Chapter

Deiodinase Enzymes and Their Activities in Graves' Hyperthyroidism

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Abstract

The origin of hyperthyroidism in Graves' Disease was displayed demonstrating the complexity of the processes. The role of stimulating TSH receptor antibodies is the one factor for the production of increased thyroidal T_3 and T_4. The T_3 and T_4 formation in colloid-embedded thyroglobulin and the activities of thyroidal deiodinases [type 1 (DIO1) and type 2 (DIO2)] play a crucial role in that. The findings of different authors were summarized with respect to highlighting the role of tissue-specific deiodinase activities. Apart from the results of experimental studies, the clinical results were brought to the front. The role of tissue-specific type 2 deiodinase activity was demonstrated according to thyroid function, the presence of autoantibodies against thyroid peroxidase (TPO), thyroglobulin (Tg) and TSH receptor. Autoantibodies against human eye muscle membrane and cytosol antigens had influencing effects on tissue-specific DIO2 activities, and the antieye muscle antibody immunoglobulin isotypes were associated with eye muscle enlargements. Antithyroid drug (ATD) therapy demonstrated relevant effects on tissue-specific DIO2 activities, which were manifested in the alterations of thyroid hormone levels. An asymptomatically appearance of autoantibodies against peptides corresponding to amino acid sequence of DIO2 was detected associating with thyroid hormone and anti-TPO, anti-Tg and TSH receptor antibody levels during the therapy.

Keywords: hyperthyroidism, Graves' Disease, type 1 and type 2 deiodinases, ophthalmopathy, autoantibodies

1. Introduction

Graves' hyperthyroidism is characterized by increased thyroid hormone levels (T_4 and T_3) with the supprimation of TSH levels, diffuse enlargement of thyroid glands and associated symptoms with orbitopathy or/and dermopathy [1, 2]. The course of disease is characterized by duality. The main autoimmune processes are manifested in thyrotoxicosis with a lymphocytic infiltration and diffuse thyroid enlargement, which can be associated with orbitopathy in 15–25% and pretibial myxedema in 0.5–4.5% [3]. The autoimmune processes are associated with the development of autoantibodies against different antigens, such as thyroid antigens [TSH receptor, thyroid peroxidase (TPO) enzyme and thyroglobulin (Tg)] and IGF-1 receptor, as well as against extraocular muscle membrane and cytosol antigens, and intracellular particles (flavoprotein subunit of mitochondrial succinate dehydrogenase, sarcalumenin, calsequestrin, collagen XII)] in thyroid-associated

ophthalmopathy [4–7]. The increased production of proinflammatory cytokines (IL-1, IL-6, TNFα), chemokines and costimulatory ligands on fibroblasts and adipocytes lead to inflammatory and infiltrating processes, and glycosaminoglycan (GAG) accumulation resulting in local tissue enlargements [8, 9]. In orbitopathy, the local infiltrating processes are responsible for the proptosis and sometimes the damage of nervi optici that can reach vision loss in the final stage. TSH receptor stimulating antibodies are kept to be the causative factors for hyperthyroidism. Autoantibodies against IGF-1 nearby receptor are involved in the edematous-infiltrative processes [10]. Antibodies against thyroid peroxidase (TPO) and thyroglobulin (Tg) are the relevant thyroid autoantibodies in Graves' Disease [11]. The binding of IgG and IgA autoantibodies to human extraocular muscle was different: IgG types bound endomysially, while IgA types bound to muscle fibers [12].

Deiodinase enzymes, DIO1, DIO2 and DIO3 are responsible for the conversion of T_4 to active T_3 hormone, the maintenance of the local T_3 levels and the inactivation of T_4 and T_3 hormones [13, 14]. Deiodinase enzymes show tissue-specific expression, which limits their functions. Many drugs, iodine and selenium supply, proinflammatory cytokines and autoantibodies can influence DIO activities [15, 16]. The increased T_4 levels are connected to the acceleration of the physiological degradation of DIO2 enzyme [17]. The common localization of DIO2 enzyme between thyroid and eye muscle tissues suggests that its autoantigenic role can be important in Graves' ophthalmopathy [18, 19]. 5′-deiodinase enzymes (DIO1 and DIO2) play a crucial role in thyroid hormone synthesis. TPO enzyme plays a role in the iodination of tyrosyl residues and their coupling to T_3 and T_4 in the colloid-embedded Tg with the interaction of hydrogen peroxide (H_2O_2) at the apical plasma membrane of thyrocytes [20]. The schematically illustrated process of thyroid hormone synthesis is exhibited in **Figure 1** highlighting the role of DIO1 and DIO2 activities.

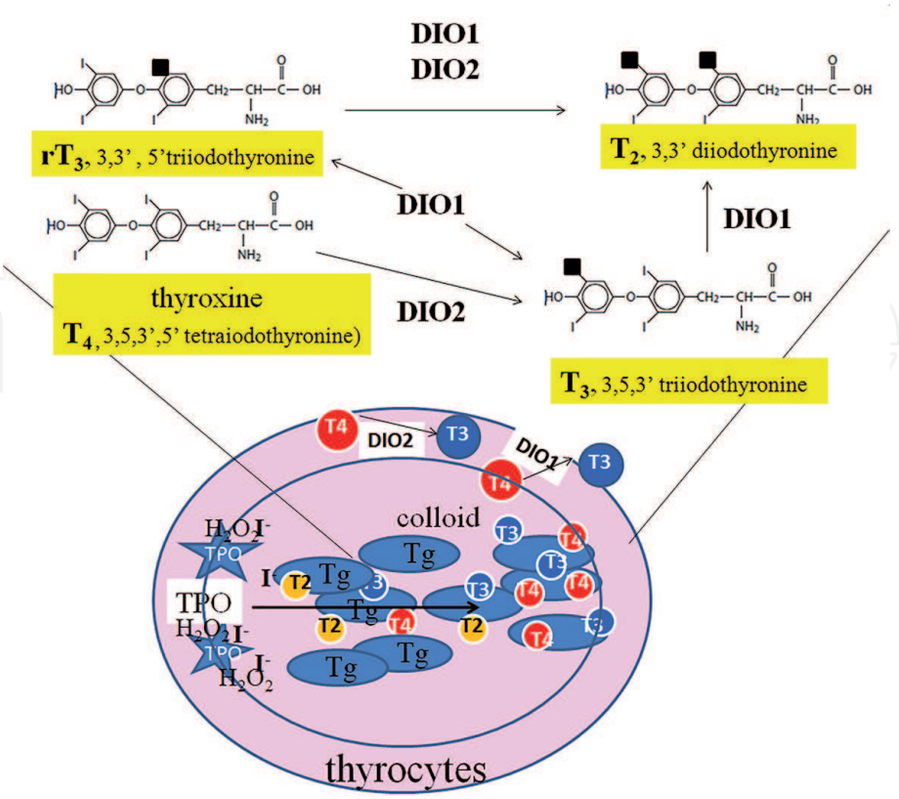

Figure 1.
Schematic illustration of thyroid hormone synthesis and thyroidal deiodinase activities (DIO1 and DIO2). DIO1: Type 1 deiodinase; DIO2: Type 2 deiodinase; Tg: Thyroglobulin; TPO: Thyroid peroxidase; T2, T3 and T4: Iodothyronines with 2, 3 and 4 iodides.

