual loss of immunity with increasing age as registered for IAA. Among IAA-positive subjects, 45.45% had Abs directed against MAP/PI epitopes; this number equaled 25% in IAA-negative children. In contrast, reactivity to MAP/ZnT8 homologs accounted for 66.67% among individuals positive to classical ZnT8A and reached 80.95% among those without anti-ZnT8 Abs. This difference, however, could be attributed to a possible future implication of autoimmune thyroiditis. Coincidence of autoimmune diseases, with AITD most frequently complicating T1D, is well known to the scientific literature[175], therefore positivity to ZnT8-derived homologous peptides may indicate an increased risk for multiple autoimmune syndrome. In fact, all patients suffering from autoimmune thyroiditis were positive to the analyzed peptides. This ratio was still high for coeliac disease (82%) but much lower in case of other concomitant symptoms such as occasional hyperglycemia, impaired glucose tolerance and/or obesity (42%). An estimated familial clustering accounts approximately for 40-50%[176] and in this study was reflected by immune reactivity to MAP-derived epitopes and their homologs. Moreover, male sex has been considered a T1D risk factor for siblings[177]; an equal ratio of positivity to the homologous peptides in males and females could point at a combined gender-related effect, even though, in contrast to autoimmune thyroiditis concerning prevalently women, boys and girls are equally affected by T1D in young populations[178].

A much higher female reactivity to MAP/ZnT8 may be predictive of a further AITD onset. Classical anti-ZnT8 Abs along with high GADA titers have been considered a risk biomarker for AITD in LADA patients[108]. Males presented a higher response to MAP-derived homologs of proinsulin in different age periods suggesting the impact of differing immune responses to early environmental

exposures. In both genders, Abs against MAP/PI always appeared accompanied by responses to MAP/ZnT8; furthermore, positivity to all four MAP/PI homologs was present only with a complete reactivity to MAP/ZnT8 hinting at a high sensitivity of the former epitopes indicating the asymptomatic phase of prediabetes.

Numerous reports evaluating genetic factors in the development of T1D confirm that HLA-DQ2 and HLA-DQ8 haplotypes strongly predispose to the disease. Yet, heterozygous individuals have increased susceptibility compared to homozygous subjects, in particular DR3/DR4 genotype, confers the highest risk for T1D[179-180]. In this view, 62.22% of the analyzed samples had low-risk or protective genotypes, however 3 out of 4 cases who progressed to overt diabetes carried a high-risk HLA genotype. Regardless epidemiological studies indicate <10% of high-risk genotypes progressing to islet autoimmunity, 58% of subjects with HLA-conferred susceptibility presented multiple reactivity to the homologous peptides. A slightly lower prevalence among children with low-risk genotypes confirms the previous association of anti-MAP Abs with HLA DQA1∗0201/DQB1∗0202 at T1D onset[181] that, together with HLA DQA1*0501/DQB1*0201, in the present study were the most frequent genotypes and corresponded to 50% of positivity to the homologous epitopes, whereas immune reactivity linked to DQA1*0201/DQB1*02 was displayed by 4 out of 5 children. Furthermore, these genotypes confer the highest risk for coeliac disease in homozygous individuals[182]. A greater number of individuals should be analyzed in order to investigate further the association of MAP with HLA genotypes and hypothesize a possible promotion of molecular mimicry between the selected peptides and islet autoantigens.

More cases positive to MAP/ZnT8 registered among at-risk children and a poor reactivity among HCs are in contrast with prevalence of Abs against MAP/PI homologs presenting a diminished at-risk subject/HCs ratio (3,31:1 for MAP/PI vs. 12,9:1 for MAP/ZnT8); the resulting low statistical significance of data relative to anti-MAP/PI responses might be improved by recruiting a higher number of participants. Possible changes of immune responses against the peptides and an early status of classical Abs should be evaluated at younger age including blood collection at birth, in order to verify whether the previous findings relative to Sardinian children at risk for T1D may be similar in other populations. At present, much higher responses to all MAP/ZnT8 homologs were observed in children younger than 10 years old; interestingly, both proinsulin epitopes followed a similar trend, whereas more cases positive to Abs against their MAP-derived homologs were detected in the 10-18 years group.

Significantly high responses to the above-mentioned peptides were further compared with seroreactivity to L5P as a specific MAP antigen. Considering the particular structure of L5P that lacks a free hydroxyl group in its core and presents an unmodified saturated fatty acid in the *N*-terminal

domain[183], several ELISA protocols were tested, however the best sensitivity has been achieved applying the same procedure and antigen concentration as for the other epitopes. In result, anti-L5P responses were higher in children at risk for T1D than in HCs but not statistically significant, in contrast to the other MAP-derived antigens. Abs prevalence to L5P and the four MAP peptides analyzed together resulted similar in HCs while differed greatly in at-risk subjects (81.25% vs. 54.12%, respectively).

When initial reactivity to L5P was compared to responses against only MAP3865c$_{133–141}$ peptide presenting the most similar positivity pattern, Abs prevalence appeared much higher in HCs (19% vs. 7%, respectively) and quite lower in children at risk for T1D (54% vs. 65%, respectively). Interestingly, seroreactivity against either single MAP3865c$_{133–141}$ or the entire group of MAP-derived and the homologous human epitopes was almost identical as that elicited by L5P antigen upon inclusion of samples relative to further time-point blood collections, resulting in the final Abs coincidence exceeding 90%. This phenomenon was due to the time-related loss of anti-MAP Abs in some children mirrored by the absence of positivity to L5P. It is plausible that MAP-derived homologs cross-react with ZnT8 and PI peptides at a young age inducing initially immune responses in subjects predisposed for T1D that are subsequently attenuated. As suggested for Crohn's disease, Abs prevalence among healthy controls and individuals who don't progress to overt diabetes may result from latent infection or past exposure to MAP in early childhood conferring some natural protection[145].

ZnT8A and IAA, together with other islet cell antigens, are standard biomarkers indicating an increased risk for T1D, especially in cases of multiple Abs positivity. Although, they cannot be detected during the first months after birth and their levels decrease with age. As previously observed in Sardinian children at risk for T1D, immune responses to MAP peptides appear before classical Abs used for T1D diagnosis, however in many cases they tend to attenuate. In this context, L5P might have a general diagnostic potential but, in the analyzed samples, it was not sensitive to changes in seroreactivity against MAP registered for the other epitopes. Nonetheless, positivity to L5P in HCs corresponded to values below the cut-off point obtained for MAP-derived and human homologous peptides, and was reflected by a significant correlation performed for MAP3865c$_{133–141}$. On the other hand, L5P may be helpful to immediately indicate the presence of anti-MAP Abs when evaluation of a more complex Abs status, achievable by the application of the homologous epitopes, is not required, avoiding false-positive results due to temporal cross-reactivity.

This study provides evidences that either protein or lipid MAP antigens may induce similar Abs responses in terms of strength, while somewhat higher specificity corresponding to the optimal positivity threshold characterized the selected MAP peptides. Since L5P has been described as

more specific than the current diagnostic test for Johne's disease based on MAP purified protein derivative[183], almost complete coincidence in responses to both types of antigens used in the present study lends support to the involvement of anti-MAP Abs in T1D. Higher prevalence of Abs against L5P and MAP-derived peptides in subjects with low and moderate T1D risk conferred by HLA haplotypes is in line with the current onset trends in less genetically predisposed individuals[161].

IV. Humoral and cell responses to MAP-derived epitopes in Polish T1D youths

This study was performed with the purpose to evaluate differences in the recognition of MAP and human homologous antigens in a population of young T1D patients with a distinct biogeographical and cultural background compared to the previously selected cohorts. Humoral and cell responses upon *in vitro* exposure to a MAP/ZnT8 peptide pair were assessed in a pilot group.

Results

Upon the analysis of seroreactivity, major differences of mean values between T1D and control subjects were registered for PI_{46-61} that corresponded to 28.38% of T1D patients above the cut-off for positivity compared to 7.69% among controls (p<0.001, AUC=0.708, Figure 13A), followed by its homolog $MAP2404c_{70-85}$ accounting for 27.03% and 11.54%, respectively (p<0.008, AUC=674). Nonetheless, statistical significance was attained only for the PI peptide when considering the numbers of positive and negative individuals in both groups (p<0.03), even though the value relative to the homologous MAP-derived epitope was close to the threshold of significance (p<0.06).

The second MAP/PI pair provided a lower Abs prevalence translated into 16.22% of positive T1D patients and only 3.85% among controls (p<0.013, AUC=0.663) for $MAP1,4\alpha gbp_{157-173}$, while the homologous PI_{64-80} was recognized in a similar fashion by the two groups (21.62% vs. 19.23%, respectively) and lacked statistical significance.

Concerning MAP/ZnT8 peptide pairs, a higher seroreactivity among patients was observed for $MAP3865c_{125-133}$ and corresponded to 25.68% of positive T1D individuals compared to 11.54% among controls. The homologous $ZnT8_{178-186}$ triggered even more elevated Abs responses accounting for 29.73% and 15.38% of positivity in the respective groups.

Serum Abs reactivity to $MAP3865c_{133–141}$ displayed higher levels among controls (19.23%), although the prevalence relative to T1D subjects was only slightly lower (17.57%). Comparable immunoreactivity among both groups was registered also for $ZnT8_{186-194}$ (16.23% for T1D vs. 15.38% for controls). Results obtained for MAP/ZnT8 pairs were not significant due to similar mean values and insufficient differences in proportions between Abs-positive and Abs-negative individuals.

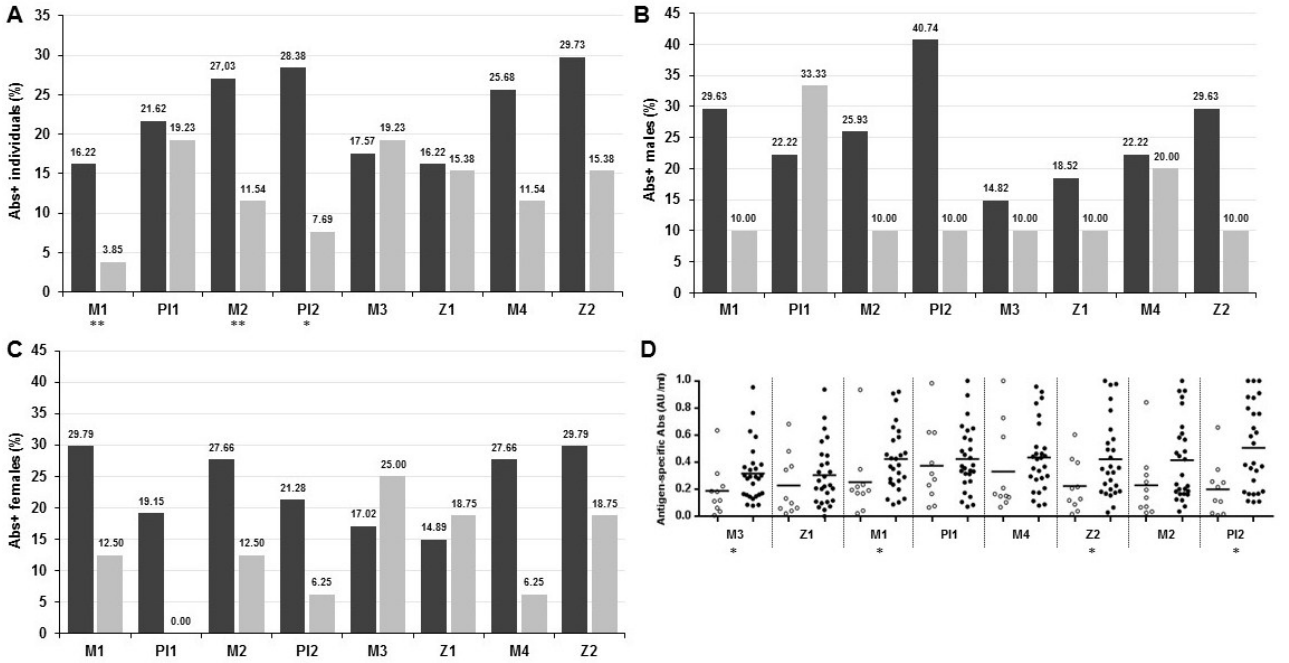


Figure 13. Prevalence of Abs against MAP-derived and homologous human antigens in whole study population (A) and separately in males (B) and females (C) with the relative percentage of positive subjects: Dark bars represent T1D patients; light grey bars correspond to HCs. (D) Distributions representative for male participants; each dot corresponds to values obtained for a single sample in separated ELISA tests with plate-coated peptides. Black points: T1D males. White circles: control males. Means (horizontal bars) and statistically significant differences are indicated (Mann-Whitney).

