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RESEARCH ARTICLE

Relationship between autoantibodies against type 2 deiodinase and antithyroid autoantibodies in Graves' disease using antithyroid drug therapy

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ABSTRACT

Background: Previously, the presence of autoantibodies against type 2 deiodinase cys- and hom-peptides was investigated in Graves' disease with relation to thyroid function, ophthalmopathy and therapy. The new design was to reveal the relationship among antithyroid antibodies involved in hormone synthesis.

Aim and scope: To demonstrate some regularity between the appearance of peptide autoantibodies and antithyroid antibodies against TSH receptor, thyroid peroxidase and thyroglobulin, the study followed the main path of hormone synthesis and the effect of antithyroid drug treatment. Has any effect of peptide autoantibodies on the titers of other autoantibodies?

Methods: The methods were described in our previous papers detailed. Autoantibodies against type 2 deiodinase peptides were detected with enzyme-linked immunosorbent assay in 78 patients with Graves' disease and 30 controls. Thyroid hormones (TSH, FT_4 and FT_3) and antibodies against TSH receptor, thyroid peroxidase and thyroglobulin were measured with chemiluminescence immunoassay.

Results: The frequency of cys-peptide antibodies was greater in the new onset of Graves' disease. Propylthiouracil treatment was associated with increased occurrence of cys-peptide antibodies ($p<0.0069$). No dose-dependency in methimazole treatment could be demonstrated for cys-peptide antibody levels. Antibodies against hom-peptide or both peptides demonstrated a strong relationship with the appearance of antithyroid antibodies. No peptide antibodies were associated with anti-thyroglobulin antibody positivity (0 cases out of 11 for hom-peptide, $p<0.0071$ and 0 cases out of 8 for both peptides, $p<0.0251$). In contrast, all cases with TSH receptor antibody positivity were positive for peptide antibodies (all cases out of 13 for hom-peptide, $p<0.0139$ and all cases out of 9 for both peptides, $p<0.0470$). TSH receptor antibody levels were relevantly decreased, when hom-peptide antibodies were present [24.35(15.68-37.8) vs 11.8(8.16-17.07) IU/l, $p<0.0437$].

Conclusion: The presence of hom-peptide antibodies was connected to the absence of antibodies against thyroglobulin and smaller anti-thyroid peroxidase antibody positivity. The presence and the high titers of TSH receptor antibodies demonstrated a close relationship with the appearance of hom-peptide antibodies. The relationship among the studied autoantibodies may depend on the alteration of thyroid hormone synthesis and the steric hindrance of targets and antithyroid drug treatment. Autoantibodies against type 2 deiodinase peptides can influence the therapeutical effectiveness.

Introduction

Graves' disease (GD) is characterized by a hormonal dysfunction with the signs of hyperthyroidism, diffuse goitre and thyroid autoimmunity associating sometimes with extrathyroidal manifestations, such as orbitopathy and dermopathy [1]. The appearance of TSH receptor stimulating antibodies is connected to the development of hyperthyroidism. The excess of T_3, which is dominant, and T_4 hormones are produced due to the increased hormone synthesis and the conversion from T_4 to T_3 catalyzed by type 1 (DIO1) and type 2 (DIO2) deiodinase enzyme activities [2, 3]. Antithyroid drug (ATD) therapy in Hungary consists of the treatment with methimazole (MMI) and propylthiouracil (PTU), which have the feature to block thyroid hormone synthesis and reduce DIO1 activity, particularly by PTU, while their inhibiting effects on DIO2 activity are questionable [4].

In the new onset of GD, the generation of main autoantibodies, such as thyroid peroxidase (TPO), thyroglobulin (Tg) and TSH receptor stimulating antibodies are associated with the increased hormonal synthesis of T_3 and T_4. TPO catalyzes the iodination of tyrosines and the coupling of iodotyrosine residues located in the colloid embded thyroglobulin producing triiodothyronine (T_3) and thyroxine (T_4) hormones [5]. Therefore, the role of deiodinase enzymes (DIO1 and DIO2) can not be excluded as targets for thyroid autoimmunity in hyperthyroid GD. The high protein expression of enzymes (TPO, DIO1 and DIO2) in hyperthyroidism can be a target for thyroid autoimmunity. The feature of thyroid autoimmunity may explain why intracellular enzymes could also be targets for the generation of autoantibodies. Antibody-dependent and/or C3 complement-mediated cytotoxicities may be the main factors how thyroid follicular cells could be destroyed making reachable the enzymes to induce autoimmune processes [6].

In our previous paper, the occurrence and the role of autoantibodies against two peptides corresponding to amino acid (aa) sequences of DIO2 were studied with relation to thyroid hormone levels and ophthalmopathy [7]. A strong relationship was demonstrated between the presence of peptide antibodies and thyroid hormone levels under ATD therapy.

In the recent study, the occurrence of these peptide antibodies was investigated in detail and their relationship with autoantibodies against TPO, Tg and TSH receptor, as well as with the new onset and relapse of hyperthyroid Graves' disease, and ATD therapy. The increased occurrence of peptide antibodies was connected to the new onset of hyperthyroid GD and PTU treatment. The production of peptide antibodies showed a close time-relation with the appearance of antibodies against Tg, TPO and TSH receptor in Graves' disease.