This review emphasizes the role of deiodinases in the hyperthyroidism of Graves' Disease with respect to the thyroid functional stages and the relationship with antithyroid autoantibodies and autoantibodies against extraocular muscle and peptides corresponding to amino acid sequence of DIO2, as well as with the antithyroid drug (ATD) therapies.

2. Three types of deiodinase enzymes are involved in thyroid hormone activation and inactivation

Three types of deiodinase enzymes (DIO1, DIO2, DIO3) are responsible for the activation and inactivation of thyroxine (T_4) and triiodothyronine (T_3) thyroid hormones [21]. Deiodinase enzymes demonstrate tissue-specific localization. DIO1 enzyme is expressed in the liver, kidney and thyroid parenchymal cells localized in the plasma membrane [22]. Its active center is found in the cytosol. T_4 plays as a prohormone for the active T_3 hormone. T_4 has four iodine bindings at the 3,3′, 5 and 5′ positions. DIO1 enzyme is able to cleave iodine from 5 (inner ring deiodination, step of T_4 inactivation) or 5′ position (outer ring deiodination, step of active T_3 hormone production). The dual effect of DIO1 enzyme plays a crucial role in the excessive thyroid hormone production, called hyperthyroidism. DIO2 enzyme is a widespread 5′-deiodinase expressed in thyroid, skeletal muscle and adipose tissues, hypothalamus, pituitary, skin, osteoblast, astroglia, retina, cochlea, placenta and endothelial cells localized in the endoplasmic reticulum [23]. Its active center is found in the cytosol. DIO1 expression can be induced transcriptionally by T_3 and TSH receptor stimulating antibodies. Fasting and chronic illnesses decrease DIO1 activity. The inhibitory effect on thyroidal DIO1 and DIO2 activities was demonstrated *in vitro* in the presence of proinflammatory cytokines (IL-1, IL-6 and TNFα) [24]. The inhibitory rate was higher on DIO2 than on DIO1 activities. The inhibitory degree was dose-dependent. Thyroidal DIO1 activity is responsible for only 6% of the daily T_3 production [25]. Propylthiouracil (PTU) inhibits its activity. DIO2 plays a crucial role in the maintenance of intracellular T_3 levels via 5′-deiodination, converting T_4 to T_3. Its increased activity is partly present in hyperthyroidism; however, its activity is decreased in nonthyroidal illness [26]. DIO2 is a posttranslationally T_4-dependent enzyme, which accelerates its proteasomal degradation. DIO2 enzyme is involved in the feedback mechanism of hypothalamic–pituitary-thyroid axis [27]. The normal development and regeneration of skeletal muscle requires DIO2 activity [28].

DIO3 is an enzyme located in the plasma membrane. It has both extra- and intracellular activity [29]. DIO3 plays a crucial role in fetal development and tissue-repair. It is expressed in placenta, uterus, neurons, skin, alveolar cells, glial cells, urothelium, gastrointestinal tract, hypothalamus and skeletal muscle [30]. DIO3 inactivates T_3 through inner ring 5-deiodination. Its increased activity is responsible for the consumptive hypothyroidism observated in hepatic hemangiomas [31]. Nether DIO2 nor DIO3 are PTU sensitive enzymes. Hypothyroidism is connected to an increase in DIO1 and DIO2 activities, but DIO3 activities are decreased [22]. Hyperthyroidism is connected to an increase in both thyroidal DIO1 and DIO2, but to a decrease in extrathyroidal DIO2 activities. Iopodic acid, the contrast material with high iodine content decreases the activities of all deiodinase enzymes. The alterations in T_4 and T_3 levels according to thyroid function have a different effect on the deiodinase enzyme activities in the living cells vs. sonicated cells [23]. No protein synthesis can happen in sonicated cells. Therefore, the sonicated cell content could be regarded as a deiodinase enzyme solution.

3. The role of DIO1 and DIO2 deiodinase enzymes in thyroid hormone production of Graves' hyperthyroidims

Hyperthyroidism is characterized by increased serum FT_4 and FT_3 levels, which can be associated with Graves' Disease, toxic goiter, destruction-induced thyrotoxicosis and subacute thyroiditis. Thyroid follicular cells possess both DIO1 and DIO2 enzymes, but not DIO3 enzyme. The amount of produced FT_4 and FT_3, and the ratio of FT_3 to FT_4 can help with the diagnosis [32]. Serum FT_3 levels are predominant and are better formed than FT_4 in hyperthyroidism connected to Graves' Disease or toxic goiter [33]. In Graves' hyperthyroidism, the increase in the daily production of T_3 and T_4 was 7-fold and 3.5-fold, respectively. Laurberg and coworkers demonstrated that the major source of excess T_3 derived from increased thyroidal DIO1 and DIO2 activities (in a ratio of 3 to 1). This is in contrast to what is found in euthyroidism, where 20% of T_3 came from thyroidal production and 80% from extrathyroidal deiodination [25]. In hyperthyroidism, a large part of T_3 levels was produced by the thyroid (in 57–77%) by way of converting T_4 to T_3 with decreased peripheral deiodination. The extrathyroidal DIO2 activities were decreased in hyperthyroidism with the exception of the thyroidal one due to the increased thyroidal formation of T_4 and T_3. Maia and coworkers supported that thyroidal DIO1 activity is responsible for 67% of T_3 production in hyperthyroidism [22]. In HEK 293 cells, which transiently expressed DIO1 and DIO2 enzymes, the effect of 2–20-200 pM T_4 was studied on these cells modeling hypo-, eu- and hyperthyroid states. DIO1 activity was continuous, but DIO2 activity was decreased by the concentration of 200 pM T_4. Salvatore and coworkers emphasized the greater role of DIO2 enzyme in the excess T_3 in Graves' hyperthyroidism [34]. Ito and coworkers suggested that thyroidal DIO1 and specifically, DIO2 could be contributed to the higher ratio of FT_3 to FT_4 in Graves' hyperthyroidism [35]. The lower ratio of T_3 to T_4 can help us with the diagnosis of destruction-induced thyrotoxicosis and subacute thyroiditis [36]. Values less than of 20 confirm the above mentioned diseases, while the values above 20 are connected to Graves' hyperthyroidism. Weetman and coworkers made the DIO1 and DIO2 activities responsible for the syndrome of low T_4 with increased T_3 levels during PTU treatment [37]. Thyroidal DIO1 activity is mainly regulated by cAMP at pretranslational levels, similarly to TSH receptor stimulating antibody-induced cAMP. Thyroglobulin and iodine contents of thyroid can influence the generation of T_4 and T_3 through the rate of hydrolysis from the colloid-embedded thyroglobulin. This condition can contribute to the alterations in the production of thyroid hormones. Very few reports could be found, which explained in detail the thyroid hormone production connecting to the formation of the coupling mechanism alone or together with deiodinase conversion. Iodide alone inhibited both thyroidal deiodinase activities rapidly decreasing the circulating T_3 by 50% and T_4 by 70% [33]. Ipodate is also a potent inhibitor for DIO1 and DIO2 enzymes due to its iodine content of 64%. Ipodate with PTU resulted in a profound decrease in serum T_3. In untreated Graves' hyperthyroidism, the T_3 content of Tg was 2-fold of what was found in euthyroidism [38]. In hyperthyroidism, local DIO2 activity is required for the intrapituitary production of T_3, which is responsible for the acute decrease in TSH levels [39].