Sex-related analysis explained the picture of general responses. T1D males displayed particularly high Abs prevalence to PI_{46-61} (p<0.005, 40.74%) and a lower percentage of positive cases compared to controls (22.22% vs. 33.33%, respectively) when considering the other PI peptide (Figures 13B and 13D). Raised Abs levels against both MAP-derived antigens homologous to proinsulin were observed among T1D subjects regardless of gender, however only males showed significant values for $MAP3865c_{133–141}$ (p<0.014). Interestingly, PI_{64-80} did not trigger elevated Abs reactivity among female controls (Figure 13C). On the other hand, obese girls presented more elevated seroreactivity to $MAP3865c_{133–141}/ZnT8_{186-194}$ than T1D females inversely to the trend observed among males and highly contributed to level off the responses seen in the general analysis for this peptide pair. Even though the difference in Abs responsiveness to $MAP3865c_{133–141}$ between T1D and HC males was not marked (14.82% vs. 10%, respectively), statistical significance of the means was on the threshold point (p<0.051). In contrast, males displayed similar Abs prevalence for $MAP3865c_{125-133}$ among both T1D and HCs but the response to its $ZnT8_{178-186}$ homolog was much higher in diabetic patients leading to significant results based on mean values (p<0.043, 29.63% vs. 10%, respectively).

In order to evaluate the cytokine expression profile, PBMCs of a pilot cohort (n=11 T1D patients and n=3 controls) were stimulated with MAP3865c$_{133–141}$ or ZnT8$_{186-194}$ peptide selected initially as a representative pair of MAP/human homologous antigens. Both antigens were able to induce particularly increased specific production of IL-1β and IL-12p40 in T1D with even higher levels registered for control subjects (Figure 14A). A similar trend was observed in the expression of INFγ upon stimulation with the MAP-derived homolog only, while ZnT8$_{186-194}$ corresponded to decreased INFγ levels among T1D patients without inducing changes in controls. Interestingly, T1D subjects expressed a diminished production of TNFα after incubation with each epitope that contrasted the results relative to the obese individuals.

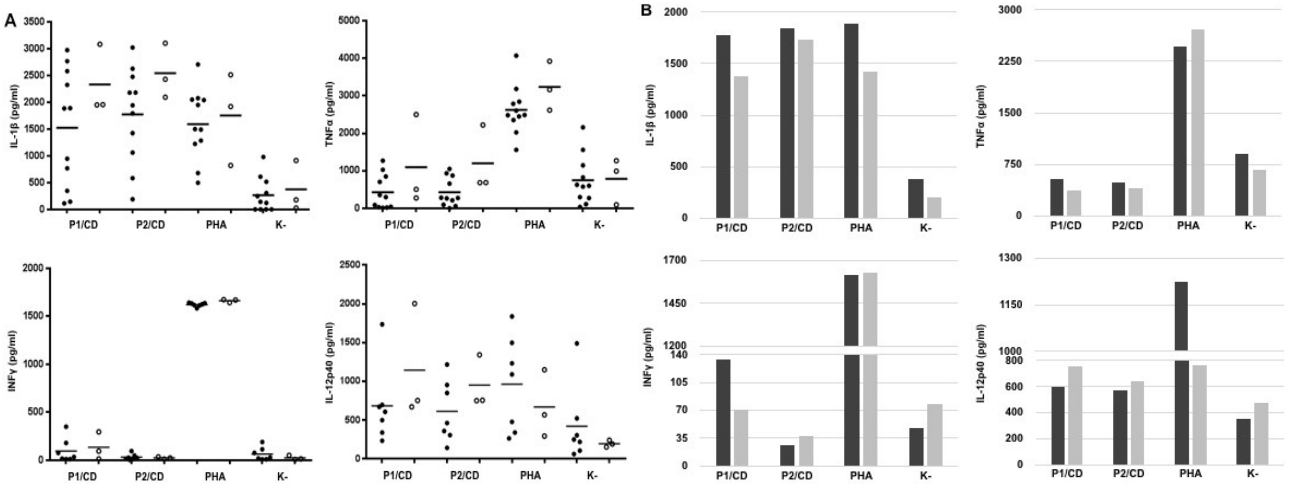


Figure 14. Cytokine expression in PBMCs following to immunostimulation with MAP3865c$_{133–141}$ (P1) and ZnT8$_{186-194}$ (P2) antigens. CD28/49d was added as a co-stimulator to wells containing the selected peptides. The concentrations were determined from cell culture supernatant after 18h for IL-1β and TNFα or 72h for INFγ and IL-12p40. (A) Analysis between T1D and control subjects. Each dot corresponds to a single sample and horizontal bars indicate means. Black points: T1D patients. White circles: control subjects. (B) Analysis based on anti-MAP positivity in T1D patients. Dark bars: MAP-positive (n=4); light grey bars: MAP-negative (n=7).

Cytokine profiles was further assessed for the T1D group in the context of Abs positivity to the antigen pair (Figure 14B). For IL-1β, the expression reached elevated levels with somewhat higher values among Abs-positive subjects. Likewise, IL-12p40 was highly induced compared to untreated samples, however a slightly lower levels corresponded to the seroreactive individuals. An incisive difference was observed for INFγ produced at raised levels in MAP-stimulated cells by patients who presented Abs against the selected homologous pairs with almost unchanged concentrations in Abs-negative subjects. In samples incubated with ZnT8$_{186-194}$, INFγ expression decreased in both groups. A comparable reduction in TNFα levels was displayed by each group regardless of reactivity to the eight epitopes.

Flow cytometry analysis performed in order to evaluate the degree of cell apoptosis following the immunostimulation showed lower number of live PBMCs in conditions including the selected peptides compared to untreated cells (Figure 15). The percentage was comparable between T1D and control individuals for MAP3865c$_{133–141}$, while for ZnT8$_{186-194}$ less live cells were registered for the obese patient. Labeling with annexin V that specifically binds to phosphatidylserine normally found on the intracellular side of the plasma membrane permitted to identify cells during early apoptosis when phosphatidylserine translocate to the external leaflet. The levels of apoptotic cells increased for all samples incubated with either MAP3865c$_{133–141}$ or ZnT8$_{186-194}$, particularly in the early stages. Necrotic cells were detected by staining with 7-AAD that has a high DNA binding constant and permits to identify cells in more advanced apoptotic processes such as the loss of membrane integrity and DNA fragmentation. However, slightly elevated necrosis corresponded to ZnT8$_{186-194}$ stimulation in the control sample only.

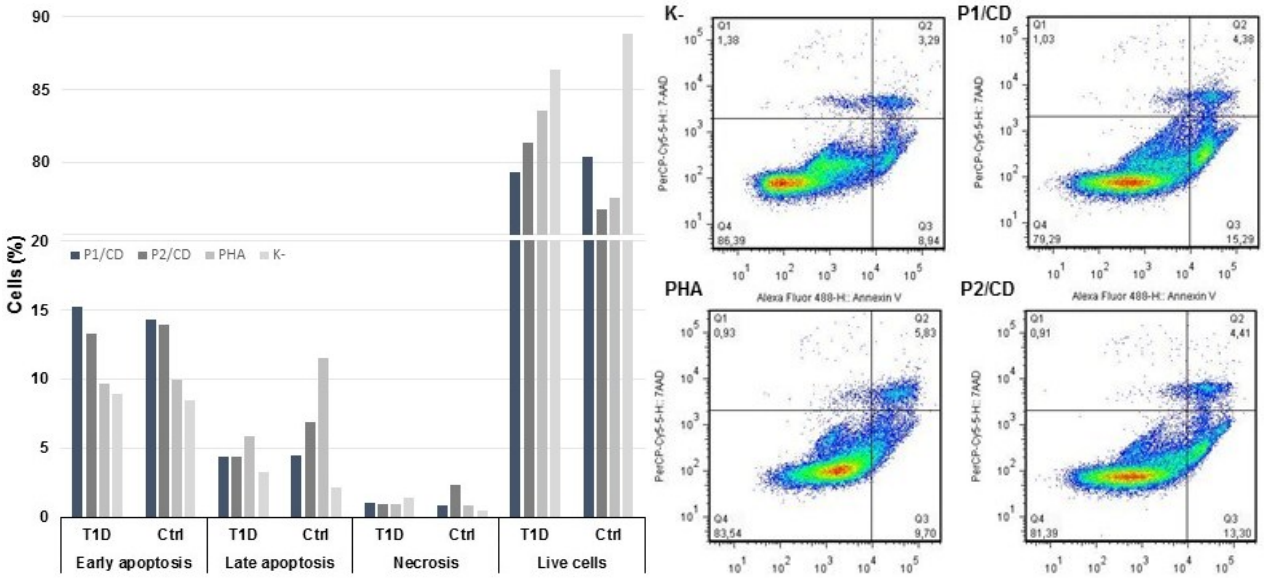


Figure 15. Cell apoptosis in PBMCs stimulated with MAP3865c$_{133–141}$ (P1) and ZnT8$_{186-194}$ (P2) antigens. The histogram shows percentage differences in the relative apoptosis phase analyzed in n=1 T1D and n=1 control subject following 72h of cell culture. Cytometry charts illustrate cell gating based on 7-ADD labeled with PerCP-Cy5-H and Alexa Fluor 488-conjugated annexin V staining in the T1D sample.

The phenotypical characterization of T-cell population showed a significantly elevated percentage of CD3+ cells in samples stimulated with each peptide compared to negative control conditions (Figure 16). T1D patients showed significantly raised CD3+ levels in comparison with control subjects, however higher values were detected for these subjects also in the untreated PBMC cultures (p<0.03 for MAP3865c$_{133–141}$ and p<0.00003 for ZnT8$_{186-194}$). When considering T1D patients positive or negative to anti-MAP Abs, the serological differences were not reflected by the

percentage of lymphocytes but still the ability of both epitopes to induce T cell proliferation was marked. Only $ZnT8_{186\text{-}194}$ induced a significant increase in CD3+ levels respect the unstimulated culture (p<0.003 for anti-MAP Abs-positive and p<0.01 for anti-MAP Abs-negative subjects).

To evaluate the responses of monocytes following the incubation with the homologous antigens, PBMCs were simultaneously stained for CD14+/CD16+ subset. Elevated levels were observed for both peptide-stimulated conditions, however neither of them permitted to attain statistical significance (Figure 16). What is more, control subjects displayed even higher cell percentage. In contrast, responses of monocytes among T1D patients positive for Abs against MAP epitopes were markedly raised for each peptide compared to seronegative subjects, although the content of CD14+/CD16+ subset was higher also in the untreated cultures. Significant difference between stimulated cells and the negative culture was obtained only for $MAP3865c_{133–141}$ (p<0.04).

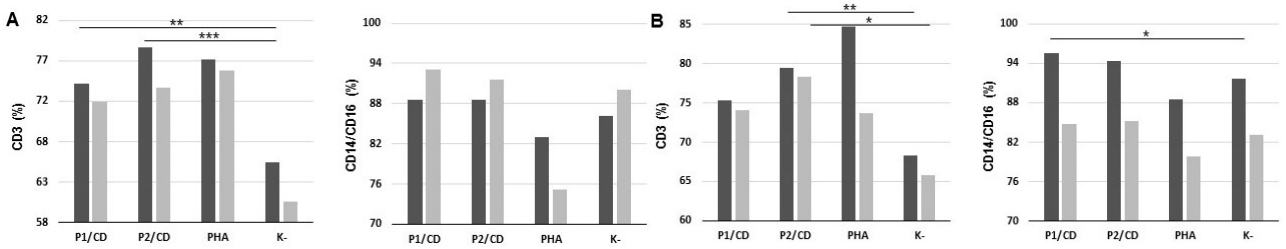


Figure 16. Immunofluorescence analysis of $CD3^+$ and $CD14^+/CD16^+$ populations in PBMCs stimulated with $MAP3865c_{133–141}$ (P1) and $ZnT8_{186\text{-}194}$ (P2) peptides to which CD28/CD49d was added as a co-stimulator. (A) Comparison between T1D patients (dark bars) and control subjects (light grey bars). (B) Assessment in T1D patients positive (dark bars) or negative (light grey bars) to anti-MAP Abs. Statistically significant results for T1D group are indicated by asterisks.