Patients and Methods

Patients

The patient groups were formed by the previously studied 78 patients with

Graves' disease (mean±SD of age: 43±13 years, 20 males, 38 cases had ophthalmopathy) and 30 controls (47±16 years, 3 males) [7]. The diagnosis of GD was based on the clinical signs of hyperthyroidism and diffuse goitre using ultrasonography to exclude toxic adenoma. The thyroid functional state was as follows: 51 and 24 cases were hyperthyroid and euthyroid, respectively; 3 cases were hypothyroid at the time of the study. ATD treatment resulted in 31 hyperthyroid and 11 euthyroid patients treated with MMI, as well as 4 hyperthyroid and 2 euthyroid patients treated with PTU; 3 hypothyroid patients were treated with MMI.

Thirty healthy persons formed the control group.

Methods

The peptides (cys- and hom-peptides) corresponding to aa sequences of DIO2 were applied for the guinea pig immunization (GenBank AAD45494-1, aa sequence from 124 to 152 for hom-peptide showed a whole homology; aa sequence from 132 to152 for cys-peptide had some change at positions of 133,136 and137, therefore showed 86% of homology). All peptides contained the active center of enzyme. The guinea pig immunization and the enzyme-linked immunosorbent assay (ELISA) methods were described detailed in our previous papers, which were applied for the production and the detection of peptide antibodies [7, 8]. The O.D. values above the control O.D.+ 2SD were regarded as positive (above 106.37 for hom-peptide and above 107.12 for cys-peptide antibodies).

Thyroid hormones (TSH, FT_4, FT_3) and autoantibodies against TPO and Tg were measured in patient sera with chemiluminescence immunoassay in a fully automated way. Autoantibodies against TSH receptor were detected with competitive enzyme immunoassay (Medizym T.R.A., Germany). The values above 1.5 IU/l were regarded as positive.

Statistics

Data were displayed in geometric mean (GM) with 95% of confidence interval (95%CI) expect for the ratio of FT_3 to FT_4. The logarithms of the skewed data were applied for the evaluation of thyroid hormone and autoantibody levels, which could be regarded as approximately normally distributed data. In figures, the data in GM with + SD and –SD were exhibited. Chi-squared test for the comparison of categorical data and Student's *t*-test for the comparison of the measured thyroid hormone and autoantibody levels were used. ROC (receiver operating characteristic) analysis was applied for the evaluation of the cut-off values for autoantibody levels against Tg, TPO and TSH receptor with respect to the positivities of antibodies againts hom-peptide and both peptides. The *p* values below 0.05 were regarded as significant, but borderline increased *p* values were also exhibited. The statistical analysis was performed with Medcalc 17.9.7. software for Windows®.

Results

1. The appearance of antibodies against peptides with relation to the new onset and relapse, as well as to antithyroid drug treatment

The occurrence of autoantibodies against cys-peptide was more frequent in the new onset of GD than in the relapse (28 out of 54 cases vs 4 out of 19 cases, $p<0.0403$) (Figure 1). PTU treatment was associated with 100 % of the presence of antibodies against cys-peptide (6 out of 6 cases with PTU treatment vs 14 cases out of 45 with MMI treatment; vs 10 cases out of 27 in cases, who did not have been treated, $p<0.0069$). Antibodies against cys-peptide were connected to the ratio of FT_3 to FT_4 above 0.41 in 38 % of the cases treated with MMI and in 100 % of the cases treated with PTU ($p<0.0176$). In the flow chart, the titers of peptide antibody levels represented case values. The increased cys-peptide antibody levels overlapped with the more increased hom-peptide antibody levels in the cases treated with PTU and who did not have been treated yet.

