Antibodies against Proinsulin and Homologous MAP Epitopes Are Detectable in Hashimoto’s Thyroiditis Sardinian Patients, an Additional Link of Association

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Abstract

Hashimoto’s thyroiditis (HT) is the prevailing organ-specific autoimmune disease in Sardina, often complicated with other autoimmune disorders, most commonly type 1 diabetes (T1D). While numerous studies describe levels of anty-thyroid antibodies (Abs) in T1D patients, few papers evaluate the status of anti-islet autoimmunity in subjects affected by HT. Previously, we portrayed Mycobacterium avium subspecies paratuberculosis (MAP) as an environmental factor strongly associated with both diseases. In this study, we analyzed plasma of Sardinian HT patients (n=177) and healthy controls (HCs; n=175) for the presence of Abs against proinsulin and MAP-derived homologous epitopes: MAP1,4αgbp157-173/PI64-80 were recognized by 5.08% and 18.64% of HT vs 0.57% and 7.43% of HCs (AUC=0.6 for both; p<0.0003 and 0.002, respectively), whereas the prevalence of Abs against MAP2404c70-85/PI46-61 peptides was higher but not significant in patients when compared to HCs. In women (n=152), Abs against MAP1,4αgbp157-173 were detected in 12.50% of HT vs 2.75% of HCs (AUC=0.63; p<0.0002), while positivity to its human homolog PI64-80 was observed in 16.42% of HT vs 6.42% of HCs (AUC=0.61; p<0.001). In men (n=25), a significant anti-PI46-61 Abs levels were detected in 4% of HT vs none of the HCs (AUC=0.7; p<0.003). Age-related analyses revealed the highest prevalence between 31-40 years old (45.83%) in the total study population and among males (33.33%); in contrast, women had a higher seroreactivity between 51-60 years (42.11%). A further follow-up and determination of anti-islet Abs levels is needed to evaluate the association of immune responses directed against the MAP/PI homologous peptides with progression to overt diabetes in HT subjects.

Introduction

Hashimoto’s thyroiditis (HT) is an autoimmune inflammation of the thyroid gland associated with alterations in glucose and insulin metabolism, therefore frequently occurring along with
type 1 diabetes (T1D). Both diseases are organ-specific, characterized by T-cell infiltration, cell-mediated immunity and production of autoantibodies (aAbs) leading to dysfunction or destruction of the target organ (thyroid gland and pancreatic islet cells, respectively). It is estimated that about 20% of T1D patients is positive to anti-thyroid aAbs with prevalence in females and, inversely, 2.3% of children with autoimmune thyroiditis (AITD) have aAbs against β-cells [1–4]. However, recommended measurements of thyroid-stimulating hormone (TSH), as well as thyroid peroxidase (TPO) and thyroglobulin (Tg) aAbs levels in T1D patients to evaluate future development of overt thyroiditis is not yet a common practice [5, 6]. Similarly to other autoimmune diseases, HT and T1D have multiple etiology resulting from an interaction between genetic predisposition and environmental risk factors. Although they have been investigated for decades, there is no clear explanation concerning mechanisms underlying the development of multiple autoimmune diseases within the same individual or family [7–9].

The functioning of the thyroid gland can be impared by deficiency of trace elements such as zinc [10], essential either in follicles of the thyroid gland or for a correct maturation and secretion of insulin stored as hexameric complexes bound by two Zn²⁺ ions [11]. ZnT8 is a zinc transporter membrane protein expressed in insulin secretory granules of pancreas. It is targeted by aAbs in T1D patients and has been identified as a novel T1D biomarker increasing diagnostic sensitivity when analysed together with other T1D aAbs (ICA, IAA, IA2A and GADA) [12]. Recently, high levels of anti-ZnT8 aAbs (ZnT8A) have been associated with the onset of autoimmune thyroiditis in LADA patients [13]. Previously, we reported a significantly high positivity to ZnT8A and antibodies against homologous peptides derived from Mycobacterium avium subsp. paratuberculosis (MAP) in HT and T1D patients that corroborates a possible association of the bacterium with both diseases [14–17]. Furthermore, our group identified MAP peptides homologous to proinsulin (PI) that were highly recognized in new-onset T1D children from continental Italy (45.7–49.1%) compared to healthy controls (3.3%), hypothesizing a cross-reaction between Abs targeting the homologous PI/MAP region [18]. PI has been described as a β-cell antigen initiating T1D pathogenesis in NOD mouse [19] and Abs against its processed form, insulin, are the first to be detected in T1D patients [20].

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 above-mentioned findings encouraged us to design the current study, aimed at verifying the presence of anti-PI and homologous anti-MAP Abs in HT patients, possibly predicting a future implication of type 1 diabetes in these subjects.