Patients with a complete data regarding demographics, clinical history and nutritional habits were selected from the study population for the correlation analysis with the presence of Abs directed against the assessed peptide set. Based on several reports associating MAP to diet and its possible transmission with contaminated food, the evaluation was restricted to variables describing patients' dietary preferences and other factors linked to predisposition for autoimmune diseases. Multivariate analysis permitted to identify a moderate association with the consumption of dairy products, use of milk formula and duration of breastfeeding (Figure 17). Interestingly, the latter conditioned not only the substitutive feeding but also the intake of milk derivatives in further life. Familial risk, comorbidities and natural/caesarean or premature delivery did not show a significant relationship with the serological status. Body mass and age formed a separated cluster that had a major impact for obese subjects. No correlation was observed with classical T1D autoantibodies and disease phase.

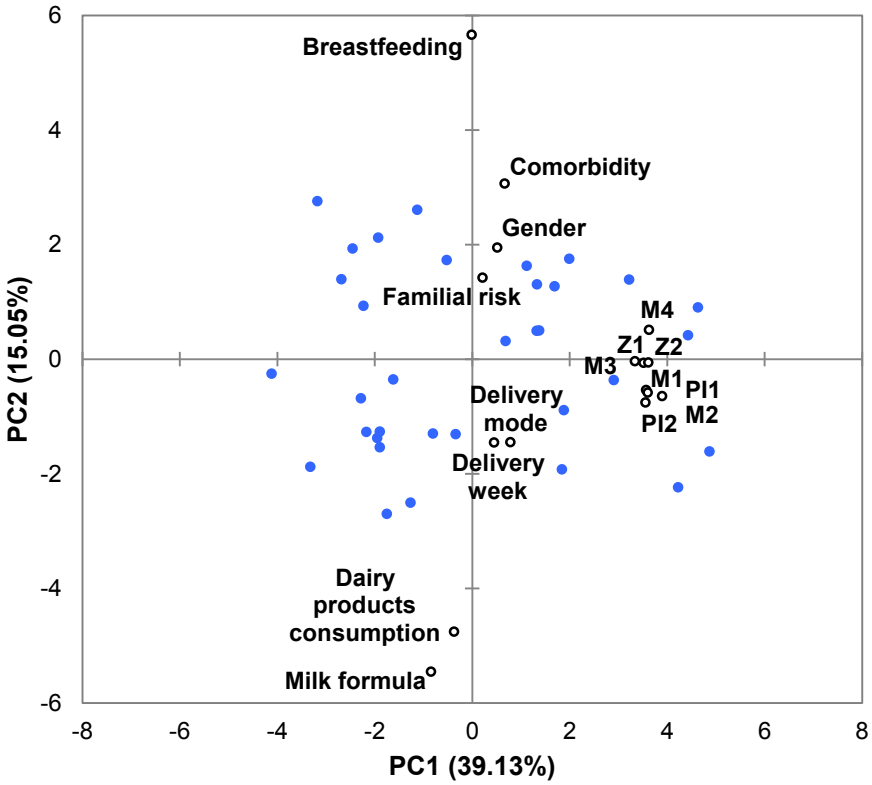


Figure 17. Bi-plot distribution of variables in relation to positivity for Abs against MAP, ZnT8 and PI peptides in T1D subjects. Circles indicate the variables relative to patients' demographics, early-life diet and associated risk factors. Blue dots correspond to single samples for which all data relative to the analyzed variables were available (n=34). The presented principal components account for 54.18% of the total variation.

Discussion

Following the investigations on anti-MAP seroreactivity in Italian youths, the question whether unrelated populations originating from biogeoraphically distinct environments may present similar Abs status was posed. In the present study, Polish T1D patients displayed an increased Abs prevalence against MAP and the homologous proinsulin peptides, however this result followed the serological pattern previously obtained in Sardinian children at a high risk for T1D rather than humoral responses observed in youths with T1D susceptibility from mainland Italy. Different immunogenicity of the selected peptides may be due to genetic predisposition towards specific antigens and cultural conditioning. Neonatal exposure to MAP may also play a significant role as adaptive immunity in early life is characterized by reduced allo-antigenic recognition, tolerogenic reactivity and poor responses to foreign antigens[184].

To better characterize the impact of MAP-derived antigens and their possible cross-reactivity with human homologs, MAP3865c$_{133–141}$ and ZnT8$_{186-194}$ peptide pair was selected for immunostimulation assays based on results previously observed in Italian youths; this choice was made prior to serological testing, therefore subsequent lack of statistical difference between T1D

and control groups as well as small number of included patients must be considered when evaluating further results. When evaluating humoral responses, the addition of each antigen to PBMCs isolated from the pilot cohort induced shifts in the production of some proinflammatory cytokines as observed in other experiments. Particularly high levels of IL-1β, especially in subjects bearing anti-MAP Abs, may indicate the activation of macrophages following to overproduction of Th1 cytokines[185].

Only slight increase of INFγ concentrations is in line with another report describing poor cytokine mRNA expression in Iranian T1D patients[186]. INFγ has been considered an important mediator of immune responses against mycobacterial infections and its secretion may be inhibited by other cytokines such as TGFβ and IL-10[187-188]. It is possible that, since IL-10 and TGF-β are already upregulated in patients with clinical T1D, cytokine balance may have been skewed towards impaired protection against MAP in antigen-stimulated cell cultures. By unknown mechanisms, MAP is able to evade proinflammatory Th1-like responses that during the subclinical phase of infection are lost and a Th2-like response, characterized by production of antibodies, predominates[189]. However, in T1D subjects positive for anti-MAP Abs, stimulation with MAP3865c$_{133–141}$ caused a high production of INFγ compared to MAP-unresponsive patients and an inverse trend was registered for ZnT8$_{186-194}$.

Similarly, the production of IL-12p40 was elevated in both patients and control subjects with higher values for either obese or T1D subjects negative for anti-MAP Abs. Experiments performed on monocyte-derived macrophages infected with MAP reported no up-regulation of IL-12p40 gene pointing again at an immune evasion strategy of the mycobacterium; at the same time, an increase in IL-12p40 production may be helpful in the suppression of T1D as demonstrated in NOD mice[190-191]. Likewise, TNFα is expressed at high levels in both *in vitro* differentiated Th1 and Th17 cells promoting autoimmunity and acts as an important mediator in inflammatory disorders including obesity[192-193]. In the present study population, untreated PBMCs of both T1D and control subjects showed raised TNFα concentrations that further increased in obese patients upon stimulation with the homologous peptides, while levels in MAP3865c$_{133–141}$-stimulated cells appeared reduced. Low TNFα production by T1D group may result from acquired antigen-specific tolerance[194]. In contrast, MAP-infected macrophages show raised expression of TNFα accompanied by the lack of IL-12 induction and limited apoptosis[136].

In the immunostimulation experiments, the effect of MAP/ZnT8 peptides on cell death leaded to highly increased early apoptosis of PBMCs. This phenomenon is widely discussed in the scientific literature due to contradictory results. Some indices suggest that through such a mechanism macrophages eliminate live mycobacteria or, on the other hand, bacteria adopt measures to

overcome host's immune responses[195]. This way, exposure to high MAP loads caused the apoptosis of infected macrophages through dose-dependent pathways, while low MAP concentrations did not induce cell death in a manner that may parallel events occurring during natural infection[196]. By maintaining macrophages in life, MAP ensures itself more time for replication and hampers the presentation of bacterial antigens that decreases the risk to be detected by the immune system. However, pro-survival signaling identified in MAP infected tissue appears more pronounced during early Abs responses without the typical cell-mediated immunity associated with MAP clearance[197].

The $CD14^+/CD16^+$ monocyte subset was selected for further evaluation as it has been linked to several conditions in autoimmune diabetes and demonstrated to promote the pathological picture in obesity[198-199]. Moreover, the increase of these monocytes was noted in tuberculosis and several bacterial sepsis[200]. Yet, expanded $CD14^+/CD16^+$ cells but not the classical monocytes showed reduced TNF expression following stimulation with LPS which probably evolved as a protective mechanism against excessive cytokine production[201]. In line with these observations, anti-MAP-positive T1D patients displayed high $CD14^+/CD16^+$ coupled with low TNFα concentrations especially in PBMCs incubated with $MAP3865c_{133–141}$. Conversely, stimulation with the homologous $ZnT8_{186-194}$ was associated to a pronounced percentage of CD3+ cells characterized by bigger differences between T1D and control subjects rather than in relation to anti-MAP seropositivity. Analysis on a more numerous group of patients with healthy subjects as reference controls would provide major details on the potential impact that MAP-derived antigens may exercise in T1D development.

Finally, the presence of anti-MAP Abs identified in patients' serum was analyzed in relation to possible links with clinical parameters, perinatal conditions and early-life diet. Interestingly, the results partially reflected associations found in Iranian CD patients whose Abs reactivity to the same MAP-derived antigens was paralleled by a high consumption of fast food meals but only partially by the intake of milk and pasteurization, while green tea and breastfeeding was inversely associated[202]. For the current cohort, information regarding dietary habits included only dairy products and nutrition in the first months after delivery. Complementary or exclusive feeding with infant formula leaded to a more frequent consumption of milk derivatives later in life which, however, was moderately correlated with anti-MAP seroreactivity. Similarly, breastfeeding showed only a partial relationship that might be dependent on cultural references based on the integration with milk formula. These results are supportive of the hypothesis seeing pasteurized dairy products as vehicles of viable MAP cells or their fragments acting as antigens cross-reactive with self-protein fragments. Further, low impact of familial risk and mode of delivery points at the importance of environmental agents in the development of T1D.

V. Detection of Abs against MAP in Sardinian LADA patients

This chapter is based on the following study:

> Niegowska M et al. Increased seroreactivity to proinsulin and homologous mycobacterial peptides in latent autoimmune diabetes in adults. PLoS One. 2017 May 4;12(5):e0176584.

The present study describes Abs responses to the selected MAP, ZnT8 and PI homologous peptides in Sardinian LADA patients with further evaluation based on age-related classification and disease phenotype performed in the attempt to investigate whether earlier results may reflect the hypothesis of common factors underlying T2D and LADA etiology[203] and help in identifying the risk for autoimmune comorbidities.

Results

After a single-peptide analysis, no significant differences in responsiveness to the analyzed antigens were found between LADA subjects and HCs with the exception of MAP2404c$_{70\text{-}85}$/PI$_{46\text{-}61}$ pair (Figure 18), for which Abs prevalence characterized by high statistical significance exceeded 17% in LADA patients and 9% among HCs (p<0.0009 for MAP2404c and p<0.0004 for the homologous PI, Figure 19A). Fisher's exact test performed based on the numbers of positive and negative subjects further confirmed the significant results when comparing LADA patients and HCs (p<0.028 for MAP2404c$_{70\text{-}85}$ and p<0.039 for PI$_{46\text{-}61}$). Moreover, a high degree of correlation (r^2=0.68; Figure 19B) was detected between Abs titers recognizing the two homologs in either patients or HCs suggestive of cross-reactivity.

Responses to the six remaining peptides were distributed evenly among LADA subjects at the level of 10.31-11.66%. HCs displayed somewhat lower Abs positivity towards both MAP-derived peptides homologous to ZnT8 accounting for 7.69%, whereas percentages of anti-ZnT8 Abs were similar to those observed in the patient group. Similarly, prevalence of Abs directed against MAP1,4αgbp$_{157\text{-}173}$/PI$_{64\text{-}80}$ epitope pair among HCs remained slightly below 10%. Analysis of separated groups defined by high and low GADA titers (LADA1 and LADA2, respectively) confirmed the above mentioned results with respect to HCs, however no differences in mean values or Abs prevalence were found between the two disease phenotypes (Figures 19C and 19D). In fact, MAP2404c$_{70\text{-}85}$/PI$_{46\text{-}61}$ homologs were recognized by 15.00-16.25% of patients in each LADA group

(p<0.0037), whereas immune reactivity towards the other selected peptides appeared leveled off at 8.47-12.50% (Figure 18B).