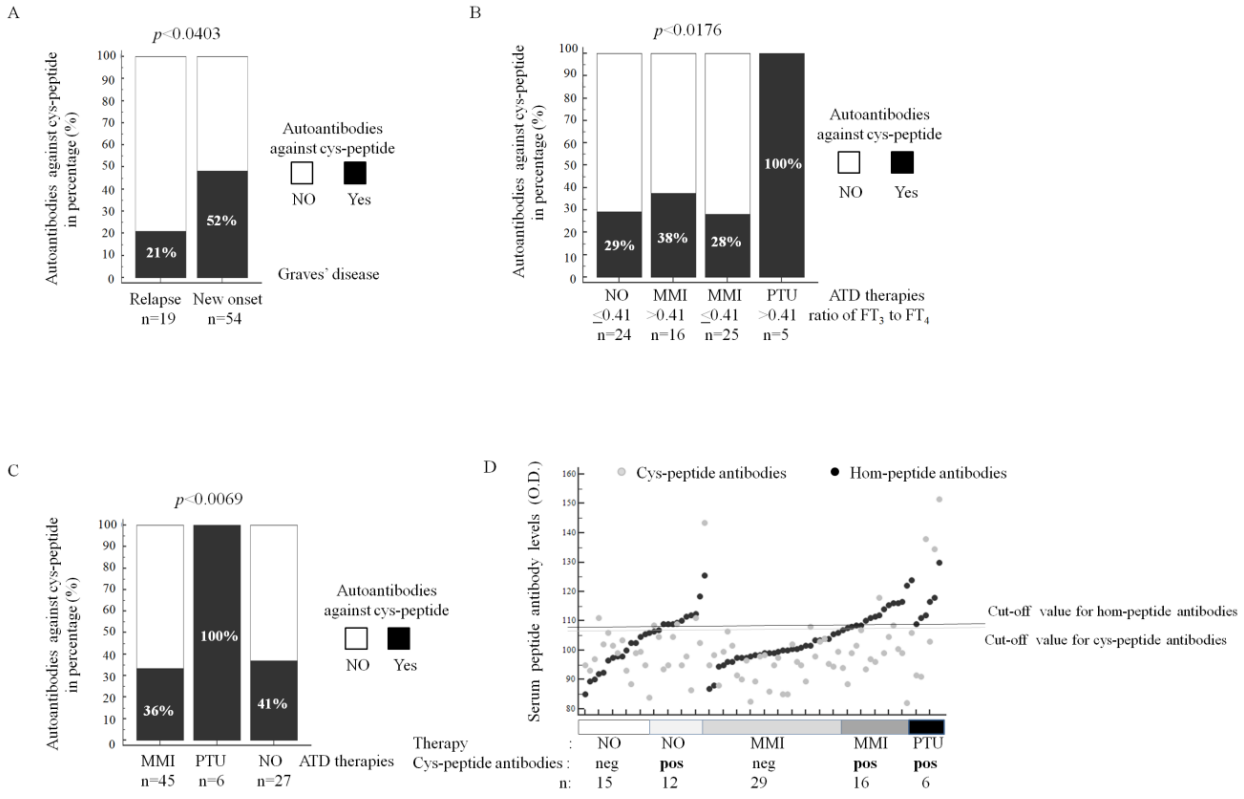

Figure 1: The appearance of antibodies against cys-peptide in percentage according to the new onset and relapse of Graves' disease (A), the ratio of FT_3 to FT_4 (B), and the antithyroid drug treatment (C). The flow chart shows the titers of antibody levels against both peptides (D) according to antithyroid drug treatment.

The effect of ATD treatment was investigated on the titers of antibodies against peptides in a dose-related manner. The cys-peptide antibody levels showed a borderline dose-related increase in patients treated with MMI and failed with PTU (Figure 2).