4. *In vitro* model for the measurement of tissue-specific DIO2 activities

In our study, homogenized (supernatant of 100 000 x g separated by centrifugations) thyroidal, skeletal and eye muscle tissue fractions, called cytosol fractions were applied for the measurements of deiodinase enzyme activities [40]. Thyroid

tissues were obtained from the removal of euthyroid goiter; the removal of skeletal muscle during accident surgery and the removal of extraocular muscle during strabismus surgery. All tissue fractions contained DIO2 enzyme, the activity of which was measured in the presence of patient sera with Graves' Disease with respect to the different thyroid hormonal stages. The DIO2 content of cytosol fractions was proofed before the study using guinea pig sera immunized with TCSS and LVFR peptides. Both peptides were corresponding to amino acid sequences of human DIO2 (GenBank AAD45494–1) and contained the selenocysteine at position 133 in the active center of the enzyme: *LVVNFGSATCPPFTSQLPAFRKLVEEFSS*. TCSS peptide (aa 132–152): T**CC**PP**TF**SQLPAFRKLVEEFSS was synthesized with double cysteins replaced at position 133, as well as amino acids reserved at positions 136 and 137. LVFR peptide (aa 124–144): LVVNFGSAT**C**PPFTSQLPAFR was 100% identical to the original amino acid sequence. The bindings of immunized sera to cytosol fractions and to tissue sections were investigated with enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry, respectively. Immunized sera against TCSS peptide resulted in more intensive positive bindings to the cytosol fractions and gave positive reactions to tissue sections.

The patient sera of hyper-, eu- and hypothyroid Graves' Disease were added to thyroidal, skeletal and eye muscle cytosol fractions, which contained DIO2 enzyme activities. The study could be considered as an *in vitro* model, in which the patient sera included the actual hormonal and autoantibody parameters. The effects of these parameters were measured on DIO2 activities. The results, after evaluating them with respect to the parameters, may contribute to gaining useful data for the course and treatment of disease. Thyroidal DIO1 activities were inhibited by 2 μM PTU. The sample mixture contained 12.5 μg protein per cytosol fraction. Radioiodine labeled T_4 (^{125}I-T_4) 1 kB/50 μl was the substrate in reducing condition [20 mM dithiothreitol (DDT)]. The results were extrapolated at 1 pmol/T_4 of patient serum. DIO2 enzyme activity was expressed as pmol of T_4 converted per mg/min of protein. The whole protocol is described in detail in our previous paper [41].

5. Recent research with tissue-specific DIO2 activities in Graves' hyperthyroidims

5.1 Measurement and evaluation of DIO2 enzyme activities: DIO2 enzyme activities were measured after adding patient sera with Graves' ophthalmopathy to thyroidal, skeletal and eye muscle cytosol fractions, all containing DIO2 enzymes. The results were then evaluated according to thyroid functional stages

Fifty-two patients with Graves' Disease, of whom 37 had ophthalmopathy, were investigated [42]. The difference in the disease duration, the ratio of FT_3 to FT_4 and the serum levels of TSH receptor antibodies was significant between the Graves' patients with and without ophthalmopathy. The difference in DIO2 activities was relevant and constant among thyroidal, skeletal and eye muscle cytosol fractions in hyper-, eu- and hypothyroidism in Graves' ophthalmopathy. The effect of increased serum FT_4 levels was 1.9 times greater on eye muscle DIO2 than thyroidal DIO2 activity. The findings demonstrated that, the tissue-specific DIO2 activities also play a crucial role in the T_3 content of peripheral tissues in hyperthyroidism. The skeletal muscle and thyroidal DIO2 activities were lower by 27% and 47%, respectively in hyperthyroidism, as well as were lower by 27% and 87%, respectively in hypothyroidism compared to eye muscle DIO2 activity. DIO2 activities of all

cytosol fractions were 6.3 times lower in hyperthyroidism and 3.5 times greater in hypothyroidism compared to those in euthyroidism (**Figure 2**). In hyperthyroidism, the thyroidal DIO2 activity was better inhibited than that of peripheral tissues. In hypothyroidism, increased thyroidal DIO2 activity could be found together with increased peripheral tissue DIO2 activities.

The effects of FT_3 hyperthyroidism were identical on DIO2 activities in all cytosol fractions, but their activities were 2 times higher in euthyroidism compared to those found by increased FT_4 levels. No increase in any DIO2 activities could be detected with respect to FT_3 levels in hypothyroidism compared to those in euthyroidism. The decrease in all DIO2 activities was the consequence of the increased FT_4 levels, which demonstrated a substrate-mediated inhibitory effect in hyperthyroidism. Note, however, that the inhibitory effect of proinflammatory cytokines (IL-6, IL-1 and TNFα) and the therapy cannot be excluded in some cases. Our previous study confirmed the role of IL-6 in Graves' ophthalmopathy with active eye signs [43]. The presence of inflammatory orbital events and a longer manifestation of ophthalmopathy were associated with increased serum IL-6 levels. Therefore, the autoimmune features of Graves' Disease can modify DIO2 activities. The increased DIO2 activities in all cytosol fractions in FT_4 hypothyroidism could be explained by the concomitantly increased serum levels of TSH receptor antibodies compared to those in FT_3 hypothyroidism. Nevertheless, serum TSH levels were not suppressed by increased serum FT_4 levels, which could be explained by the pituitary resistance to T_4 [44]. No similar results could be demonstrated for increased T_3 levels. Contrary to FT_4 hypothyroidism, the lack of increased DIO2 activities in FT_3 hypothyroidism support that in this condition the active protein synthesis of DIO2 enzyme is needed for increasing their activities. The partly increased skeletal and eye muscle DIO2 activities in both FT_4 and FT_3 hyperthyroidism excluded a relevant inactivating role of DIO3 in muscle cytosol fractions.