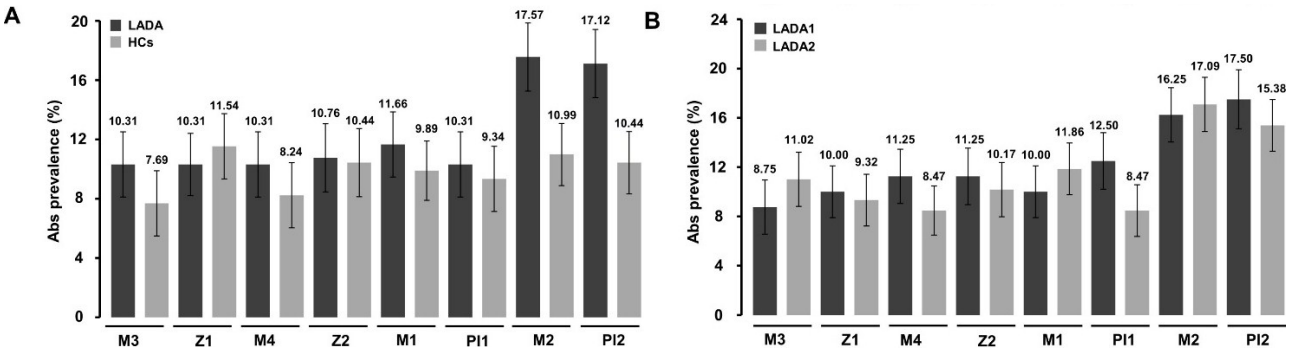
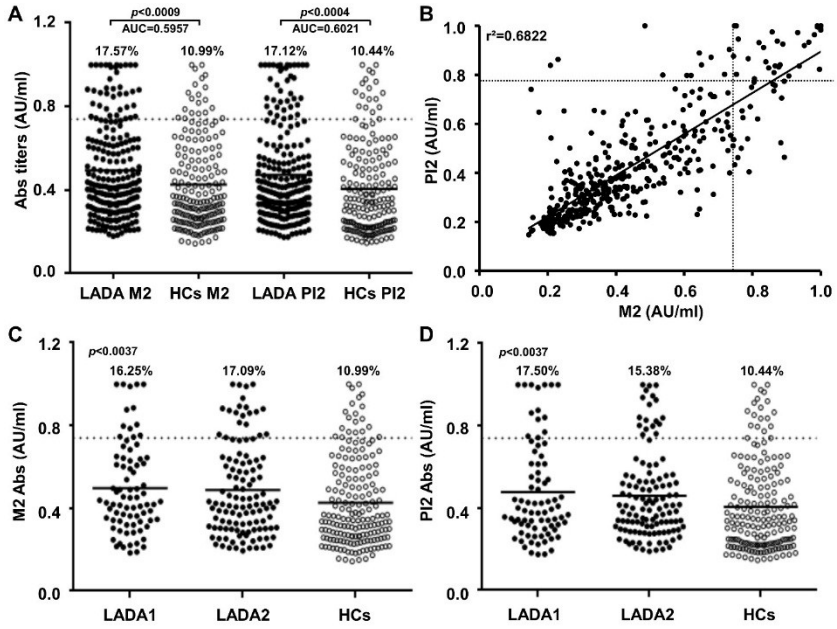


Figure 18. Prevalence of Abs against MAP, proinsulin and ZnT8 homologous epitopes in LADA patients and HCs. The histograms represent percentages of subjects with Abs positivity to selected epitopes upon single-antigen analysis; sera were tested in duplicate for their reactivity against plate-coated peptides. (A) Dark bars represent LADA patients; light grey bars correspond to HCs. (B) Abs prevalence among LADA 1 (dark bars) and LADA 2 (light grey bars) patient groups. Relative percentages and standard deviations are reported for each bar.

Figure 19. Prevalence and correlation of Abs specific for MAP2404c$_{70\text{-}85}$/PI$_{46\text{-}61}$ peptide pair in LADA patients and HCs. (A) Distribution of Abs values for MAP2404c$_{70\text{-}85}$ (M2) and PI$_{46\text{-}61}$ (PI2) antigens. Horizontal bars specific for LADA and HCs groups correspond to means. AUC and p values (CI 95%) are indicated above the distributions. (B) Correlation between Abs recognizing MAP2404c$_{70\text{-}85}$ and PI$_{46\text{-}61}$ homologous epitopes in LADA subjects and HCs. Each dot represents Abs detected in one sample; in both graphics the dotted lines indicate positivity thresholds established for each assay based on the ROC analysis. (C) Distributions relative to Abs titers of M2 and (D) PI2 in LADA1 and LADA2 patients with HCs as reference; p values calculated by Kruskal-Wallis test as well as percentages of Abs prevalence in each group are reported above the distributions.

Only statistically significant results are presented.



Data relative to other islet autoantibodies were available for 188 patients; among them 43 individuals resulted positive to IA-2A, of which 12 (30.23%, Table 3) had anti-MAP Abs and those directed against MAP2404c$_{70-85}$/PI$_{46-61}$ epitopes were detected in 9 subjects (25.58%). IA-2A-negative patients showed 18.71% of general anti-MAP Abs prevalence that decreased to 16.77% when only the statistically significant pair of homologs was considered. After division of patients in disease subtypes, we obtained 8 IA-2A-positive LADA1 and 35 LADA2 patients among which 3 and 10 subjects, respectively, with anti-MAP/PI Abs. Patients without elevated IA-2A titers had Abs against MAP/PI antigens in 13 cases for LADA1 and 15 for LADA2 (19.70% and 18.99%, respectively).

Table 3. Reactivity MAP, ZnT8 and PI antigens in relation to IA-2A titers.

	IA-2A	Patients	Anti-MAP Abs	M2/PI2 Abs*
LADA	+	43	13 (30.23%)	11 (25.58%)
	-	155	29 (18.71%)	26 (16.77%)
LADA1	+	8	3 (37.50%)	3 (37.50%)
	-	72	14 (19.44%)	13 (18.06%)
LADA2	+	35	10 (28.57%)	8 (22.86%)
	-	82	16 (19.51%)	14 (17.07%)

*Antigens for which statistically significant values were obtained.

74 LADA1 and 114 LADA2 subjects with complete clinical data were selected from the study population and further classified in four age groups: 32-41, 42-51, 52-61 and ≥62 years, for which Abs prevalence was calculated either to all peptides together or to the homologous pair MAP2404c$_{70-85}$/PI$_{46-61}$ separately (Table 4). Seroreactivity was evaluated between patients and HCs as well as in distinct LADA phenotypes with regard to age at onset and at the time of blood collection. The youngest and the oldest patients were both diagnosed with LADA1 at 32 and 77 years old, respectively. In all disease groups, the highest reactivity to at least one of the analyzed epitopes was registered in the youngest individuals, even though only slightly lower prevalence was observed after 61 years for LADA2; the weakest responses corresponded to the 42-51 age range except for LADA1 for which the lowest seroreactivity occurred in the oldest subjects. When Abs against only MAP2404c$_{70-85}$/PI$_{46-61}$ homologs was taken into account, the highest prevalence was assigned to the 32-41 group for LADA1 and ≥61 year old patients for LADA2; for both phenotypes quite high positivity with significant values was maintained in the 52-61 group (p<0.0001). Independently from the analyzed peptides, HCs presented a progressive loss of Abs with age.

Clinical history regarding insulin-free period after LADA diagnosis was complete for 142 patients. Most of them (n=127; 63.68% with LADA2 phenotype) required insulin therapy within 4 consecutive years and only 23 subjects undergone insulin treatment within 12 months following LADA onset (69.57% with LADA2). Differences in anti-MAP positivity between periods of insulin independence reached 1% until 48 months when both LADA phenotypes were analyzed together (Table 4) with the exception of insulin treatment started after 13-24 months characterized by the highest seroreactivity; after splitting of patients according to LADA subtypes, LADA1 subjects showed Abs prevalence increasing with length of insulin-free periods from 14.28% to 25%, while opposite trends were observed for LADA2 decreasing from 18.75% to 10% of seropositive individuals. However, a few positive cases in each period do not permit to draw significant conclusions.

Table 4. Seroreactivity to MAP, ZnT8 and PI epitopes in different age groups and during insulin independence period. Percentages of patients positive to the analyzed epitopes are reported along with the relative numbers of seroreactive subjects given in brackets. Mann-Whitney *U* test was employed to calculate statistical significance.

Age group (years)	LADA patients		LADA 1		LADA 2		HCs
	Onset[a]	Sample collection[b]	Onset	Sample collection	Onset	Sample collection	Sample collection
	Abs prevalence for 8 peptides (%)						
32-41	35.30 (11)	38.70 (13)	40.00(5)	45 .00(5)	35.29 (6)	28.57 (6)	42.31 (21)
42-51	8.00 (4)	17.31 (6)	10.00(2)	25.00 (4)	6.67 (2)	16.00 (4)	18.46 (12)
52-61	25.90 (22)	20.30 (14)	27.58 (8)	23.53 (4)	25.00 (14)	20.51 (8)	10.64 (5)
>61	19.35 (6)	22.73 (10)	6.25 (1)	15.38 (4)	33.33 (5)	27.28 (9)	11.12 (2)
	Abs prevalence for MAP2404c$_{70-85}$/ PI$_{46-61}$ (%)						
32-41	23.53 (7)	25.1 (8)	35.71 (4)	36.37 (4)	17.65 (3)	16.67 (3)	28.85 (15)
42-51	8.00 (4)	17.31 (6)	10.00 (2)	20.00 (4)	6.67 (2)	16.00 (4)	12.31 (8)
52-61*	24.70 (21)	18.64 (13)	27.59 (8)	23.53 (4)	23.21 (13)	17.95 (7)	6.38 (3)
>61	19.35 (6)	22.73 (10)	6.25 (1)	15.38 (4)	33.33 (5)	27.28 (9)	0.00
Insulin-free period[c]	**Abs prevalence for MAP2404c$_{70-85}$/ PI$_{46-61}$ (%)**						
≤12	21.74 (5)	-	14.28 (1)	-	25.00 (4)	-	-
13-24	17.64 (4)	-	0.00	-	33.33 (4)	-	-
25-36	18.19 (2)	-	16.67 (1)	-	20.00 (1)	-	-
37-48	18.42 (14)	-	18.75 (6)	-	18.19 (8)	-	-
>48	13.33 (2)	-	25.00 (1)	-	10.00 (1)	-	-

* Statistically significant. [a] Samples collected at the time of LADA diagnosis. [b] Samples collected during a control medical visit following LADA onset. [c] Expressed in months.

Although sex-related analysis did not reveal associations with high titers of Abs against the selected peptides, the number of males and females varied among LADA phenotypes based on patients' age at onset. Men accounted for 60% of LADA1 subjects between 32 and 51 years old, whereas proportions in older groups were close to 1:1. In contrast, individuals assigned as LADA2 and aged 42-51 or older than 61 years at diagnosis were characterized by 1:2 male to female ratio.

Discussion

The study cohort selected from Sardinian population displayed increased prevalence of Abs targeting a portion belonging to a MAP-derived putative regulator for proline utilization (MAP2404c) and its human PI homolog with high sequence identity corresponding to contiguous fragments of B chain and C-peptide. Responses to the same epitope pairs were even higher in Sardinian infants at risk for T1D and new-onset children from mainland Italy. In a similar fashion, increased seroreactivity to ZnT8 transmembrane regions and their homologous MAP peptides (MAP3865c) has been observed in either Sardinian or Italian cohorts at different disease stages[149-150, 204-205]. These results are in line with the ubiquitous presence of MAP in the environment and the frequency of exposure resulting in a straightforward transmission pathway to humans. Detection of Abs may indicate past contact with MAP following to consumption of contaminated food; most likely the intracellular form of MAP acquires enhanced virulence for humans after passage through bovine macrophages present in milk and cheese[206]. On the other hand, raised anti-MAP Abs titers have been found in individuals at continual interaction with infected animals such as veterinarians or livestock breeders; seropositivity among HCs and subjects at-risk for diabetes who do not develop clinical symptoms can be explained by exposure in early childhood to the extracellular phenotype of MAP that may confer natural immunity protective against mycobacterial infection[145].

Unlike T2D patients who do not display significant anti-MAP responses[153], LADA2 was characterized by similar Abs titers as LADA1 subtype. This outcome confirms the hypothesis involving MAP in the pathogenesis of autoimmune diabetes and sustains distinct mechanisms underlying the development of LADA2 with prevailing autoimmune component despite etiological factors shared with T2D. Among genetic risk factors, single nucleotide polymorphisms (SNPs) in *TCF7L2*, resulting in increased expression of the transcriptional factor, have been linked to T2D and LADA phenotype with low GADA titers[207-208], whereas other authors have not reported any association[209-210]. Interestingly, *TCF7L2* down-regulation has been observed in MAP-infected bovine

monocyte-derived macrophages[211]. Data relative to *TCF7L2*-conferred risk were not available for the study cohort, however a future assessment based on the combination of these two factors in Sardinian population, considered its genetic homogeneity[172], may provide additional elements to complete the present thesis.