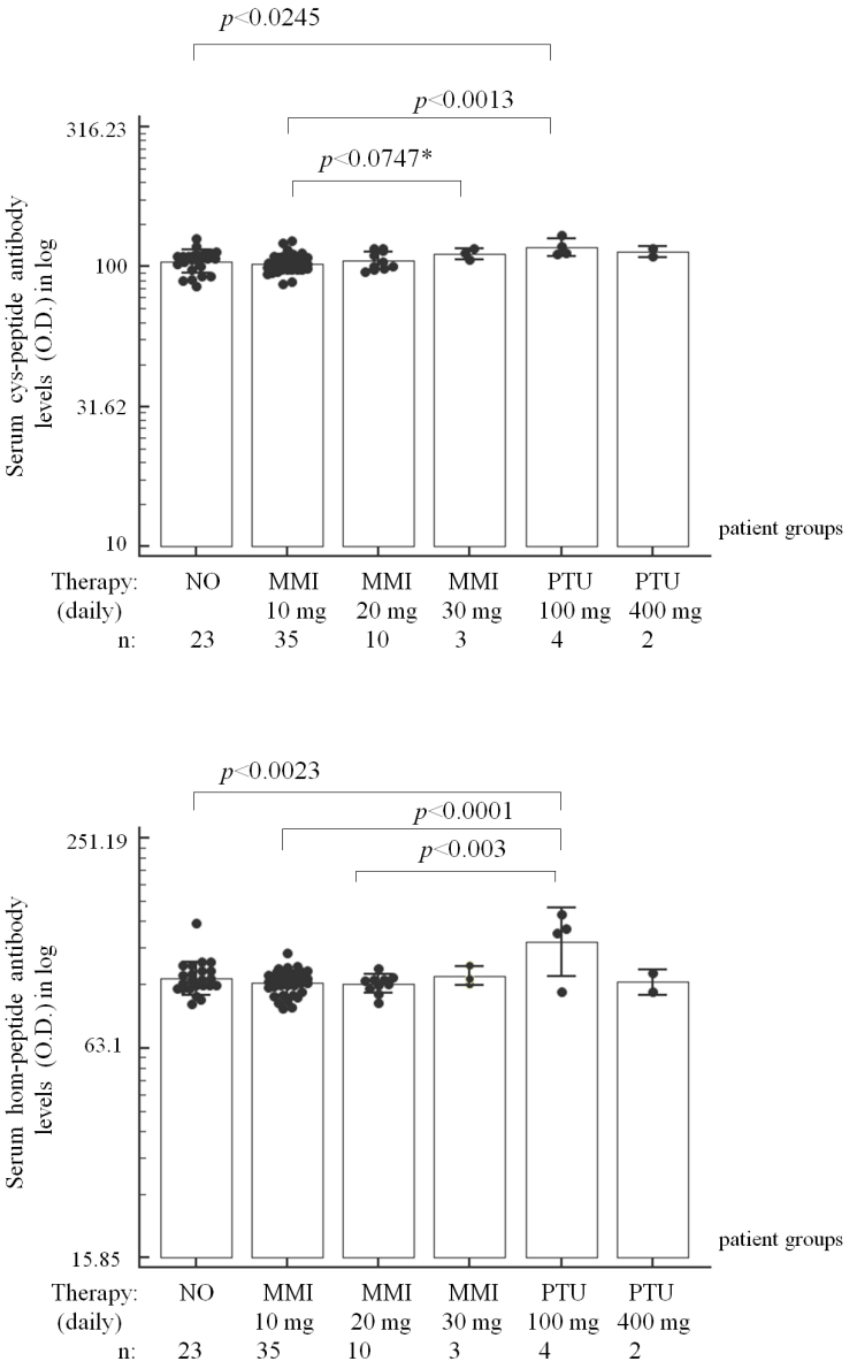

Figure 2: The dose-related effect of antithyroid drugs on the serum hom- and cys-peptide antibody levels.

The frequency of cys-peptide antibodies increased in dose-related manner in cases with MMI treatment: 6/35, 4/10 and 2/3 at doses of 10, 20 and 30 mg, respectively. The difference in cys-peptide antibody levels was borderline between 10 mg and 30 mg of MMI [102.32(88.2-118.71) vs 111,09(101.68-121.38), p<0.0747]. 100 mg of PTU was connected to more increased cys-peptide antibody levels compared to those treated with 10 mg of MMI [117.51(101.94-135.46) vs , p<0.0013] and who did not have been treated yet [vs 104.14(86.33-125.62), p<0.0245]. The increase in hom-peptide antibody levels was greater in cases treated with 100 mg of PTU compared to those treated with 10 mg and 20 mg of MMI [126.48(81.32-196.71) vs 96.84(82.72-113.37), *p*<0.0001, and vs 96.13(85.09-108.61), *p*<0.003, respectively], and who did not have been treated yet [vs 99.76(80.47-123.68), *p*<0.0023]. The frequency of cys-peptide antibodies was higher in the cases with the ratio of FT_3 to FT_4 above 0.41 compared to those who had below that (9/13 vs 17/58, *p*<0.073).

2. Relationship between antibodies against peptides and antithyroid antibodies

ROC analysis was applied to demonstrate the cut-off values for anti-TPO and anti-Tg antibodies, and TSH receptor antibodies with respect to antibody positivities found againts hom-peptide and both peptides (Figure 3). The values of the area under the ROC curve were significant. The Youden index was relevant for anti-Tg antibodies regarding to antibodies against hom-peptide (Y index:0.5738) or both peptides (Y index:0.5469), and for TSH receptor antibodies regarding to antibodies against both peptides (Y index: 0.5700). A relevant significance could be demonstrated between the frequencies of hom-peptide antibodies and antibodies against Tg, TPO and TSH receptor in crosstabs. Surprisingly, no peptide antibodies were associated with anti-Tg antibody positivity vs negativity (0 cases out of 11 vs 26 cases out of 61 for hom-peptide, *p*<0.0071 and 0 cases out of 8 vs 26 cases out of 64 for both peptides, *p*<0.0251). In contrast, all cases were positive for peptide antibodies in patients with TSH receptor antibody positivity vs negativity (all cases out of 13 vs 43 cases out of 65 for hom-peptide, *p*<0.0139 and all cases out of 9 vs 47 cases out of 69 for both peptides, *p*<0.0470). The frequency of peptide antibodies was smaller in the patients with anti-TPO positivity vs negativity (4 cases out of 12 vs 42 cases out of 65 for hom-peptide, *p*<0.0437 and 2 cases out of 8 vs 44 cases out of 69 for both peptides, *p*<0.0355).

Variables	Classification variables	Youden index	Sensitivity	Specificity	Cut-off values	AUC value*	P
Anti-Htg antibodies	hom-peptide antibodies	**0.5738**	100	57.38	≤ 28.5 IU/ml	0.745	0.0001
TSH receptor antibodies	hom-peptide antibodies	0.4462	92.31	52.31	> 3 IU/l	0.689	0.0014
Anti-TPO antibodies	hom-peptide antibodies	0.4077	50	90.77	≤ 12.8 IU/ml	0.756	0.0006
Anti-Htg antibodies	Antibodies againts both peptides	**0.5469**	100	54.69	≤ 28.5 IU/ml	0.736	0.0001
TSH receptor antibodies	Antibodies againts both peptides	**0.5700**	88.89	52.31	> 5.8 IU/l	0.709	0.0007
Anti-TPO antibodies	Antibodies againts both peptides	0.4457	75	69.57	≤ 44.2 IU/ml	0.747	0.0104