DIO2 activities in all cytosol fractions were significantly lower in Graves' ophthalmopathy with increased serum FT_3 levels keeping the proportional discrepancies constantly among thyroidal, skeletal and eye muscle DIO2 activities. However, Graves' sera without ophthalmopathy resulted in a 5-fold increase in all

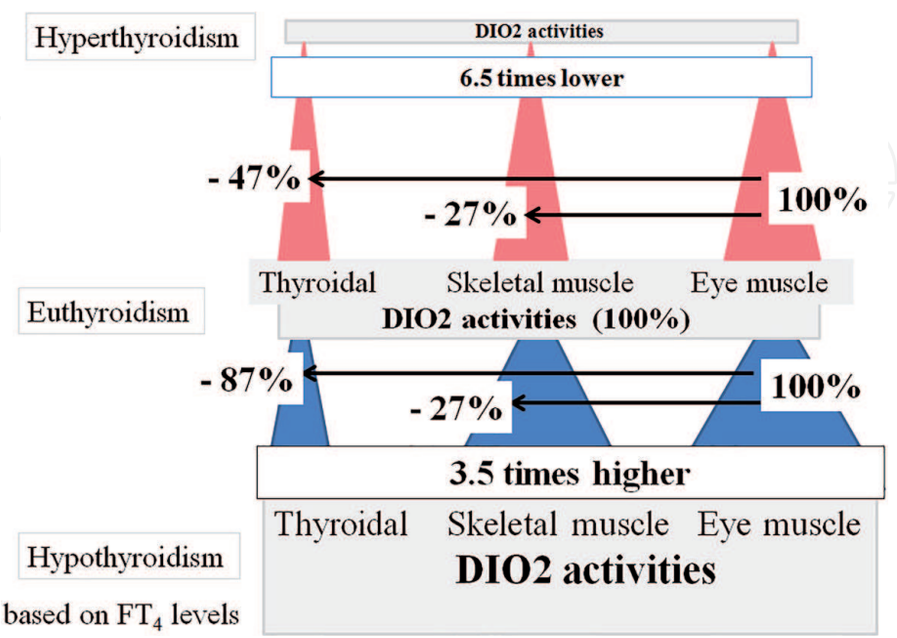

Figure 2.
The effect of patient sera with Graves' Disease on thyroidal, skeletal and eye muscle DIO2 activities with respect to thyroid functional stages based on FT_4 levels. DIO2: Type 2 deiodinase.

DIO2 activities still keeping the proportional discrepancies constant among thyroidal, skeletal and eye muscle DIO2 activities. The elevation in TSH receptor antibody levels did not associated with DIO2 elevation, but it did with TSH suppression and with a lower ratio of FT_3 to FT_4. Note that due to the small number of patients without ophthalmopathy, the conclusions can be limited.

5.2 Effects of antithyroid and antiextraocular muscle autoantibodies on thyroidal, skeletal and eye muscle DIO2 activities

5.2.1 Thyroidal, skeletal and eye muscle DIO2 activities with respect to anti-TPO and TSH receptor autoantibodies in FT_3 hyperthyroid Graves' Disease

The effects of autoantibodies against thyroid peroxidase (TPO) and TSH receptor were investigated on thyroidal, skeletal and eye muscle DIO2 activities in FT_3 hyperthyroid Graves' Disease. A greater number of patients with ophthalmopathy (n = 11) demonstrated anti-TPO antibodies than those without (n = 4) [42]. Anti-TPO antibody positive patients without ophthalmopathy exhibited 5 times greater DIO2 activities in thyroidal and skeletal muscle cytosol fractions, and even 12 times greater in eye muscle cytosol fraction compared to those with ophthalmopathy (**Figure 3**). DIO2 activities were compared between anti-TPO antibody positive and negative patients. The difference in all DIO2 activities were significant between the patients with and without ophthalmopathy in FT_3 hyperthyroid Graves' Disease, as well as between anti-TPO antibody negative and positive patients. DIO2 activities increased 17 times in patients without ophthalmopathy, but decreased by 39% in patients with ophthalmopathy in the presence of anti-TPO antibodies compared to those who were negative for these autoantibodies (**Figure 4**). The alterations could be explained by the greater increased serum FT_4 levels in anti-TPO antibody positive patients with Graves' ophthalmopathy in contrast to the patients without ophthalmopathy. The patients without ophthalmopathy showed reduced FT_4 levels, which were below the normal range, concomitantly with the elevated serum TSH levels. These result are limited by the small patient number of Graves' Disease without ophthalmopathy.

In FT_3 hyperthyroidism, TSH receptor antibody positivity was greater in Graves' ophthalmopathy (n = 11, and 9 out of 11 cases were anti-TPO antibody positive)

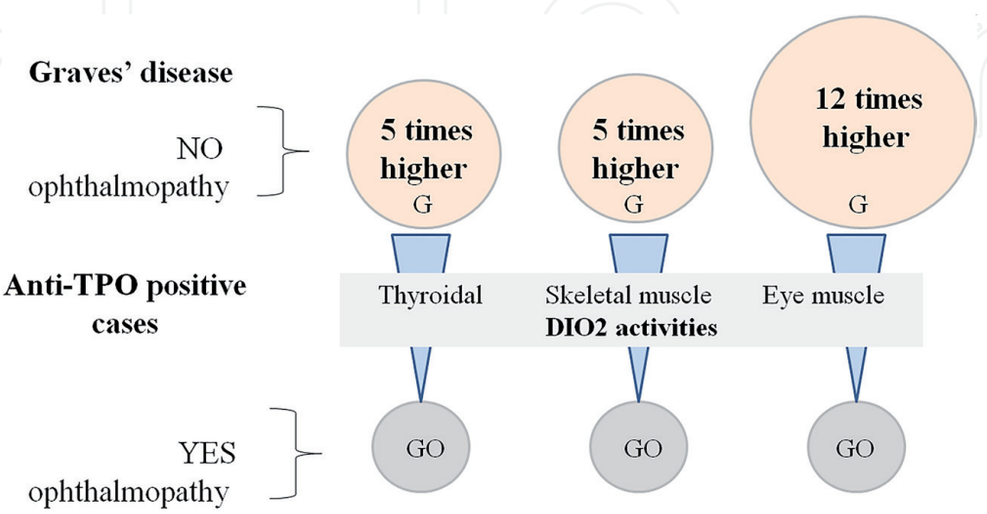

Figure 3.
The effect of patient sera with Graves' Disease on thyroidal, skeletal and eye muscle DIO2 activities in anti-TPO antibody positive patients between with (GO) and without (G) ophthalmopathy. DIO2: Type 2 deiodinase; TPO: Thyroid peroxidase.