Age-related analysis showed major prevalence of anti-MAP positivity for all the eight peptides in the youngest group (32-41 years) of LADA patients assessed either regardless or depending on GADA titers. When only the significant MAP/PI epitope pair was envisaged, this trend switched to the group over 61 years old for LADA2, while remained unvaried for LADA1 and for the general study population, even though the latter presented high Abs titers also in older groups. It is plausible that anti-mycobacterial Abs remain detectable at high levels even after 20 years following LADA diagnosis in a similar way to GADA titers[212], indicating a possible appearance of their peak values at a younger age. In the present study, responses to MAP antigens were not gender-associated, however Abs positivity in predisposed subjects, especially those assigned as LADA1, may suggest an increased risk for autoimmune thyroiditis. Comorbidity of both diseases has been already described and high GADA titers in males seem to be predisposing for thyroid autoimmunity[115]. Moreover, males aged 32-51 years at disease onset accounted for 60% in LADA1 subgroup reflecting findings observed in case of T1D that confirm male excess in populations with high incidence such as Sardinia[15]. Male to female 1:2 ratio among LADA2 subjects was similar to distributions described for T2D.

Further assessment of patients' reactivity to the selected peptides in correlation with time interval from LADA diagnosis to insulin dependence showed Abs prevalence relative to the significant MAP/PI epitope pair distributed homogeneously among subjects with insulin-free period from 18 months to 4 years (within the range 17-18%); the percentage was slightly higher among patients requiring insulin treatment between 13-24 months (23.53%) and much lower in those without therapy after 4 years from LADA onset (14.28%). Surprisingly, after separated analysis of the two disease phenotypes, the decreasing anti-MAP Abs positivity trend was maintained for LADA2 subgroup and showed an inverted picture among LADA1 individuals. These results may suggest a possible association of MAP with a more severe disease course in LADA2 phenotype leading to a sooner need for insulin dosage, however a small number of patients assigned to each insulin-free period did not permit to obtain statistical significance. Moreover, no relation with GADA titers has been found in previous studies. A deeper insight should be dedicated to this question given the increased anti-MAP responses detected among IA-2A-positive subjects.

This work provides evidence of increased prevalence of antibodies directed against MAP epitope and its homolog derived from human proinsulin in LADA patients. Furthermore, it is in line with our previous findings highlighting elevated positivity to MAP in pediatric and adult populations. Further study is needed to address genetic predisposition in correlation with anti-MAP reactivity and the presence of non-islet organ-specific autoantibodies as a possible indication of comorbidities. Yet, information relative to risk factors in dietary habits may be useful in determining any correlation of LADA with sources of putative exposure to MAP.

VI. Prevalence of anti-MAP Abs in Sardinian HT adults

This chapter is based on the following study:

> Niegowska M et al. Antibodies against Proinsulin and Homologous MAP Epitopes Are Detectable in Hashimoto's Thyroiditis Sardinian Patients, an Additional Link of Association. PLoS One. 2015 Jul 20;10(7):e0133497.

Similarly to T1D, HT is an organ-specific disease characterized by T-cell infiltration, cell-mediated immunity and production of autoantibodies that lead to dysfunction or destruction of the target organ. It is estimated that about 20% of T1D patients is positive to anti-thyroid Abs and, inversely, 2.3% of children with AITD have Abs against β-cells[175, 213-214]. While the scientific literature includes numerous studies describing levels of anti-thyroid Abs in T1D patients, only few papers evaluate the status of anti-islet autoimmunity in subjects affected by AITD, in particular related to specific epitopes. The current study, inspired by the above-mentioned findings along with the detection of seroreactivity against MAP/ZnT8 antigens observed also in subjects with autoimmune diabetes[150], aimed at verifying the presence of anti-PI and homologous anti-MAP Abs in HT patients, possibly predicting a future implication of T1D in these subjects.

Results

Among 177 HT patients, 20.34% (n=33) showed positivity to at least one of the four assessed peptides (Figure 20A). 48.5% of positive subjects had Abs against at least two peptides, 87.5% of which were positive to any of the homologous peptide pairs. One HT patient was reactive to all analyzed peptides. When single-peptide analyses of Abs reactivity were performed, 9 HT patients resulted positive to MAP1,4αgbp$_{157-173}$ (5.08%) compared to only 0.57% of HCs (AUC=0.6, p<0.0003) (Figure 21A). The homologous PI$_{64-80}$ peptide was recognized by 33 patients (18.64%) and 7.43% of HCs (AUC=0.6, p<0.002, Figure 21B).

Serum Ab reactivity to MAP2404c$_{70-85}$ and its homolog PI$_{46-61}$ was observed in 6 (3.39%) and 7 (3.95%) HT patients, respectively, with no Ab against both peptides detected in HCs, however the differences were not significant.

Considering positive cases, a higher immunoreactivity to both homologous peptide pairs was found among women, accounting for 90.48% for MAP1,4αgbp$_{157-173}$, 96.15% for PI$_{64-80}$, 100% for MAP2404c$_{70-85}$ and 66,66% for PI$_{46-61}$ (Figure 20B). Upon sex-based analysis of the study population, prevalence of positivity to MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ and MAP2404c$_{70-85}$ was markedly higher among females, while responses to PI$_{46-61}$ were slightly higher in men.

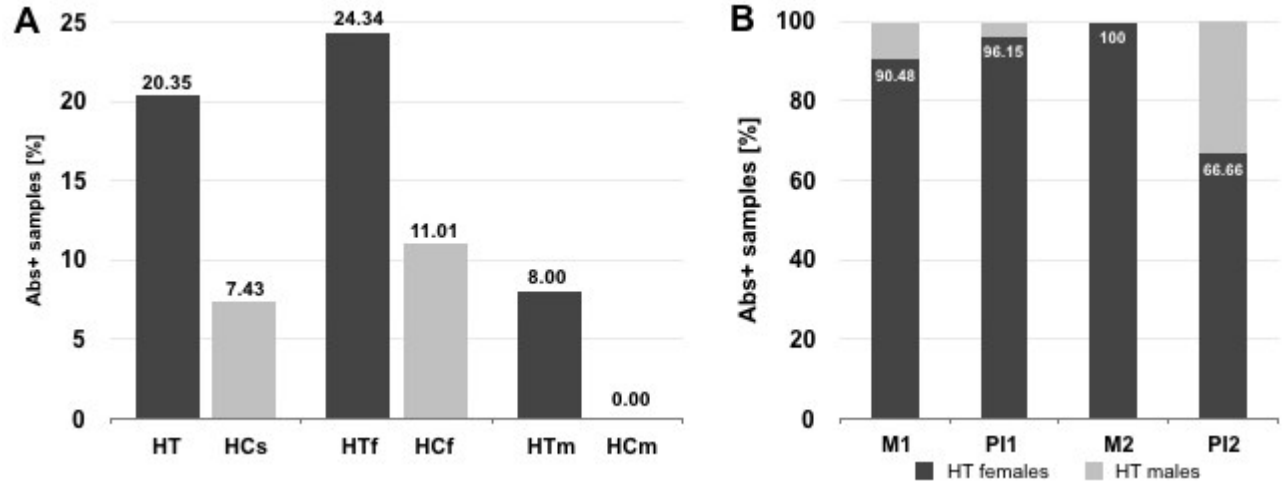
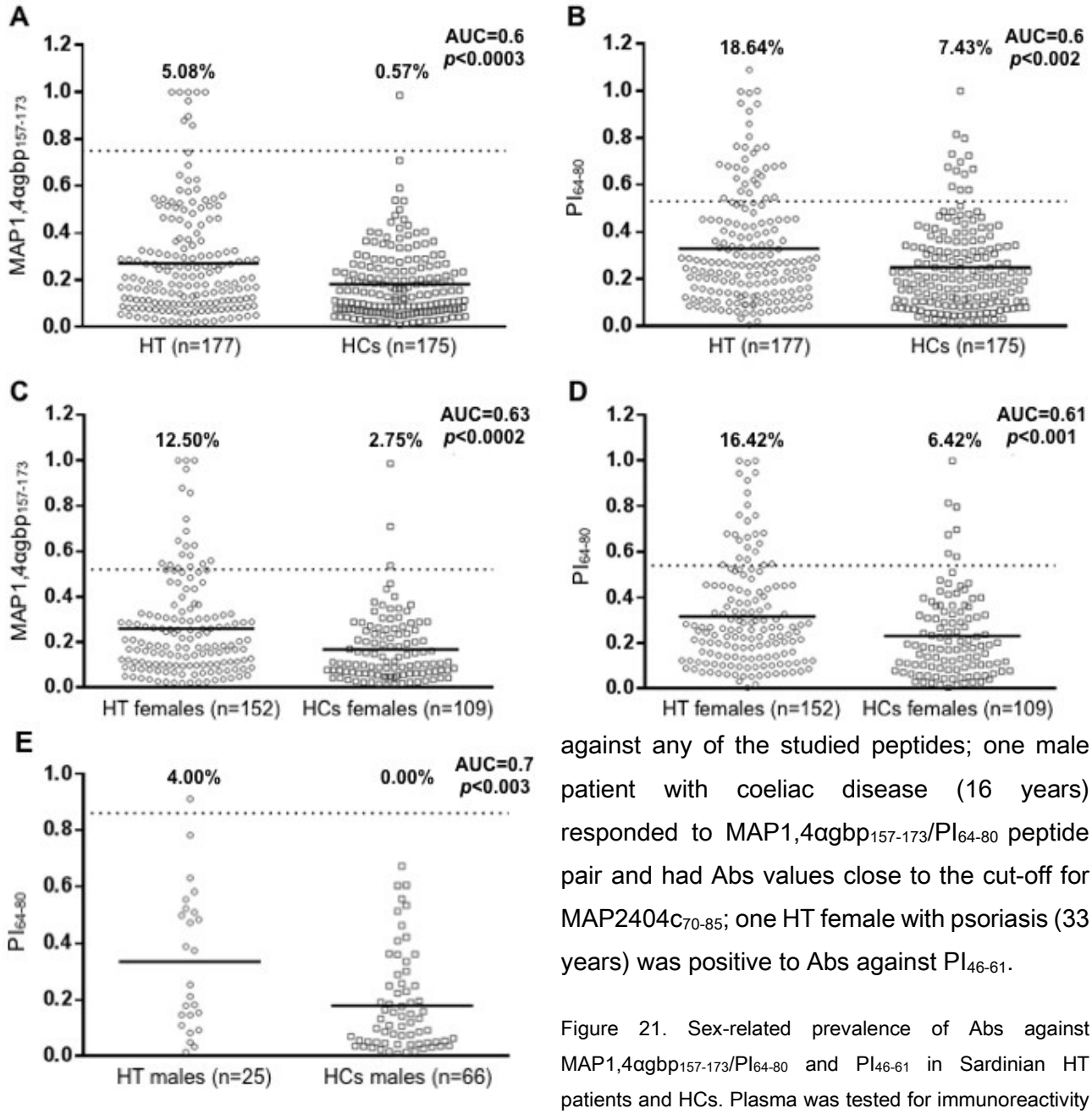


Figure 20. Prevalence of serum Ab positivity to MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ and MAP2404c$_{70-85}$/PI$_{46-61}$ homologous epitopes in Sardinian HT patients and age/sex-matched HCs. HTf: HT females. HTm: HT males. HCf: HC females. HCm: HC males.

In women (n=152), Abs against MAP1,4αgbp$_{157-173}$ were detected in 12.50% of HT subjects and in 2.75% of HCs (AUC=0.63, p<0.0002, Figure 21C). Positivity to its human homolog PI$_{64-80}$ was observed in 16.42% of patients, compared to 6.42% of HCs (AUC=0.61, p<0.001, Figure 21D). Even though anti-MAP2404c$_{70-85}$ Abs were identified in 9.21% of HT patients and in 4.59% of HCs, while 1.32% of female patients and none of sex-matched HCs resulted positive to the homologous PI$_{46-61}$, statistical significance was not attained for this peptide pair.

Males (n=25) displayed higher but not significant levels of Abs against the homologous MAP1,4αgbp$_{157-173}$ and PI$_{64-80}$ giving 8% and 4% of positive patients, respectively, compared to HCs among which no Abs were detected. Serum reactivity was not observed for MAP2404c$_{70-85}$ either in HT patients or HCs, whereas 4% of male patients and none of the HC subjects resulted positive to the homologous PI$_{46-61}$ peptide (AUC=0.7, p<0.003, Figure 21E).