* AUC : area under the ROC curve

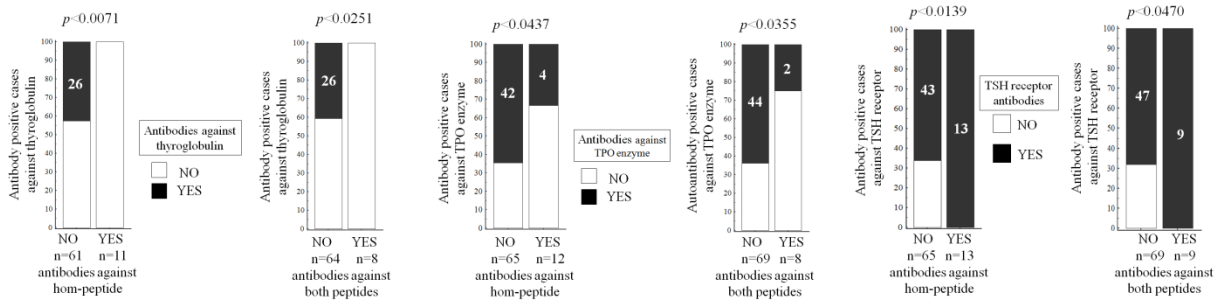

Figure 3: The cut-off values, Youden indices (sensitivity and specificity), AUC values and the significance levels using ROC (receiver operating characteristic) analysis, which was calculated from the positive cases of antibody levels against thyroglobulin (Tg), thyroid peroxidase (TPO) and TSH receptor (A) using crosstabs with the positive cases of antibodies againts hom-peptide and both peptides (B).

Next, hom- and cys-peptide antibodies were grouped according to the cut-off value given by ROC analysis (28.5 UI/ml for anti-Tg antibodies and 12.8 IU/ml for anti-TPO antibodies). These cut-off values were in the negative ranges of the detection assays. The cut-off values for the detection assays were as follows: 115 UI/ml for anti-Tg antibodies and 65 IU/ml for anti-TPO antibodies. The gradually increasing serum anti-Tg antibody levels demonstrated a borderline decrease in hom-peptide antibody levels between the subgroups of anti-Tg antibody levels below 28.5 IU/ml and above 115 IU/ml [100.96(77.25-113.17) vs 95.9(85.19-107.97), $p<0.0681$] (Figure 4). No relevant differences could be found in cys-peptide antibodies. The gradually elevating anti-TPO antibody levels demonstrated also a borderline decrease in hom- and cys-peptide antibody levels between the subgroups of anti-TPO antibody levels below 12.8 IU/ml and the range of 12.8-65 IU/ml [106.87(71.41-159.93) vs 96.26(81.88-113.17), $p<0.051$ for hom-peptide and 109.81(89.32-135) vs 104.2(91.91-118.13), $p<0.0886$ for cys-peptide antibodies; or the subgroup of anti-TPO antibody levels above 65 IU/ml [vs 103(87-121.96), $p<0.0335$ for cys-peptide antibodies]. Remarkably, serum FT_4 levels were in hyperthyroid range, but decreased regarding to the increased anti-Tg antibody levels. No such decrease in FT_4 levels could be seen regarding to increased anti-TPO antibody levels.