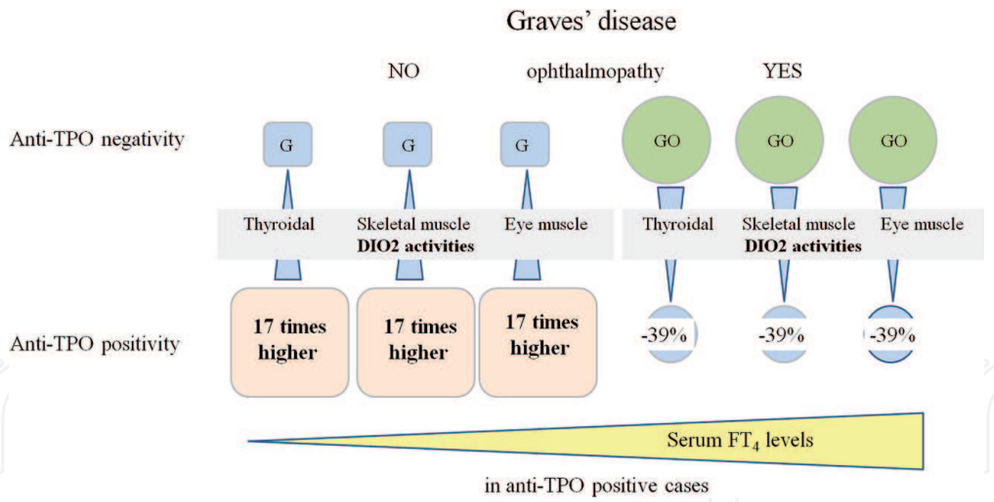

Figure 4.
The effect of patient sera with Graves' Disease on thyroidal, skeletal and eye muscle DIO2 activities between anti-TPO antibody negative and positive patients with (GO) and without (G) ophthalmopathy. DIO2: Type 2 deiodinase; TPO: Thyroid peroxidase.

than in those who had no ophthalmopathy (n = 2) [42]. DIO2 activities were significantly increased in all cytosol fractions (increased by 3.6 times) for TSH receptor antibody positive patients compared to TSH receptor antibody negative patients with Graves' ophthalmopathy, but the opposite was true for patients without ophthalmopathy (**Figure 5**). Surprisingly, in the absence of ophthalmopathy, TSH receptor antibody positive patients demonstrated relevantly decreased DIO2 activities, which were 10 times lower than those found in TSH receptor antibody negative patients in all cytosol fractions, concomitantly with the increased serum TSH levels.

5.2.2 The effects of IgG and IgM isotype antieye muscle cytosol and membrane autoantibodies on eye muscle DIO2 activity in Graves' ophthalmopathy

Next, the effects of antieye muscle cytosol and membrane autoantibodies on eye muscle DIO2 activity were examined in Graves' ophthalmopathy. Before the study, the binding reactivity of sera to human eye muscle membrane and cytosol antigens in tissue sections was controlled. In our previous results using immunohistochemistry and immunoblotting methods, antibodies against TCSS peptide, corresponding to amino acid sequence of DIO2 and eye muscle cytosol or membrane antigens (supernatant or pellet fractions of 100 000 x g, separated by centrifugations) were demonstrated, which gave intensive binding reactions to human thyroid, skeletal and eye muscle tissue sections [40].

The binding of guinea pig sera immunized by TCSS peptide could be inhibited by patient sera added in advance, which sera gave positive reactions to eye muscle tissue sections. Toyoda and coworkers investigated the DIO1 enzyme activity in FRTL-5 rat thyroid cells in the presence of IgG type immunoglobulins derived from untreated hyperthyroid Graves' patients and controls [45]. They demonstrated a relevant increased DIO1 activity, which could be completely abolished by the addition of cycloheximide.

Based on the previously mentioned results we wanted to measure the effects of antieye muscle cytosol and membrane autoantibodies on eye muscle DIO2 activity, as well as to compare DIO2 activity to eye muscle enlargements. The hypothesis was, that antieye muscle autoantibodies may affect DIO2 activity, which can lead to eye muscle enlargement. We investigated the role of antieye muscle antibodies in Graves' Disease [46]. In turn, the appearance of these autoantibodies was not

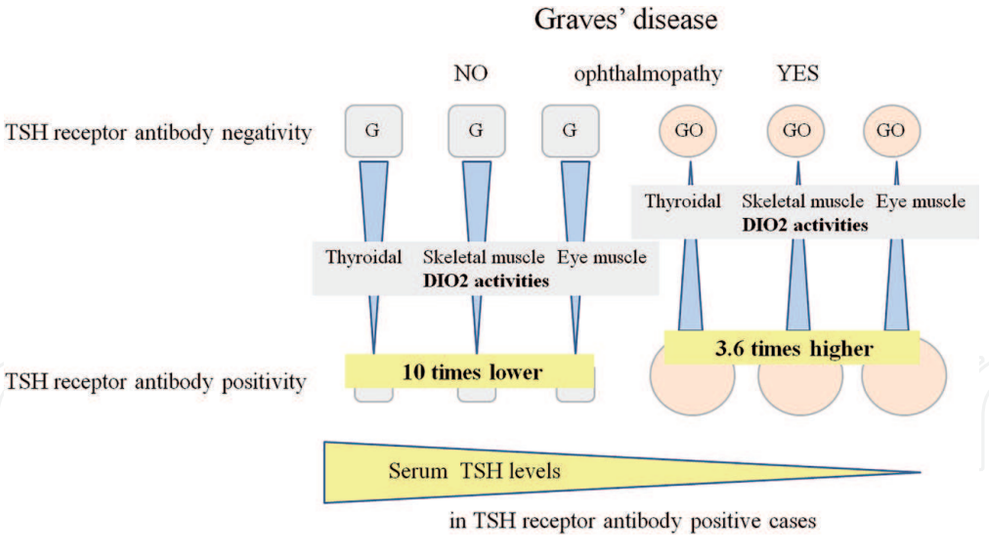

Figure 5.
The effect of patient sera with Graves' Disease on thyroidal, skeletal and eye muscle DIO2 activities between TSH receptor antibody negative and positive patients with (GO) and without (G) ophthalmopathy. DIO2: Type 2 deiodinase.

only connected to ophthalmopathy, but they could also be found in a small part of patients without ophthalmopathy. IgG, IgA and IgM isotype antieye muscle membrane and cytosol autoantibodies were measured with enzyme-linked immunosorbent assay (ELISA) in 32 patients with hyperthyroid Graves' Disease, of whom 20 cases had ophthalmopathy. None of IgA isotype autoantibodies could be detected against membrane and cytosol antigens in any of the patients. A greater number of patients with ophthalmopathy demonstrated IgM (n = 10) and IgG (n = 5) antieye muscle autoantibodies than those without ophthalmopathy, of whom 3 cases had IgG type and 3 cases had IgM type autoantibodies. Surprisingly, the addition of serum containing IgG isotype antieye membrane (EyeM) or cytosol (EyeC) autoantibodies resulted in 6.4 times or 3.9 times increased eye muscle DIO2 activity, respectively compared to those found with IgG negative sera (**Figure 6**). Conversely, the effect of IgM type antieye muscle membrane or cytosol autoantibodies was associated with 3 times or 1.9 times lower eye muscle DIO2 activity, respectively. The presence of IgG type anti-EyeC autoantibodies resulted in 1.5 times greater eye muscle DIO2 activity than anti-EyeM autoantibodies. A similar increase in DIO2 activity could be demonstrated in the presence of IgM type anti-EyeC autoantibodies compared to those with anti-EyeM autoantibodies. In this instance, the increase in eye muscle DIO2 activitiy was 2 times greater. Furthermore, the increase in eye muscle DIO2 activity was 7 times and 5 times higher in the presence of IgG type anti-EyeM and anti-EyeC autoantibodies compared to those in the presence of IgM type anti-EyeM and anti-EyeC autoantibodies, respectively. Eye muscle DIO2 activities strongly correlated with IgG type anti-EyeM and anti-EyeC autoantibody levels. It seems, the autoantibody binding to eye membrane could mediate a signal towards the cytosolic DIO2 enzyme. The findings between eye muscle DIO2 activity and eye muscle enlargement suggest this idea. IgG type anti-EyeM autoantibodies were associated with increased eye muscle enlargement, although the difference was not significant. However, IgM type anti EyeM autoantibodies were associated with a significant decrease in eye muscle enlargement. The fact that IgM type anti-EyeM autoantibodies could play a role in DIO3 activity, could not be excluded. The eye muscle DIO2 activity was more greater in patients with the absence of ophthalmopathy compared to those in the presence of that. Our results suggest that autoantibodies against eye muscle