Materials and Methods

Subjects

177 subjects (n = 25 males, n = 152 females; mean age 44.94±15.65 years) attending the Department of Endocrinology, University Hospital of Sassari (Italy), affected by HT and 175 age/sex-matched healthy controls (HCs; n = 66 males, n = 109 females; mean age 42.18±13.23 years) were enrolled in this study. HCs were blood donors at the University Hospital of Sassari with no clinical evidence of T1D or other autoimmune diseases. The patients were diagnosed based on the presence of anti-thyroid peroxidase (TPO) and anti-thyroglobulin (TG) Abs, as well as levels of thyroid-stimulating hormone (TSH), free triiodothyronine (FT3) and thyroxine (FT4). Plasma was separated by sedimentation method from venous blood samples collected in EDTA-coated BD Vacutainer tubes, and stored at -20°C.
Peptides

MAP1,4αgbp157-173 (GTVELGGPLAHFPQPL) and MAP2404c70-85 (RGFVVLPVTRR DVT) with their respective homologs PI64-80 (GQVELGGPGAGSLQPL) and PI46-61 (RGFYTPKTRREADL) were synthesized at 85.58–92.70% purity (LifeTein, South Plainfield, NJ 07080, USA) assessed by HPLC and stored in single-use aliquots at -20°C.

ELISA

Indirect enzyme-linked immunosorbent assays (ELISA) to detect antibodies against MAP/PI homologous peptides in plasma samples were performed as described previously [18]. Highly positive samples with reactivity set at 1.000 arbitrary units (AU)/ml were used to normalize the OD values. The statistical analyses of the assay were performed using Version 6.0 Graphpad Prism software and their significance was determined through the Mann-Whitney U test for not normally distributed data or the Student’s t-test (95% CI).

Auto-antibodies assays and thyroid hormone levels

Anti-thyroid Abs titers were measured by chemiluminescence methodology in the serum of all HT patients, using the Liaison Anti-TPO kit (DiaSorin, Italy) for anti-TPO assay and the Liaison Anti-TG kit (DiaSorin, Italy) for anti-TG assay with normal values ranging from 0–100 UI/mL and 0–10 UI/mL, respectively, according to the manufacturer’s instructions.

Levels of FT4 (ng/dl), FT3 (pg/ml), TSH (μU/ml) were measured by the LIAISON Analyzer family (DiaSorin S.p.A., Vercelli Italy).

Correlation analyses were performed with Version 6.0 Graphpad Prism software.

Ethical statement

The current study protocol was approved by the Bioethical Committee of the University of Sassari. All participants were enrolled upon signing written informed consent. All governmental and institutional regulations regarding the ethical involvement of human volunteers in clinical studies were respected.

Results

Prevalence of Abs against MAP1,4αgbp and MAP2404c/PI epitopes in HT subjects and age/sex-matched HCs

Among 177 HT patients, 20.34% (n = 33) showed positivity to at least one of the four assessed peptides (Fig 1A). 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 analysed peptides. When single-peptide analyses of Ab reactivity were performed, 9 HT patients resulted positive to MAP1,4αgbp157-173 (5.08%) compared to only 0.57% of HCs (AUC = 0.6; p < 0.0003) (Fig 2A). The homologous PI64-80 peptide was recognized by 33 patients (18.64%) and 7.43% of HCs (AUC = 0.6; p < 0.002) (Fig 2B).

Serum Ab reactivity to MAP2404c70-85 and its homolog PI46-61 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 MAP1,4αgbp157-173, 96.15% for PI64-80, 100% for MAP2404c70-85 and 66.66% for PI46-61 (Fig 1B). Upon sex-based analysis of the
Fig 1. Prevalence of serum Ab positivity to MAP1,4αgbp_{157-173}/Pl64-80 and MAP2404c_{70-85}/Pl46-61 homologous epitopes in Sardinian HT patients and age/sex-matched HCs.

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Fig 2. Sex-related prevalence of Abs against MAP1,4αgbp157-173/PI64-80 and PI46-61 in Sardinian HT patients and HCs. Plasma was tested for immunoreactivity against plate-coated homologous peptides MAP1,4αgbp157-173/PI64-80 and MAP2404c70-85/PI46-61. 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.

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study population, prevalence of positivity to MAP1,4αgbp157-173/Pl64-80 and MAP2404c70-85 was markedly higher among females, while responses to Pl46-61 were slightly higher in men.

In women (n = 152), Abs against MAP1,4αgbp157-173 were detected in 12,50% of HT subjects and in 2,75% of HCs (AUC = 0,63; p<0,0002) (Fig 2C). Positivity to its human homolog Pl64-80 was observed in 16,42% of patients, compared to 6,42% of HCs (AUC = 0,61; p<0,001) (Fig 2D). Even though anti-MAP2404c70-85 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 Pl46-61, statistical significance was not attained for this peptide pair.

Males (n = 25) displayed higher but not significant levels of Abs against the homologous MAP1,4αgbp157-173 and Pl64-80 giving 8% and 4% of positive patients, respectively, compared to HCs among which no Abs were detected. Serum reactivity was not observed for MAP2404c70-85 either in HT patients or HCs, whereas 4% of male patients and none of the HC subjects resulted positive to the homologous Pl46-61 peptide (AUC = 0,7; p<0,003) (Fig 2E).