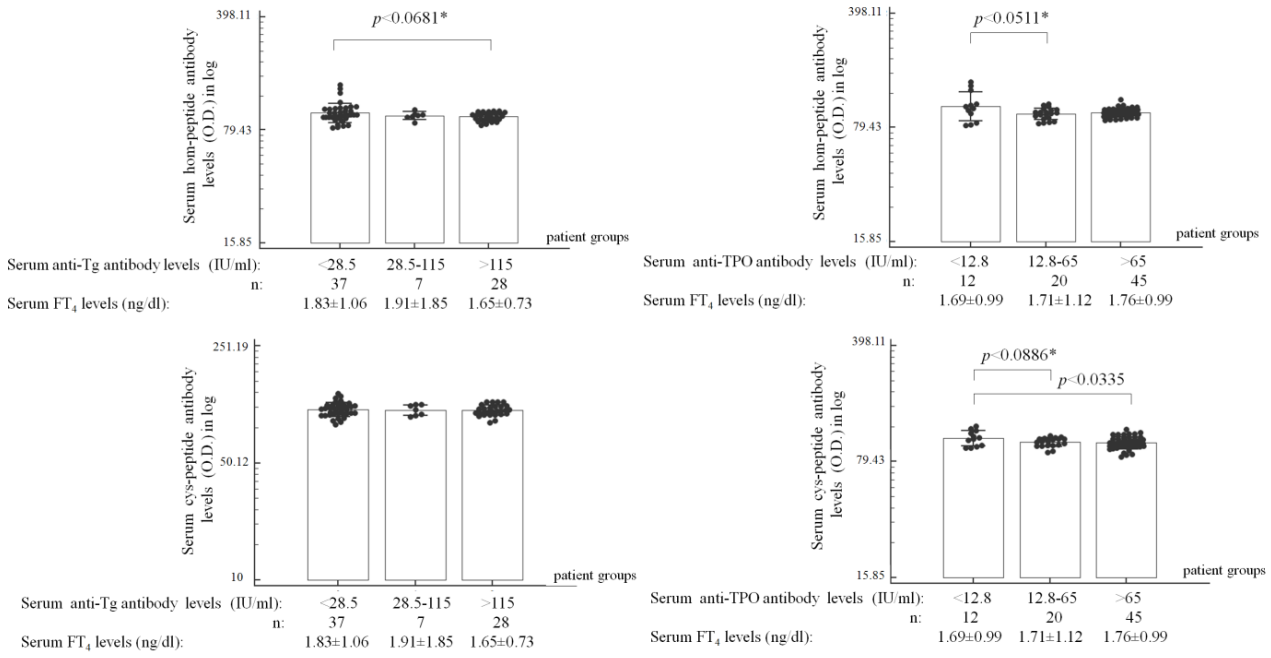

Figure 4: Changes in serum cys- and hom-peptide antibody levels according to the subgrouped anti-Tg and anti-TPO antibody levels with the coincidence of serum FT_4 levels.

The patients with TSH receptor antibody levels above 5.8 IU/l and who were negative for hom-peptide antibodies demonstrated relevantly increased anti-Tg and anti-TPO or TSH receptor antibody levels with decreased cys-peptide antibody levels compared to those who were positive for hom-peptide antibodies [77.44(3.81-1574.45) vs 21.21(16.65-27.02) IU/ml, $p<0.0268$ for anti-Tg antibodies and 195.32(14.13-2700.35) vs 41.27(2.55-668.28) IU/ml, $p<0.0121$ for anti-TPO antibodies; or 24.35(4.07-145.6) vs 11.8(4.97-28.05) IU/l, $p<0.0437$ for TSH receptor antibodies, and 102.78(90.99-116.11) vs 114.8(98.02-134.44), $p<0.0007$ for cys-peptide antibodies] (Figure 5). The appearance of hom-peptide antibodies was associated with the normal range of anti-Tg and anti-TPO antibodies, or with remarkably decreased titers of TSH receptor antibodies, particularly in the presence of antibodies against both peptides.

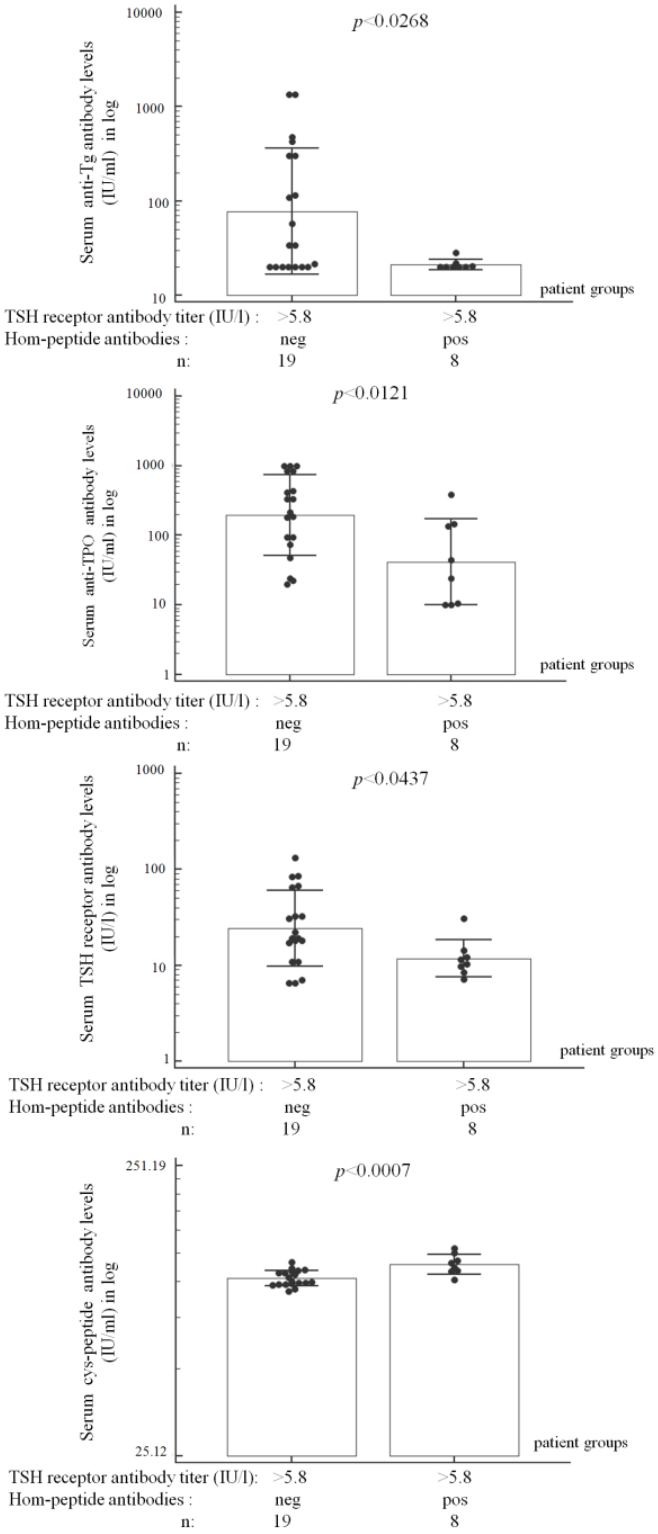

Figure 5: Changes in serum antibody levels against thyroglobulin (Tg), thyroid peroxidase (TPO), TSH receptor and cys-peptide with respect to the absence and the presence of hom-peptide antibodies. All patients had TSH receptor antibody levels above 5.8 IU/l.