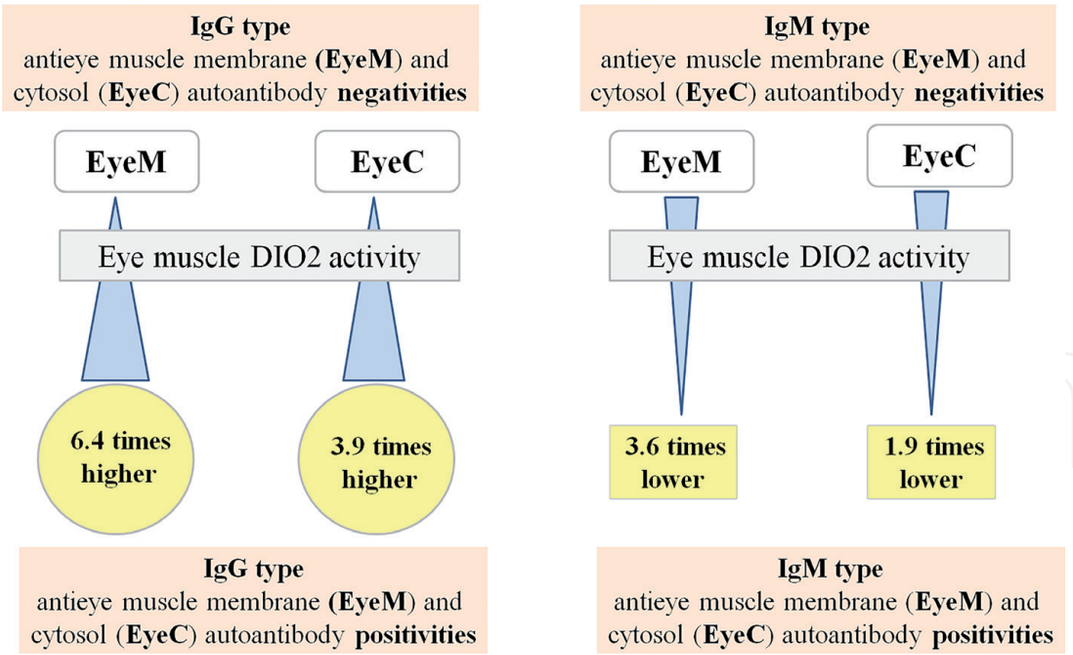

Figure 6.
The effect of patient sera containing IgG and IgM type antieye muscle membrane (EyeM) and cytosol (EyeC) autoantibodies on eye muscle DIO2 activity in Graves' ophthalmopathy. DIO2: Type 2 deiodinase.

antigens have a role in the development of ophthalmopathy through the eye muscle enlargements. The limitation of this study was the small patient number containing IgM isotype anti-EyeM and IgG isotype anti-EyeC antibodies. Another limitation could be the measurement method of the eye muscle enlargements, which was done using ultrasound in the absence of CT or MRI possibilities.

5.3 The effect of antithyroid drugs on tissue-specific DIO2 activities, as well as their role in the induction of autoantibodies against DIO2 peptides

5.3.1 The effect of antithyroid drugs on thyroidal, skeletal and eye muscle DIO2 activities

Antithyroid drugs (ATD) are used very often in the therapy of Graves' hyperthyroidism. Methimazole (MMI) and propylthiouracil (PTU) are the medicines used to block the synthesis of thyroid hormones in Hungary. ATDs are thioamide derivates with the binding reaction to DIO1 enzyme forming an intermediary selenyl-iodide-DIO1 enzyme complex (presumably the same is true for DIO2 also). In addition, they inhibit the activity of TPO enzyme due to the impairment of H_2O_2 generation and the coupling of iodotyrosines. MMI may be a selective DIO1 blocker and inhibits thyroidal H_2O_2 generation. However, MMI has no remarkable effect on DIO2 activity. PTU is a very strong inhibitor for DIO1 activity. None of the patients were treated with PTU in the tissue-specific DIO2 activity study.

The difference in thyroidal DIO2 activities was significant between those with and without ophthalmopathy in FT_3 hyperthyroidism who did not undergo MMI therapy [42]. MMI therapy was associated with a greater increase in thyroidal, skeletal and eye muscle DIO2 activities in both patients without and with ophthalmopathy (the increase was 17 times and 4 times higher, respectively) compared to the increases in patients who were not treated with MMI. MMI therapy was associated with greater TSH levels and greater ratio of FT_3 to FT_4 in patients without ophthalmopathy, and greater TSH receptor antibody levels in patients with ophthalmopathy (**Figure 7**).

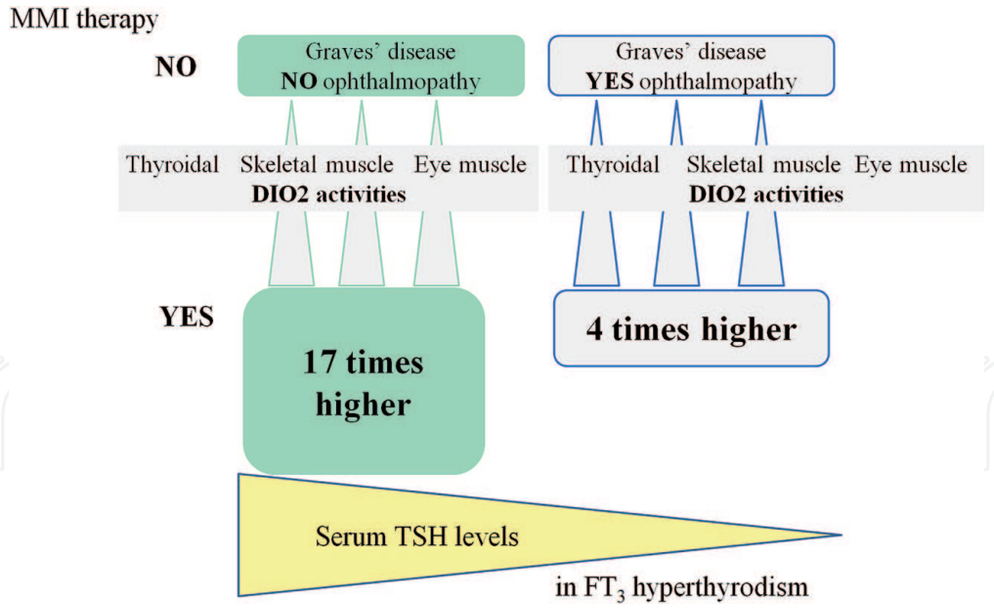

Figure 7.
The effect of patient sera who were treated with methimazole (MMI) on thyroidal, skeletal and eye muscle DIO2 activities in hyperthyroid Graves' Disease with and without ophthalmopathy. DIO2: Type 2 deiodinase.