Six patients had diseases of non-autoimmune origin complicating HT: one patient positive to the first peptide pair had hepatitis C; one out of three HT women with hypertension responded to MAP1,4αgbp$_{157-173}$ and showed anti-PI$_{64-80}$ reactivity slightly below the fixed positivity threshold; two patients with T2D did not result positive to any of the anti-MAP/PI Abs. Three HT patients had concomitant autoimmune diseases: one with allergic asthma did not show significant levels of Abs



against any of the studied peptides; one male patient with coeliac disease (16 years) responded to MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ peptide pair and had Abs values close to the cut-off for MAP2404c$_{70-85}$; one HT female with psoriasis (33 years) was positive to Abs against PI$_{46-61}$.

Figure 21. Sex-related prevalence of Abs against MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ and PI$_{46-61}$ in Sardinian HT patients and HCs. Plasma was tested for immunoreactivity against plate-coated homologous MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ and MAP2404c$_{70-85}$/PI$_{46-61}$ peptides.
Statistical analyses were performed for all peptides separately in the context of entire study population (A, B), female (C, D) and male (E) HT subjects and HCs. The dotted lines indicate thresholds of positivity relative to each assay calculated by ROC analysis. The percentage of Abs-positive patients is reported on top of each distribution; horizontal bars correspond to means. AUC and p values (CI 95%) are indicated in the top right corner. Only statistically significant data are reported.

Analyses of trends in age prevalence relative to Abs directed against any of the studied peptides revealed differences in positivity among HT patients' age groups: <20 years (22.22%), 20-

30 years (15.38%), 31-40 years (45.83%), 41-50 years (10,42%), 51-60 years (23.81%) and >60 years (13.33%). Detailed data are presented in Table 5.

Table 5. Age-related trends in prevalence of anti-MAP/PI Abs+ Sardinian HT patients.

HT subjects	Peptides	Age groups (years)									
		<20	20-30	PR	31-40	PR	41-50	PR	51-60	PR	>60
Total study population	MAP/PI 4 peptides	22.22	15.38	1.12	45.83	3.01	10.42	2.5	23.81		13.33
	MAP1,4αgbp$_{157-173}$	16.67	-		4.16		2.08		7.14		3.33
	PI$_{64-80}$	22.22	15.38	1.12	48.67	3.2	10.42	2.5	21.43		10.00
	MAP2404c$_{70-85}$	-	7.69		-		2.08		2.38		6.67
	PI$_{46-61}$	11.11	-		4.16		2.08		4.76		3.33
Females	MAP/PI 4 peptides	18.18	16.67	0.83	33.33	2.08	17.07	2.56	42.11	8.85	11.54
	MAP1,4αgbp$_{157-173}$	18.18	8.33	2.08	14.28	3.57	7.31		23.68	4.97	3.85
	PI$_{64-80}$	18.18	16.67	1.04	33.33	2.78	9.76		21.05		7.69
	MAP2404c$_{70-85}$	-	8.33	1.04	4.76	1.19	9.76	1.46	15.79		7.69
	PI$_{46-61}$	9.09	-		-		-		-		3.85
Males	MAP/PI 4 peptides	14.28	-		33.33		-		-		-
	MAP1,4αgbp$_{157-173}$	14.28	-		33.33		-		-		-
	PI$_{64-80}$	14.28	-		-		-		-		-
	MAP2404c$_{70-85}$	-	-		-		-		-		-
	PI$_{46-61}$	14.28	-		-		-		-		-

Values are reported as percentage of positive cases. PR: prevalence ratio calculated in relation to HCs; PR is not indicated when Abs+ cases were not found among HCs. Absence of Abs+ cases is marked by hyphens.

Anti-PI$_{64-80}$ Abs were prevalent in each age group showing the highest positivity (48,67%) in patients 31 to 40 years comparing to 15.22% among HCs (AUC=0.65, p<0.03), followed by 21.43% of immunoreactivity among 51-60 year-old HT subjects (AUC=0.74, p<0.0002) and no responses registered for HCs. The homologous MAP1,4αgbp$_{157-173}$ peptide was recognized by 4.16% (AUC=0.64, p<0.05) and 7.14% (AUC=0.73, p<0.0004) of patients from the respective age groups, while positive cases were not detected among HCs.

For the same age groups, 4.16% (AUC=0.64, p<0.04) and 4.76% (AUC=0.63, p<0.04) of HT patients displayed positivity to PI$_{46-61}$. Abs directed against its homolog MAP2404c$_{70-85}$ were not

observed among 31-40 year-old patients (AUC=0.67, p<0.01), but 2.38% of HT subjects 51 to 60 years resulted reactive (AUC=0.69, p<0.004). All HCs were negative for this peptide pair.

In female patients, the highest prevalence of positivity to the studied peptides occurred for 51-60 years group and accounted for 42.11%. In particular, a significant immunoreactivity to MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ homologs was observed in 23.68% (AUC=0.68, p<0.016) and 21.05% (AUC=0.69, p<0.008) of HT women, respectively, compared to 4.76% of positivity to MAP1,4αgbp$_{157-173}$ and no anti-PI$_{64-80}$ Abs detected among female HCs. Highly significant results of Ab prevalence for the same peptide pair were obtained for 41-50 years group with 7.31% of female patients positive to MAP1,4αgbp$_{157-173}$ (AUC=0.68, p<0.009) and 9.76% to the homologous PI$_{64-80}$ (AUC=0.7, p<0.003), whereas responses to these peptides were not detected in HC women.

Seropositivity among HT males was highest (33.33%) between 31 to 40 years of age due to the presence of anti-MAP1,4αgbp$_{157-173}$ Abs (AUC=0.9, p<0.02). Positive cases were not detected among HC men for any of the analyzed peptides in all age groups.

Further analysis involved the association between the selected peptides and the titers of diagnostic HT autoantibodies. Even though 80% of HT patients reactive to MAP/PI peptides had anti-TPO Abs levels above positivity threshold, prevalence among anti-TPO negative subjects accounted for 60% compared to 27% among anti-TPO positive ones. A higher prevalence (36%) was observed for anti-TPO 100-1000UI/mL range and decreased to 15% for >1000UI/mL values.

All HT patients positive to MAP/PI peptides had anti-TG Abs above the 10UI/mL cut-off with 32% prevalence and a homogeneous distribution up to 5000UI/ml.

67% of MAP/PI immunoreactive HT subjects had TSH levels below or above the normal range values (0.46-4.68μU/ml) with an overall prevalence of 29%. Prevalence relative to <0.46μU/ml TSH accounted for 20% and for 32% when values exceeded 4.68 μU/ml. Patients with normal TSH values showed 19% prevalence of anti-MAP/PI Abs positivity.

Only 7% of HT patients responding to MAP/PI peptides had FT3 levels exceeding the 5.26pg/ml threshold, however the prevalence reached 33% compared to 21% for subjects with normal FT3 values.

FT4 levels fell outside the normal range of 0.77-2.19 ng/dl in 14% of MAP/PI-positive HT subjects, with 33% and 50% prevalence for values below and above the reference thresholds, respectively. Prevalence of MAP/PI reactivity among patients with normal FT4 values equaled 21%.

Anti-TPO Abs concentrations moderately correlated with both MAP peptides in MAP/PI positive HT subjects, however statistical significance was not attained. Upon sex-related analyses, males presented a high correlation with PI$_{46-61}$ (0.89, p<0.042); the remaining peptides correlated

moderately or even highly for MAP1,4αgbp$_{157-173}$, yet without significance. Moderate but not significant correlation with MAP epitopes was found among females.

Although age-related analysis did not produce significant results, a moderately high correlation of anti-TPO Abs with MAP1,4αgbp$_{157-173}$/PI$_{64-80}$ homologous peptides was detected in the 31-40 year-old group. No correlation with anti-TG Abs concentrations and levels of TSH, FT3 and FT4 was found for any of the analyzed peptide pairs.

Discussion

HT is the most frequent organ-specific autoimmune disorder complicating T1D by involvement of common immunological processes shared by different autoimmune diseases[175]. In Sardinian population, AITD is a prevailing autoimmune disease, while T1D is well known to have the second highest prevalence worldwide[212]. Previous outcomes showed a markedly significant association of MAP with T1D analyzed through detection of seroreactivity against identical MAP/PI homologous peptides as those used in the present study. Positivity to both peptides in HT subjects is identified in most cases within the same person providing another indication that homologous MAP and PI regions may be cross-recognized by human Abs. Even though comorbidity and familial aggregation of autoimmune diseases envision common genetic determinants, substantial co-existence within siblings compared to incidence between successive generations highlights environmental impact[215]. In this study, 54.38% of patients with complete clinical data had HT history in family. This ratio reached 70% among HT subjects positive to any of the analyzed peptides, suggesting a possible transmission of MAP, MAP-derived peptides or anti-MAP Abs within family.

While women are 5-10 times more affected by HT[216], there is no T1D sex-prevalence in childhood, although morbidity among men of European origin is more common in early adulthood[217]. The current results are partially consistent with these trends, accounting for almost 86% of women in the HT study population and showing a higher prevalence of anti-MAP and anti-PI Abs among adult females. 5% prevalence of GADA in HT non-diabetic patients was reported not to impair insulin action or secretion[218]. Prevalence to three out of four peptides analyzed in this study was up to 5%, however positivity to PI$_{64-80}$ exceeding 18% of HT patients could increase the risk for adult-onset diabetes. A high prevalence of seropositivity to glutamic acid decarboxylase (GAD) has been documented in Japanese patients with AITD when compared to HCs and patients with thyroiditis of non-autoimmune origin, however the major contribution was attributed to Grave's disease (GD), whereas HT patients presented only slight but not significant GADA prevalence[219]. As suggested, AITD patients with high GADA titers and positivity to multiple islet autoantibodies are at risk for the development of T1D. Similarly, no significant positivity for IAA and anti-insulin receptor Abs (AIRA)

has been reported in HT patients but high levels of both autoantibodies were typical for GD patients[220]. In contrast, anti-IAA were detected by other authors[221-223], even though a further study describes high responses to GADA in non-diabetic HT patients with negative association to IAA[224].

Interestingly, age-related analyses revealed a markedly higher prevalence of anti-MAP/PI Abs in HT patients 31 to 40 years old indicating a possible association with latent autoimmune diabetes in adults (LADA), even though IAA tend to be undetectable in late-onset T1D. This fact points at a putative involvement of MAP as an environmental agent contributing to the development of autoimmunity and permits to hypothesize a cross-reaction between the homologous peptides. In HT females, this trend was switched to the period of 51-60 years old, although a significant prevalence was already visible in 41-50 age group. This trend is typical for age-related incidence of T2D that lacks the association with MAP[153]; similarly, two HT patients with concomitant T2D included in the present study resulted negative to both MAP/PI peptide pairs. High prevalence of anti-MAP Abs in older groups of Irish cattle affected by Johne's disease[225] may indicate a similar time-depending natural scenario of immune responses in MAP-positive subjects. Finally, the onset of autoimmune diseases in males is supposed to occur earlier compared to females and is characterized by acute inflammation and the appearance of aAbs[226]. In the enrolled study population, mean anti-MAP/PI Abs titers among positive patients were higher in men.

Regardless a lower prevalence of anti-MAP/PI Abs in children and youth (<20 years), two HT patients had very high Abs values for three peptides. The same Abs are demonstrated to appear before the classical anti-islet autoantibodies in Sardinian children at onset of T1D, therefore, may suggest a developing β-cell autoimmunity. The presence of anti-MAP/PI Abs in two out of three HT patients with concomitant autoimmune diseases may point at prediction of multiple autoimmune syndrome (MAS), however such an association should be investigated in a wider group. Since dermatological conditions have an important place in MAS, it would be of particular relevance for patients with psoriasis, the most frequent autoimmune disease affecting Sardinian men.

The analysis of clinical data showed reactivity to MAP/PI epitopes among HT patients to be associated with the presence of anti-TG Abs and high TSH levels. Although FT3 and FT4 presented a high prevalence outside the reference range, only a few cases fell within this group. Anti-TPO Abs were detected in 80% of MAP/PI-positive patients and revealed a moderate correlation with concentrations of anti-MAP Abs; a high prevalence of Abs against the homologous peptides among anti-TPO-negative HT subjects requires evaluation in a wider number of patients. For this thyroid biomarker, correlation with proinsulin became even stronger when analyzed separately in males. Interestingly, low anti-TPO Abs concentrations corresponded to higher values obtained for Abs directed against MAP peptides; this combination may indicate a possible protective link against

raising anti-TPO Abs titers, especially between 31 and 40 years, but could be confirmed following to further investigation of Abs dynamics in the analyzed patients. Conversely, Abs against proinsulin correlated positively with increasing anti-TPO Abs concentrations in men suggesting a probable risk for development of autoimmune diabetes in adolescents and young adults.