The changes in the titers of TSH receptor antibodies were detailed in the flow chart with respect to the presence of cys- and hom-peptide antibodies (Figure 6). The high titers of TSH receptor antibodies were relevantly decreased when antibodies against both peptides were present and did not change remarkably in the presence of cys-peptide antibody positivity.

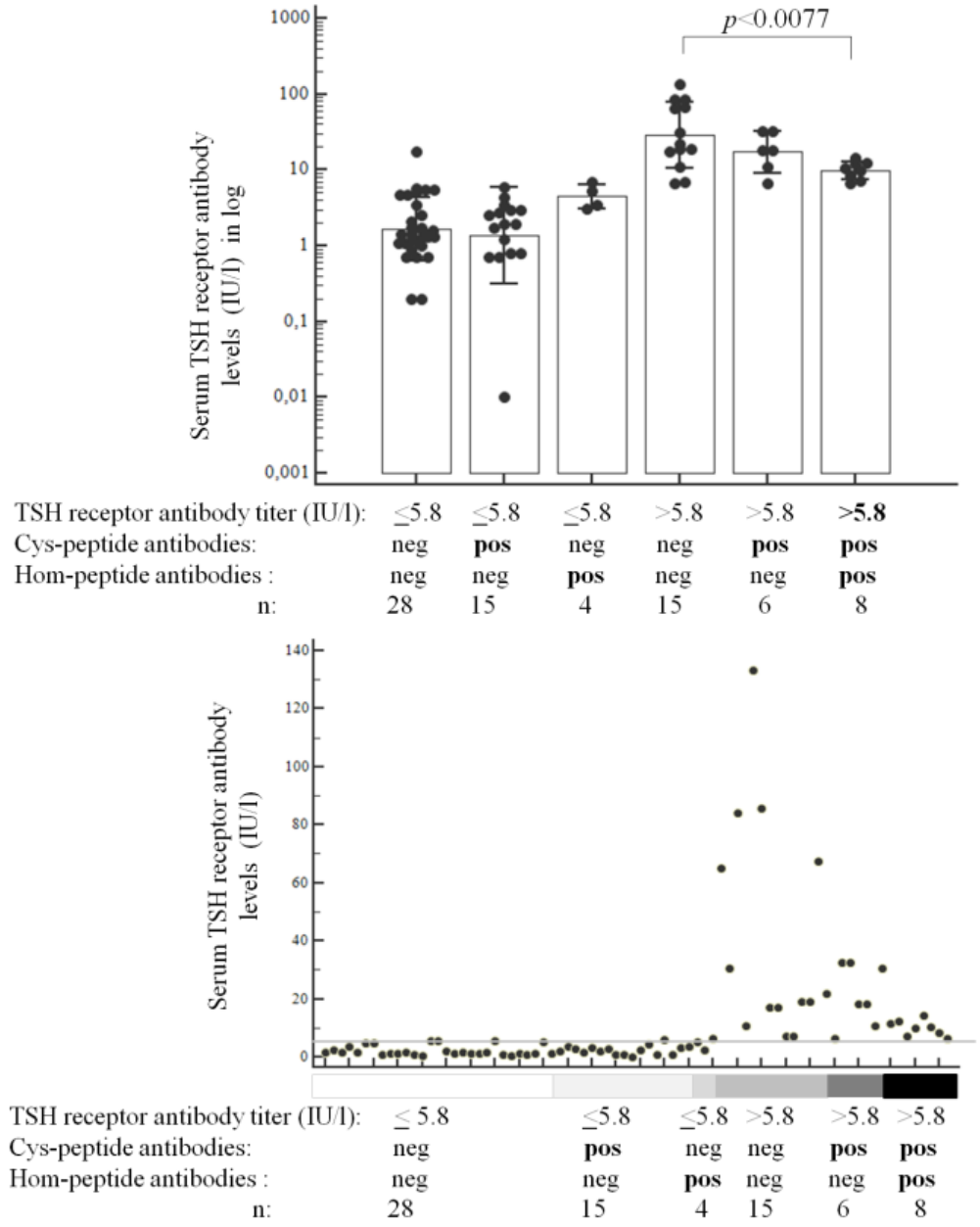

Figure 6: The alterations in TSH receptor antibody levels in the presence of cys- and/or hom-peptide antibody levels. The flow chart demonstrated the case values of TSH receptor antibodies influenced by the presence and absence of anti-peptide antibodies.