5.3.2 Occurrence of antibodies against peptides corresponding to amino acid sequence of DIO2 during antithyroid drug therapy and the efficacy of therapy

In another study, the occurrence of autoantibodies against DIO2 peptides, such as TCSS (cyspeptide) and LVFR (hompeptide) peptides were investigated in 78 patients with hyperthyroid Graves' Disease [47]. The relationships were examinated among ATD therapies, antibodies against TPO, thyroglobulin (Tg) and TSH receptor, as well as thyroid hormone levels. The appearance of autoantibodies against cys-, hompeptide or both peptides could be detected in 24, 4 or 9 cases, respectively, in Graves' Disease. The appearance of these autoantibodies was not associated with the clinical signs of urticaria or ANCA-associated vasculitis. These anticys- and antihompeptide antibodies could be demonstrated in 12 and 3 cases in hyperthyroidism, and in 10 and 1 cases in euthyroidism. A significant difference was found in the occurrence of anticyspeptide antibodies between PTU (n = 3 out of 3 cases) and MMI (n = 13 out of 42 cases) therapies. The frequency of antipeptide antibodies was smaller in Graves' ophthalmopathy (9 cases for anticyspeptide antibodies and 1 for antihompeptide antibodies). The exact mechanism is not clear, but ATDs are thioamide drugs with the binding feature to DIO and TPO enzymes blocking the T_4 conversion to T_3, and the iodination with the phenolic coupling of iodothyrosine residues. Their higher binding features are connected to their greater reactivity with free radicals. Not only the asymptomatic occurrence of autoantibodies against cys- and/or hompeptide was surprising in hyperthyroid Graves' Disease, but also their strong relationship with decreasing anti-TPO and increasing TSH receptor antibody levels (**Figure 8**). In hyperthyroidism, two antipeptide antibodies possessed distinct features with relation to the occurrence of anti-TPO, anti-Tg and TSH receptor antibody levels, as well as to the thyroid hormone levels and the ratio of FT_3 to FT_4. Antibodies against cyspeptide were rather stimulating: Positive correlation could be demonstrated between anticyspeptide antibodies and serum FT_4 levels; the ratio of FT_3 to FT_4 was increased when those antibodies were present compared to when they were absent. In Graves' ophthalmopathy, the serum FT_4 and FT_3 levels were lower in the presence of antibodies against cyspeptide compared to when those antibodies were absent. The ratio of FT_3 to FT_4 was increased in patients

without ophthalmopathy compared to those when it was present (**Figure 9**). Antibodies against hompeptide and both peptides were rather inhibiting: anti-TPO and anti-Htg antibodies levels were reduced in their presences compared to when they were absent. In hyperthyroid Graves' ophthalmopathy, antibodies against both peptides were associated with reduced antibody levels against TPO and Tg, but with increased TSH receptor antibody levels, particularly when the clinical activity score (CAS) was above 4. In FT_4 hyperthyroidism, MMI treated Graves' patients without ophthalmopathy, demonstrated significantly increased FT_3 to FT_4 ratio with the occurrence of anticyspeptide autoantibodies.

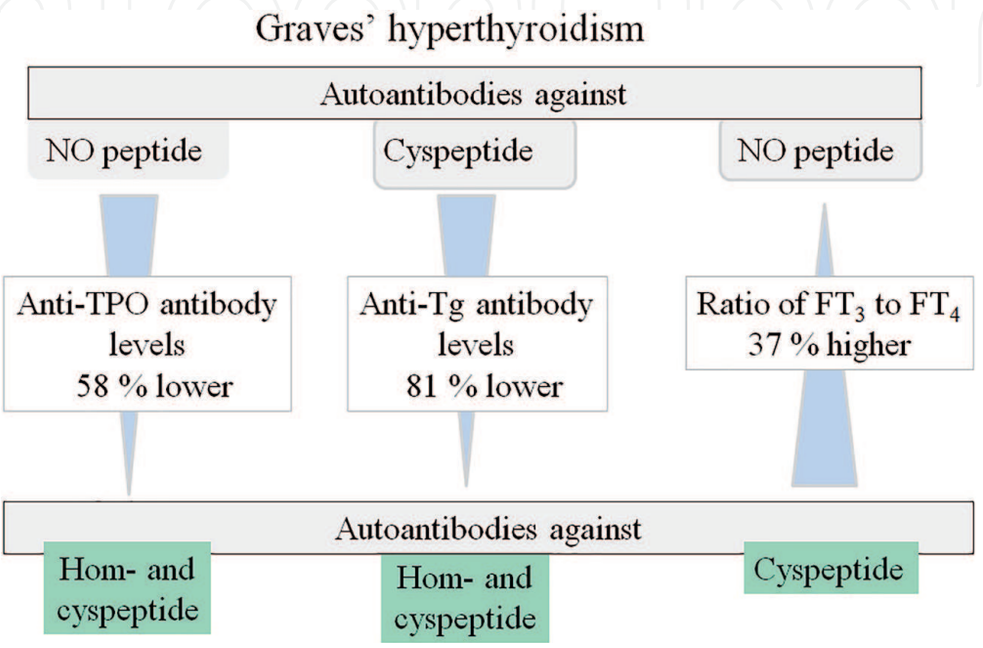

Figure 8.
The effect of patient sera containing autoantibodies against peptides (hom – and/or cyspeptide) corresponding to amino acid sequence of DIO2 on the levels of anti-TPO and anti-Tg autoantibodies, as well as on the ratio of FT_3 to FT_4 in hyperthyroid Graves' Disease. DIO2: Type 2 deiodinase; TPO: Thyroid peroxidase; Tg: Thyroglobulin.