VII. Conclusions

A more frequent occurrence of Abs against homologous MAP-derived and human antigens in T1D patients compared to healthy population provides indices for a strong environmental component in the development of autoimmunity. There are no proofs whether MAP infects predisposed hosts as a causative agent or takes advantage of favourable conditions that arise with the physiological imbalance leading to the loss immune tolerance. An additional link of association given by a high prevalence of anti-MAP Abs in Hashimoto's thyroiditis suggests at least partially common mechanisms governing the onset of several autoimmune diseases that tent to appear in concomitance. The increasing prevalence of T1D in developed countries during last century goes hand in hand with the introduction of intensive breeding which facilitates intraherd MAP infections and contamination of animal products. Indeed, detection of viable MAP in retail meat, fresh milk and derivatives including infant formula hints at a straightforward transmission route. While human exposure to MAP is potentially easy and frequent since early life, peculiar characteristics of the mycobacterium such as extremely low growth, asymptomatic latency periods and tropism to intestinal macrophages make difficult the investigation. For a deeper understanding of the interplay between genetic susceptibility to MAP infections and effective presence of mycobacterial cells in T1D population, NOD2/CARD15 mutations should be evaluated in relation to anti-MAP seropositivity, detection of specific *IS900* gene and isolation of viable MAP from patients. Further steps should also focus on in depth assessment of cell-mediated immunity with regard to disease phase and particular attention on MAP-correlated shifts in the self-tolerance equilibrium. These results will not definitively solve the question in a similar manner to Crohn's disease that despite a strong evidence obtained over decades of research lacks the decisive proof, but certainly will add elements to this complicated puzzle.

VII. Methods

Subjects

In the framework of the TRIGR study, 23 Sardinian children at risk of T1D (i.e. with a first-degree relative affected by T1D; mean age 2.8±2.7 years) attending the Department of Diabetes, St. Michele Hospital of Cagliari, Italy were enrolled in the study and followed in time in order to identify a correlation between the presence of Abs and the onset of diabetes. The first blood samples were collected at birth with further annual collections up to 10 years, giving in total 139 samples over the period of 2002-2012. In addition, all at-risk subjects were analyzed for the presence of ICA, GADA, IA-2A and IAA (Table 6). Reference control samples of age-matched healthy volunteers (n=22, mean age 4.3±2.1 years) were provided by the Tor Vergata University Hospital of Rome.

Table 6. Demographic characteristics and pattern of islet autoantibodies in T1D at-risk subjects.

ID	Age at first blood collection (months)	Age at detection of islet Abs (months)	ICA	IAA	GADA	IA2A
2	4	-	-	-	-	-
3	0	-	-	-	-	-
4	3	-	-	-	-	-
5	3	17	+	+	+	+
6	0	-	-	-	-	-
8	0	-	-	-	-	-
9	6	48	+	-	-	-
10	3	97	+	-	-	-
12	24	48	+	+	-	+
14	11	18	+	+	+	+
16	24	-	-	-	-	-
20	24	85	+	-	-	-
22	18	-	-	-	-	-
24	9	-	-	-	-	-
26	0	-	-	-	-	-

ID	Age at first blood collection (months)	Age at detection of islet Abs (months)	ICA	IAA	GADA	IA2A
27	12	-	-	-	-	-
32	24	-	-	-	-	-
33	12	-	-	-	-	-
34	0	-	-	-	-	-
38	0	-	-	-	-	-
39	0	-	-	-	-	-
40	0	-	-	-	-	-
43	0	-	-	-	-	-

0: blood collected at birth

54 children and youth (n=27 males and n=27 females, mean age 9.42±3.84 years) at risk for T1D verified by the presence of disease familiarity and/or high risk HLA genotype, and reference control samples of age-matched healthy volunteers (n=42, mean age 6.94±3.58 years) without known history of autoimmune disorders or recent inflammatory episodes were enrolled in the present study following to periodical visits at the Tor Vergata University Hospital of Rome. Diagnosis of T1D onset was performed upon analyses for the presence of classical islet autoantibodies (ZnT8A, GADA, IA-2A and IAA) and levels of glycated haemoglobin according to the American Diabetes Association criteria[227]. For 27 patients, further time-point collections were performed, giving in total 105 samples. Detailed patients data are provided in Table 7.

From this dataset, 32 subjects at risk for T1D (n=19 males and n=13 females, mean age 8.90±3.52 years) and age-matched healthy volunteers (n=42, mean age 6.90±3.55 years) were selected in blind for the evaluation of seroreactivity against L5P.

Table 7. Demographic and clinical characteristics of Italian T1D at-risk subjects.

ID	Age[a]	Gender[b]	HLA genotype	Risk factor	ZnT8[c]	IAA[d]	GADA[e]	IA2A[f]
R01	12.65	M	DQ2 (DQA1*0201-DQB1*0202) + DQ8	coeliac disease familiarity (brother)	0.00	0.1	0.25	0.11
R02	9.22	F	DQ2 (DQA1*0201-DQB1*02)	coeliac disease	**77.99**	0.00	**1.13**	0.00
R03	4.08	F	DQ2 (DQA1*0201-DQB1*02)	coeliac disease	0.00	**0.51**	**4.10**	**0.90**
R04	5.61	M	DQ2 (DQA1*0501-DQB1*0201)	T1D familiarity (brother)	**45.50**	-	-	-
R05	18.89	M	DQ2 (DQA1*0201-DQB1*0202)	occasional hyperglycemia	**30.00**	0.00	0.00	-
R06	15.18	F	DQ2 (DQA1*0501-DQB1*0201)	T1D familiarity (brother)	3.00	0.3	0.47	0.23
R07	4.94	F	DR4 + DQ8	T1D familiarity (brother)	0.00	**1.52**	**22.00**	**13.80**
R08	8.61	F	DQ2 (DQA1*0201-DQB1*02)	T1D familiarity (brother)	**56.80**	-	-	-
R09	4.91	M	DQ2 (DQA1*0201-DQB1*02)	T1D familiarity (brother)	**69.86**	-	-	-
R10	4.76	F	DQ2 (DQA1*0501-DQB1*0201)	T1D familiarity (brother)	0.00	0.03	0.16	0.27
R11	9.71	F	-	coeliac disease	0.00	0.1	0.09	**1.56**
R12	8.15	M	low risk	T1D familiarity (brother)	**39.40**	0.4	0.25	0.44
R13	5.31	M	-	coeliac disease	0.00	-	-	-

R14	5.24	M	DQ2 (DQA1*0501-DQB1*0201) + DQ8	coeliac disease, autoimmune thyroiditis	**39.99**	**5.98**	**54.70**	0.32
R15	1.92	M	-	suspected coeliac disease	14.00	0.1	0.01	0.03
R16	7.16	F	DR3	occasional hyperglycemia, impaired glucose tolerance	**50.30**	0.00	0.00	**1.00**
R18	7.03	M	DQ8 (DQB1*0302)	T1D familiarity (brother)	0.00	-	-	-
R19	13.16	F	-	coeliac disease, occasional hyperglycemia	0.00	-	-	-
R20	8.33	M	-	coeliac disease	**92.33**	**2.80**	0.31	0.11
R21	6.50	M	DQ2 (DQA1*0501-DQB1*0201)	T1D familiarity (brother)	14.22	0.02	0.1	0.21
R22	7.97	M	DQ2 (DQA1*05-DQB1*02)	coeliac disease, hyperthyrotropinemia, occasional hyperglycemia	**46.44**	-	0,00	**1.64**
R23	12.87	M	DQ2 (DQA1*05-DQB1*02)	coeliac disease	0.00	0.04	**1.00**	**0.91**
R24	5.74	M	DQ2 (DQA1*0201-DQB1*0202)	coeliac disease, occasional hyperglycemia	**56.16**	0.02	**1.22**	**1.00**
R25	8.28	F	DQ2 (DQA1*0201-DQB1*0202)	coeliac disease, autoimmune thyroiditis	**93.48**	-	-	-
R26	6.32	F	DQ2	autoimmune thyroiditis	**63.45**	-	**4.57**	-
R27	7.18	M	DR3 + DR4 + DQ2 (DQA1*0501-DQB1*0201/030503)	T1D familiarity (brother)	**74.30**	**0.52**	**1.16**	0.10
R29	14.65	F	DQ2 (DQA1*0501-DQB1*0201)	coeliac disease, vitiligo and autoimmune thyroiditis familiarity	**93.54**	-	-	-
R30	-	F	-	T1D familiarity (brother)	**30.00**	-	-	-
R31	12.92	M	low risk	T1D familiarity (brother)	**47.62**	0.02	0.25	0.53
R32	8.03	F	DQ2 (DQA1*0501-DQB1*0201)	T1D familiarity (brother)	8.00	0.09	**4.93**	0.23
R33	15.12	M	DR3 + DQ2 (DQA1*0501-DQB1*0201)	T1D familiarity (brother)	22.36	-	-	-
R34	5.09	F	DQ2 (DQA1*0501-DQB1*0201) + DQ8	coeliac disease	**33.81**	**8.66**	**33.77**	**13.51**
R35	8.12	F	DQ2 (DQA1*05- DQB1*02) + DR3 + DR4	coeliac disease	0.00	**1.10**	**39.10**	**7.00**
R36	3.97	M	DQ8	T1D familiarity (brother)	**30.00**	-	-	-
R37	12.11	M	low risk	T1D familiarity (brother)	0.00	0.00	0.00	0.00
R38	7.83	F	DQ2 (DQA1*0201-DQB1*0202)	occasional hyperglycemia	**63.36**	0.00	0.00	0.00
R40	11.83	F	DQ2 (DQA1*0501/0201-DQB1*0202/0201)	T1D familiarity (brother)	**71.39**	**9.13**	**18.90**	**19.05**
R41	17.87	M	-	coeliac disease	**30.00**	-	-	-
R43	10.40	M	DQ8 (DQA1*0201-DQB1*0302)	T1D familiarity (brother)	-	0.00	0.00	0.00
R45	14.17	F	low risk	occasional hyperglycemia	**30.00**	0.00	**2.00**	**0.80**
R46	13.48	M	-	occasional hyperglycemia, obesity, T2D familiarity	**34.00**	0.00	0.00	**0.88**
R47	4.82	F	DR7/DR4 + DQ8 (DQA1*0501-DQB1*030503)	T1D familiarity (brother)	**86.14**	0.00	0.00	0.00
R48	11.86	F	DQ2	T1D familiarity (brother)	**30.00**	0.00	**1132.00**	0.00
R49	11.00	F	DQ2 (DQA1*0201-DQB1*02)	coeliac disease, impaired glucose tolerance	0.00	0.00	0.00	0.00
R50	14.15	M	DQ8	T1D familiarity (brother)	0.00	0.00	0.00	0.00
R52	9.12	M	DQ2/DQ8	T1D familiarity (brother)	**1532.05**	**0.90**	0.33	**1.00**
R53	10.02	F	DQ2 (DQA1*05-DQB1*02) + DQ8 (DQB1*0302)	TAG, occasional hyperglycemia	**41.00**	0.00	0.00	0.00
R54	14.06	F	DQ2	T1D familiarity (father), coeliac and Grave's disease familiarity (sister)	**150.75**	**1.82**	**3.12**	**1.70**
R55	11.41	F	DQ2/DQ8	T1D familiarity (brother)	0.00	0.20	0.52	**1.20**
R56	9.84	M	DQ8	coeliac disease	0.00	**9.13**	**3.46**	0.16
R57	9.99	M	DQ2	occasional hyperglycemia	**30.50**	0.14	**23.02**	**4.73**

R58	7.36	F	DQ2	T1D familiarity (brother)	0.00	0.00	0.29	0.18
R59	13.64	M	DQ2 (DQA1*05-DQB1*02) + DQ8 (DQB1*0302)	T1D familiarity (brother)	-	0.00	0.00	0.00
R60	5.75	F	DQ2 (DQA1*05-DQB1*02) + DQ8 (DQB1*0302)	T1D familiarity (brother)	-	0.00	0.00	0.00

[a] Age at blood collection. [b] F=females, M=males. [c] Positive when >30U/mL. [d] Positive when >0.4U/mL. [e] Positive when >0.9U/mL. [f] Positive when >0.75U/mL. Positive Abs values are shown in bold. Hyphens indicate no measurements performed. ID of subjects who progressed to T1D are highlighted by bold characters; subjects with a high T1D risk conferred by the classical Abs status developed upon follow-up analyses are marked by italics. Age and Abs levels are representative for the initial blood collection when multiple time-point samples were available.