Discussion

The relationship between antibodies against peptides (cys- and hom-peptides) corresponding to DIO2 aa sequences and thyroid hormone levels has been investigated in these Graves' patients [7]. The presence of peptide antibodies was mainly associated with hyper- and euthyroidism. ATD treatment influenced their appearance differently associating mainly with PTU than with MMI. The presence of cys-peptide antibodies was increased in Graves' disease with the absence of ophthalmopathy and the higher values of the ratio of FT_3 to FT_4. Cys-peptide antibodies correlated inversely with serum FT_4 levels in all patients. MMI treatment decreased the serum FT_3 levels relevantly in Graves' ophthalmopathy.

In this new reevaluation, the relationship among antibodies against peptides, TPO, Tg and TSH receptor and the appearance of peptide antibodies with relation to the onset of disease were studied in the same patients with Graves' disease treated with ATDs. The role of synthetic peptides has been investigated in thyroid autoimmunity, where TSH receptor peptides were used for antigen-specific immunotherapy in Graves' hyperthyroidism [9]. In this preliminary study, the treatment with peptides (termed ATX-GD-59) was safe and well-tolerated resulting in reduced serum TSH receptor antibody levels in 70 % of patients with Graves' hyperthyroidism. An association between hom-peptide and anti-pituitary antibodies (APA) was demonstrated in thyroid autoimmunity [10]. Several mechanism is involved in the development of thyroid autoimmunity [11]. The loss of immune tolerance to thyroid antigens, such as TPO, Tg, TSH receptor and DIO2 can be the crucial. The major histocompatibility complex (MHC) with a complex of specific autoantigenic peptides induced T cell activation of T helper cells (Th) and regulatory lymphocytes (Treg) [12]. CD4+ T cells can differentiate into various types of Th and Treg cells under cytokine and cytotoxin effects. The binding of TSH receptor peptides, containing aa sequences of immunodominant and non-immunogenic T cell epitopes, was investigated to DR3 class II HLA molecule [13]. Most autoantigenic peptides showed intermedier or low binding affinity to class II HLA molecule. In Graves' disease, the study with cyclic peptides derived from B cell epitopes (loops of TSH receptor A domain) did not induce immune reaction, but decreased the thyroid size, the elevated FT_4 levels and the degree of retro-orbital fibrosis [14, 15]. The studies with synthetic peptides helped us to understand better the link between the immune and hormonal processes in thyroid autoimmunity and to reveal their therapeutical effectiveness in the management of diseases.

Cys- and hom-peptide antibodies demonstrated a strong linkage with the development of antibodies against TPO, Tg and TSH receptor, as well as with the onset and the course of Graves' disease. The new onset and the ratio of FT_3 to FT_4 above 0.41 were associated with greater frequency of cys-peptide antibodies. All patients who were

treated with PTU had cys-peptide antibodies. No dose-dependency could be demonstrated in the titers of peptide antibodies with respect to ATD therapy. Autoantibodies against hom-peptide showed a strong relationship with the autoantibody levels against Tg and TSH receptor. The appearance of antibodies against Tg was connected to the absence of hom-peptide antibodies. While the occurrence of hom-peptide autoantibodies was associated with relevantly smaller anti-TPO antibody positivities. The presence and the titers of TSH receptor antibodies demonstrated a close relationship with the presence of hom-peptide antibodies. A remarkable relationship could be demonstrated between hom-peptide and anti-Tg or anti-TPO antibodies in the cases who had increased TSH receptor antibody levels. Surprisingly, the titers of TSH receptor antibodies depended on the appearance of antibodies against hom-peptide or both peptides.

The exact cause of the generation of autoantibodies against DIO2 peptides is not clarified. They seem to be different from antineutrophyl cytoplasmic antibodies (ANCA), which can be associated with the side-effect of ATD [16]. The drug oxidation can be one of the factors playing as a hapten. MMI and PTU are thiourea-based compounds having the features to bind to DIO enzymes resulting in intermedier selenyl-iodide-DIO enzyme complex [17, 18]. Therefore, this complex is able to inhibit TPO enzyme activity, iodination and the coupling mechanism on the face of thyroglobulin. PTU is able to block directly DIO1 enzyme activity leading to increased DIO2 activity, which feature may explain the high levels of peptide antibodies under its therapy. Antibodies directing to enzyme can lead to a reduction of this enzyme activity mainly due to the steric hindrance [19]. The generation of immuno-proteasome may be involved in the facilitation of antigen presentation for class II MHC and T cell receptor [20, 21]. DIO2 is localized in the endoplasmic reticulum and degregated by the ubiquitin system [22, 23]. The processes, during abnormal conditions and defective clearance of apoptotic bodies, may direct to the development of autoantibodies.

Conclusion

Autoantibodies against enzymes (TPO and DIO2), TSH receptor and Tg, which are the participating factors for thyroid hormone synthesis, can be associated with Graves' hyperthyroidism. The generation of autoantibodies may depend on the alteration of thyroid hormone synthesis and the steric hindrance of the targets for autoantibodies, as well as on ATD treatment. Autoantibodies against DIO2 aa sequences can be produced in Graves' hyperthyroidism affecting the ratio of FT_3 to FT_4 and the titers of TSH receptor antibodies. Autoantibodies against DIO2 peptides can influence the therapeutical effectiveness.

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