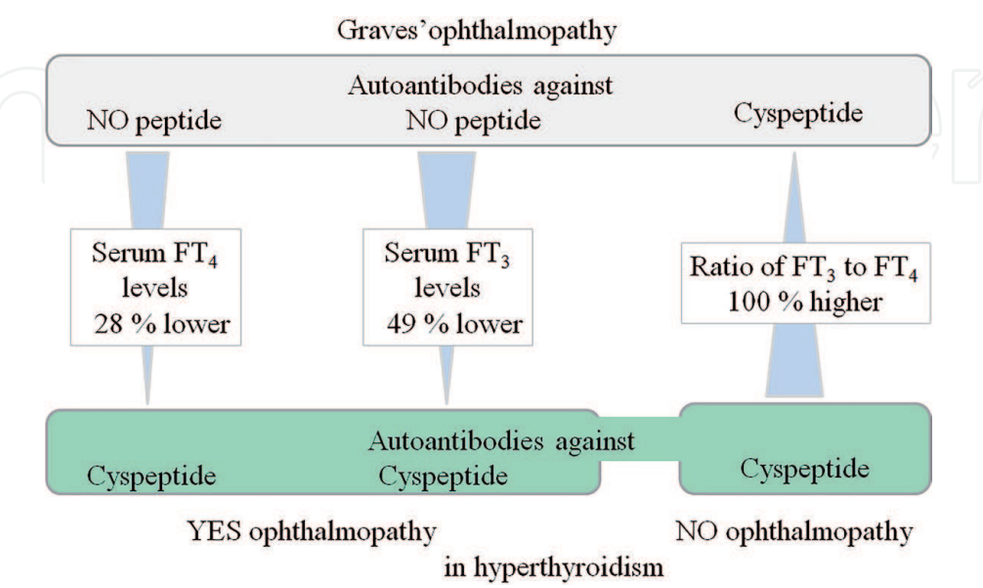

Figure 9.
The effect of patient sera containing autoantibodies against cyspeptide corresponding to amino acid sequence of DIO2 on serum FT_4 and FT_3 levels, as well as on the ratio of FT_3 to FT_4 in hyperthyroid Graves' ophthalmopathy and between the presence and absence of ophthalmopathy. DIO2: Type 2 deiodinase.

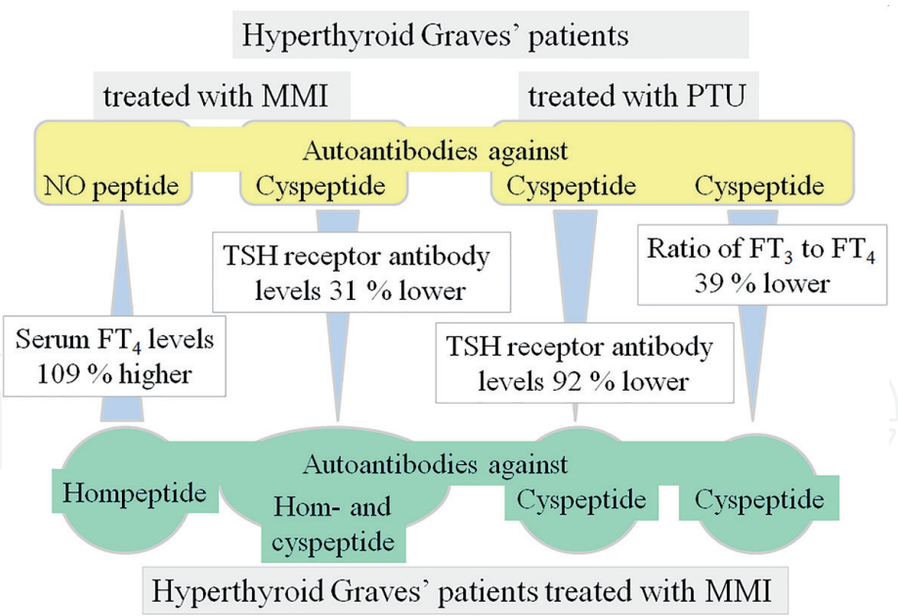

Figure 10.
The effect of patient sera containing autoantibodies against peptides (hom- and/or cyspeptide) corresponding to DIO2 amino acid sequence on serum FT_4 and TSH receptor antibody levels, as well as on the ratio of FT_3 to FT_4 in hyperthyroid Graves' Disease treated with methimazole (MMI) and propylthiouracil (PTU). DIO2: Type 2 deiodinase.

The results in thyroid hormone levels supported that the presence of antipeptide antibodies before the treatment and their absence during the treatment with MMI may contribute to the worsening of orbital processes in hyperthyroid Graves' ophthalmopathy. Antibodies against DIO2 peptides may influence the therapeutic efficacy during the treatment (**Figure 10**). In MMI treatment, the presence of antibodies against hompeptide only was connected to increased serum FT_4 levels, but when autoantibodies against both hom- and cyspeptide were present, it resulted in a relevant decrease in TSH receptor antibody levels. MMI treatment demonstrated lower TSH receptor antibody levels and lower ratio of FT_3 to FT_4 in the appearance of anticyspeptide autoantibodies compared to those treated with PTU. The exact role of antipeptide antibodies and their relationship with antithyroid autoantibodies, as well as the possibility of the occurrence of autoantibodies against other amino acid sequence of the whole DIO2 protein need futher investigations.

6. Conclusions

In hyperthyroid Graves' Disease the thyroid hormone excess is dominantly T_3. The thyroidal production of T_3 and T_4 excess can derive from the thyroidal T_4 and T_3 formation in the colloid-embedded Tg mediated by thyroidal TPO, and the additional production of T_3 due to deiodinase enzymes mediated conversion from T_4 resulting in the ratio of 3 to 1 for DIO1 and DIO2 activities in the cytosol, respectively. The results using *in vitro* model for the study of tissue-specific DIO2 activities confirmed the dominance of thyroidal DIO1 activity, but the thyroidal DIO2 activity seemed to be more reduced compared to skeletal and eye muscle DIO2 activities. The degree of DIO2 activities was tissue-specific, but the extent of their decreases in hyperthyroidism and increases in hypothyroidism was identical. The findings highlighted that the increase in tissue-specific DIO2 activity needed an active protein synthesis only in FT_3, but not in FT_4 hypothyroidism. The appearance of anti-TPO, TSH receptor, and antieye muscle membrane and cytosol autoantibodies modified the tissue-specific DIO2 activities, which manifested in both

increased and decreased serum TSH levels and sometimes in eye muscle enlargements. Besides the effect of ATDs on tissue-specific DIO2 activities, autoantibodies against peptides corresponding to amino acid sequences of DIO2 also appeared asymptomatically in Graves' Disease. Furthermore, they were also detectable before ATD therapy, and the therapy increased their occurrences. The antipeptide autoantibodies were associated with alterations in serum FT_4 and FT_3 levels, as well as in the levels of autoantibodies against TPO, Tg and TSH receptor. In Graves' ophthalmopathy, the tissue-specific DIO2 activities were much more reduced and they were connected to a lack of appearance of antipeptide autoantibodies. The occurrence of anticyspeptide autoantibodies was associated with lower serum FT_4 and FT_3 levels compared to those in patients who were negative for these autoantibodies. Although, autoantibodies could be demonstrated against eye muscle cytosol antigens, which inhibited the binding of antipeptide antibodies derived from guinea pig immunization to eye muscle in immunohistochemical studies, these antieye muscle and antipeptide autoantibodies had no pathognomonic role in Graves' ophthalmopathy. These findings above explain why the duality of features causes a greater complexity of hyperthyroidism in Graves' Disease.

Conflict of interest

The author declares no conflict of interest.

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