75 Polish children and youth at onset or with established T1D (n=48 females and n=27 males, mean age 13.01±3.52) were enrolled at the Department of Diabetology and Endocrinology of the Childrens' Memorial Health Institute in Warsaw, Poland. The diagnosis was defined based on the circulating ICA, GADA and IA-2A, C-peptide concentrations and HbA1c levels. All T1D patients were under insulin pump or pen therapy at the moment of blood collection. Due to the poor availability of healthy subjects, 26 patients with obesity (n=16 females and n=10 males, mean age 12.54±2.99, mean BMI=27.35±4.53) but not affected by autoimmune disorders and free from any therapy were recruited at the same hospital unit as reference controls. The levels of inflammation were measured through CRP tests. All participants or their legal tutors the interview including information on the familial risk for autoimmunity, consumption of dairy products and infant milk formula, breastfeeding and comorbidity.

223 LADA patients (n=104 men and n=119 women, mean age 55.22±10.71 years) and 182 age-matched healthy blood donors (n=102 males and n= 80 females, mean age 48.13±10.35 years) attending the Transfusion Medicine Department, University Hospital of Sassari, were enrolled in the present study. LADA patients have been referred as a part of a multicentric study, from five Diabetic Units of the island of Sardinia, Italy. The clinical features, autoimmune markers, and progression toward insulin dependence in these patients have been described in detail previously[48]. Any history of autoimmune or inflammatory disorders preceding blood collection from HCs was unknown. Diagnosis of LADA was performed upon analyses for the presence of circulating classical islet autoantibodies (GADA and IA-2A), age at onset and period of insulin independence according to the Immunology of Diabetes Society criteria. All patients were free from insulin therapy at the moment of blood collection and none of them developed insulin dependence for at least 6 months following diagnosis.

177 HT subjects (n=25 males, n=152 females; mean age 44.94±15.65 years) attending the Department of Endocrinology, University Hospital of Sassari, and 175 age/sex-matched healthy controls (n=66 males, n=109 females; mean age 42.18±13.23 years) were enrolled in this study. HCs were blood donors with no clinical evidence of T1D or other autoimmune diseases. The patients were diagnosed based on the presence of TPO and anti-TG Abs, as well as levels TSH, FT3 and FT4.

Venous blood samples were collected in EDTA-coated tubes after obtaining written informed consent for all study participants. A parent or a caretaker signed the participation consent for underage subjects. The study protocols were approved by the University of Sassari and respective enrollment centers in accordance with the Declaration of Helsinki. All methods were performed in accordance with institutional and governmental guidelines and regulations.

T1D-related autoantibodies and clinical testing

Levels of Abs specific to the ZnT8 C-terminal region (268–369, 325R or 325W) were determined in the sera by protein A-radioimmunoprecipitation assays according to the protocol of Lampasona et al.[228]. Abs positivity threshold was set at the 99th percentile of 100 HCs obtaining the cut-off value exceeding 30U/ml. Inter-assay coefficient of variation (CV) accounted for 14%, whereas an intra-assay CV equaled 11%.

Abs to insulin, GAD_{65}, and IA-2 were measured by radioligand assays using commercial kits (CentAK® IAA RT, CentAK® anti-GAD65, and CentAK® anti-IA2, Medipan, Germany) according to the manufacturer's instructions. Values are expressed in arbitrary units with the respective Abs thresholds of >0.4, >0.9 and >0.75 U/ml. Positivity to ICA was determined in a binary classification.

GADA titers were used to classify LADA patients in two groups based on a threshold established as previously reported[229], defined as LADA1 for subjects with high GADA values and LADA2 subtype for individuals with low GADA levels.

HLA genotype was determined for children and youth at T2D risk according to Knip M et al.[63].

Peptide antigens

Epitopes derived from MAP proteins and their homologous human antigens used to assess humoral responses in the sera of patients with autoimmune diseases and HCs, as well as two peptides derived from Helicobacter pylori used as negative controls (Table 8) were synthesized at >90% purity (LifeTein, USA) assessed by HPLC. After reconstitution to 10mM concentration in sterile DMSO, the peptides were stored at -20°C in single-use aliquots for further assays. Specificity of the peptides has been tested previously through competitive inhibition assay[149].

Synthetic L5P prepared as previously described[183] was kindly provided by Frank Biet (INRA Centre de Tours, Nouzilly, France). Due to its hydrophobic nature, the peptide was reconstituted in methanol to obtain 1mg/ml stock solution, incubated for 2 hours with occasional gentle mixing to improve the solubilization and stored at -20°C in single-use aliquots protected from evaporation.

Table 8. List of peptide antigens with respective amino acid sequences, percentage of sequence identity, source organism and confirmed or putative function.

Peptide name	Abbreviation	Amino acid sequence	Identity	Definition	Source
MAP1,4αgbp$_{157-173}$	M1	GTVELLGGPLAHPFQPL	64%	glucan-branching enzyme	MAP
PI$_{64-80}$	PI1	GQVELGGGPGAGSLQPL		proinsulin	human
MAP2404c$_{70-85}$	M2	RGFVVLPVTRRDVTDV	62%	putative regulator for proline utilization	MAP
PI$_{46-61}$	PI2	RGFFYTPKTRREAEDL		proinsulin	human
MAP3865c$_{133–141}$	M3	LAANFVVAL	78%	cation efflux membrane protein	MAP
ZnT8$_{186-194}$	Z1	VAANIVLTV		zinc transporter type 8	human
T2T4W3_HELPX$_{99-105}$		AGIVLTV	71%	preprotein translocase subunit	*H. pylori*
MAP3865c$_{125-133}$	M4	MIAVALAGL	66%	cation efflux membrane protein	MAP
ZnT8$_{178-186}$	Z2	MIIVSSCAV		zinc transporter type 8	human
J0I929_HELPX$_{1-11}$		MIIGGGVSGCA	63%	quinone oxidoreductase	*H. pylori*
L5P		$C_{19}H_{39}$CO-DF-L-NMeV-L-I-L-F-L-AOMe		cell wall lipopentapeptide	MAP

Homologous peptide pairs are evidenced by grey background. Homology between *H. pylori* and ZnT8 antigens is indicated by lateral bars. Alignment identity includes fully conserved residues and those with strongly similar properties. DF: D-phenylalanine. NMe: N-methylated. AOMe: O-methylated alanine.

Detection of Abs directed against peptide antigens

Indirect ELISAs to detect Abs specific for the peptide antigens were performed on serum samples separated from whole blood by sedimentation or density gradient method and stored at

-20°C. For each assay, Nunc MaxiSorp™ flat-bottom 96-well plates (ThermoFisher Scientific, USA) were coated with 50µl of 10µg/ml peptide solutions in carbonate-bicarbonate buffer and incubated at 4°C overnight according to the in-house developed protocol. The solution was then eliminated and non specific binding sites were blocked for 1 hour with 5% skim milk in PBS-T. The plates were washed twice with 200µl of PBS-T for the subsequent addition of 100µl serum diluted 1:100 in PBS-T into each well and incubation at room temperature for 2 hours. After washing with 200µl of PBS-T five times, the plates were incubated for 1 hour with 100µl of anti-human IgG whole molecule antibody conjugated to alkaline phosphatase (Sigma-Aldrich, USA). The wells were washed five times with 200µl of PBS-T for further addition of 200µl Sigmafast™ p-Nitrophenyl phosphate substrate (Sigma-Aldrich, USA) in order to enhance colorimetric reaction with the enzyme. OD values were read within 10-15 minutes at 405nm on SpectraMax Plus 348 microplate reader (Molecular Devices, USA). To subtract background noise, blank assays were performed following the same procedure without including serum sample.

The obtained data were expressed as the mean of three separated tests and normalized to a strongly positive control serum included in all assays with Abs reactivity set at 1.0 AU/ml arbitrary units. Optimal cut-off values to discriminate between positive and negative samples were identified based on the ROC curves with specificity set at ≥88.46% for the Polish cohort and at ≥90% for the other study populations.

In vitro stimulation of PBMCs with MAP3865c$_{133–141}$ and ZnT8$_{186-194}$ peptides

PBMCs were isolated from whole blood within 6h after collection through density gradient separation method and diluted with RPMI with 5% of heat inactivated filter-sterilized human serum in order to obtain an average concentration of 1.5x10^6 cells/ml. Cell viability was assessed through trypan blue exclusion tests on Luna II cell counter (ThermoFisher Scientific, USA). 500µl of cell suspension were incubated at 37°C 5% CO_2 in presence of MAP3865c$_{133–141}$ or ZnT8$_{186-194}$ peptide with co-stimulatory molecules CD28/CD49d (BD Biosciences, USA). PHA was used as a highly stimulating standard. Incubation time was dependent on further analysis (Table 9). The culture supernatant was collected and stored at −20°C until cytokine analysis. The cells were treated immediately according to protocols for cell surface phenotype expression.

Table 9. PBMC culture conditions and control setup for immunostimulation.

Negative control (K-)	Positive control	MAP3865c$_{133–141}$ (P1)	ZnT8$_{186-194}$ (P2)	Incubation time	Analysis
cells only	1µl PHA	7.5µl P1 + 2.5µl CD28/49d	7.5µl P2 + 2.5µl CD28/49d	18 hours	cytokines monocytes
				72 hours	cytokines lymphocytes cell apoptosis

Volumes for 500µl of cell suspension if not specified otherwise. Cell cultures were performed on 24-well plates.

Detection of cytokine levels

Cytokine concentrations in PBMC cell culture supernatants were estimated following 18h (IL-1B and TNFα) or 72h (IL-12p40 and INFγ) of stimulation by means of commercially available ELISA kits (OptEIAtm Set, BD Biosciences, USA). Each sample was diluted prior to the test according to producer's specifications and the final results were obtained by appropriate multiplication in the following ranges: from 3.9 to 250pg/ml for IL-1B, from 4.7 to 300pg/ml for INFγ and from 7.8 to 500 pg/ml for TNFα and IL-12p40. The protein level in the diluted sample was calculated from a reference curve generated for a respective assay by using reference standards containing known concentrations of appropriate protein.

Flow cytometry analysis of cell populations and apoptosis

Cells were collected from culture plates and centrifuged at 300 x g for 5 minutes at 4°C. The obtained cell pellet was then resuspended in 1ml of FBS stain buffer (BD Pharmingen, USA) and incubated for 30 minuets at 4°C. After repeating the centrifugation and discarding the supernatant, cells were resuspended in 100µl of PBS and transferred to polystyrene vials containing 7µl of mouse anti-human monoclonal Abs conjugated to fluorescence-based indicators specific for cell surface receptors expressed on monocytes or lymphocytes (Table 10). Following to incubation for 20 minutes at 4°C in dark conditions, 1ml of PBS was added to each sample and the vials were centrifuged as previously. To evaluate the apoptosis, cells were subsequently labeled with annexin V and 7-AAD using a commercially available apoptosis detection kit (Beckman Coulter, USA). Finally, 2-3 drops of PBS were added to cellular sediment destinated for cell population analysis upon removal of the supernatant.

FACSCanto II (Becton Dickinson, USA) was employed for flow cytometric acquisition on 30000 events and the obtained data were analyzed using FlowJo software (version 7.6.1, Tree Star, USA). The results are reported as a percentage of cells showing expression of the studied receptors.

Table 10. Cell-specific receptors and respective fluorescence-based indicators.

Cell receptor	Indicator	Volume (µl)	Cell gating
CD14 CD16	APC PE	7.0 4.0	monocytes
CD3	PE	7.0	lymphocytes

Statistical analysis

Statistical significance of the data was determined through the unpaired student's t-test or the Mann-Whitney U test (95% CI, two-tailed) for not normally distributed values using Graphpad Prism software (version 6.02, GraphPad Software Inc., USA). Fisher's exact test was employed to establish p-values when percentages of positive and negative subjects were compared between patients and HCs. Pairwise correlation analyses were performed in order to evaluate the reactivity against homologous peptides.

For LADA, variances of separated groups of LADA1 and LADA2 patients along with HCs were compared employing one-way ANOVA on ranks.

Relationship between variables correlated with positivity to L5P and homologous peptide pairs was assessed through principal component analysis employing XLSTAT software (version 2017, Addinsoft, France). The variable relative to HLA-inferred genetic susceptibility was classified in three groups according to low-, moderate- and high-risk T1D haplotype. Milk consumption was evaluated based on low/absent, moderate and high frequency. The cut-off for variable loadings describing the degree of correlation between variables and principal components was arbitrarily set at ≥0.45 as described elsewhere[230], with higher values considered major contributors.

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