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 MAP1,4αgbp157-173 and showed anti-Pl64-80 reactivity slightly below the fixed positivity threshold; two patients with type 2 diabetes did not result positive to any of the anti-MAP/Pl 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 MAP1,4αgbp157-173/Pl64-80 peptide pair and had Abs values close to the cut-off for MAP2404c70-85; one HT female with psoriasis (33 years) was positive to Abs against Pl46-61.

Prevalence of Abs against MAP1,4αgbp and MAP2404c/Pl peptides in HT and sex-matched HC age groups

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 1.

Anti-Pl64-80 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 MAP1,4αgbp157-173 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 Pl46-61. Abs directed against its homolog MAP2404c70-85 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 MAP1,4αgbp157-173/Pl64-80 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 MAP1,4αgbp157-173 and no anti-Pl64-80 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 MAP1,4αgbp157-173 (AUC = 0,68; p<0,009) and 9,76% to
the homologous PI64–80 (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-MAP1,4αgbp157–173 Abs (AUC = 0.9; p < 0.02). Positive cases were not detected among HC men for any of the analysed peptides in all age groups.

Prevalence of anti-MAP/PI Abs in correlation with anti-TPO and anti-Tg Abs, levels of TSH, FT3 and FT4 in HT subjects

Even though 80% of HT patients reactive to MAP/PI epitopes had anti-TPO Abs levels above positivity threshold, prevalence of MAP/PI peptides 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 100–1000 UI/mL range and decreased to 15% for >1000 UI/mL values.

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

67% of MAP/PI immunoreactive HT subjects had TSH levels below or above the normal range values (0.46–4.68 μU/ml) with an overall prevalence of 29%. Prevalence relative to <0.46 μU/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 5.26 pg/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 PI46-61 (0.89; \(p<0.042\)); the remaining peptides correlated moderately or even highly for MAP1,4\(\alpha\)gbp157-173, 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 MAP1,4\(\alpha\)gbp157-173/PI64-80 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**

We investigated the presence of Abs directed against proinsulin and homologous MAP-derived peptides in Sardinian HT patients. The present study demonstrated a significant association of anti-PI and anti-MAP Abs in the analysed subjects, suggesting a possible role of MAP in T1D pathogenesis when occurring as an associated disorder of HT.

HT is the most frequent organ-specific autoimmune disorder complicating T1D by involvement of common immunological processes shared by different autoimmune diseases [2]. In Sardinian population, AITD is a prevailing autoimmune disease, while T1D is well known to have the second highest prevalence worldwide [21]. Our group previously described a markedly significant association of MAP with T1D in Italian pediatric patients, analyzed through detection of seroreactivity against identical MAP/PI homologous peptides as those used in the present study [18]. Positivity to both peptides in HT subjects is detected 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 coexistence within siblings compared to incidence between successive generations highlights environmental impact [22]. In the present 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 [23], there is no T1D sex-prevalence in childhood, although morbidity among men of European origin is more common in early adulthood [24]. Our 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 [25]. Prevalence to three out of four peptides analyzed in our study was up to 5%, however positivity to PI46-80 exceeding 18% of HT patients could increase the risk for adult-onset diabetes. Moriguchi et al. documented a high prevalence of seropositivity to glutamic acid decarboxylase (GAD) 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 [26]. The authors suggested that AITD patients with high GADA titers and positivity to multiple islet autoantibodies are at risk for the development of T1D. Similarly, Nuovo et al. reported no significant positivity for IAA and anti-insulin receptor Abs (AIRA) in HT patients but high levels of both aAbs were typical for GD patients [27]. In contrast, anti-IAA were detected by other authors [28–30], even though a further study describes high responses to GADA in non-diabetic HT patients with negative association to IAA [31].
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 type 2 diabetes, however we previously demonstrated no association of MAP with T2D [32]; 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 [33] 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 [34]. In the 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 aAbs in Sardinian children at onset of T1D (unpublished data), therefore, may suggest a developing β-cell autoimmunity.

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.

In conclusion, we here provided the first evidence of significantly high reactivity to proinsulin and MAP-derived homologous peptides in the context of HT, with distinct prevalence in 51–60 year-old women and in 31–40 year-old men. A prospective follow-up of positive patients and analyses of classical anti-islet aAbs levels will permit to evaluate the association of immune responses directed against the homologous peptides with progression to overt T1D in HT subjects. Likewise, diet composition, in particular consumption of dairy products and iodine rich food, as well as genetic polymorphisms need to be assessed in relation to anti-MAP/PI positivity. Moreover, detection of anti-MAP/PI Abs in T1D adult patients would provide an additional view on age/sex-related prevalence.
Author Contributions
Conceived and designed the experiments: LAS. Performed the experiments: MN DP. Analyzed the data: LAS MN. Contributed reagents/materials/analysis tools: LAS MP CB. Wrote the paper: MN LAS.

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