Graves' disease: latest understanding of pathogenesis and treatment options

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Abstract

Graves' disease (GD) is the most common cause of hyperthyroidism in iodine-sufficient areas. The main responsible mechanism is related to autoantibodies that bind and activate the thyrotropin receptor (TSH-R). Although Graves' hyperthyroidism (GH) is relatively common, no causal treatment options are available. Established treatment modalities are anti-thyroid drugs (ATD), which reduce thyroid hormone synthesis, radioactive iodine (RAI), and surgery. However, emerging drugs that target the main autoantigen (monoclonal antibodies, small molecules, peptides) or block the immune pathway have been recently tested in clinical trials. GD can involve the thyroid exclusively, or it can be associated with extrathyroidal manifestations, among which Graves' orbitopathy (GO) is the most common. The presence of GO can change the management of patients. An established treatment for moderate-to-severe GO is intravenous glucocorticoids. However, recent advances in understanding GO pathogenesis have allowed the development of new target-based therapies, by blocking proinflammatory cytokine receptors, lymphocytic infiltration, or the insulin-like growth factor receptor (IGF-1R), with several clinical trials providing promising results. This article aims to review the new discoveries in the pathogenesis of GH and GO that are offering several important tools in patient management.

Introduction

Graves' Disease (GD) is an autoimmune disease characterized by an enlarged and overactive thyroid gland[1]. The main mechanism responsible for Graves' hyperthyroidism (GH) is the activation of the thyroid-stimulating hormone receptor (TSH-R) by autoantibodies that act as agonists, causing thyrocyte proliferation and hyperfunction[1,2]. Genetic and environmental factors contribute to loss of immune self-tolerance, ultimately triggering the pathogenic process [1,3,4]. New insights into GD have been provided, including the role of microbiota and polymorphisms of elements involved in immune checkpoints and immune response, namely protein tyrosine phosphatase non-receptor type 22 (PTPN22), cytotoxic T lymphocyte antigen-4 (CTLA-4), cluster of differentiation 40 (CD-40), and Fc receptor-like protein 3 (FCRL3)[5-14]. Furthermore, antigen-specific immunotherapy with two TSH-R peptides has been proposed[15]. Although conventional treatments of GH with antithyroid drugs, surgery, and radioactive iodine (RAI)are commonly used in clinical practice, novel tools are on the way (Table 1) (Figure 1).

GD affects primarily the thyroid, but extrathyroidal manifestations can be present (Box 1)[16,17]. Graves' orbitopathy (GO) is an autoimmune syndrome affecting orbital fibroadipose tissue, due to an interplay between cellular and humoral immunity against TSH-R and possibly other autoantigens expressed by thyroid epithelial cells (TEC) and orbital fibroblasts (OFs)[18]. Treatment of GO is currently changing due to new insights into its pathogenesis (Table 1) (Figure 2).

This review aims at providing an overview of epidemiology, pathogenesis, and conventional treatment of GH and GO, and discussing the most recent research acquisitions that have allowed novel treatment modalities. Relevant studies published between Jan 1, 2000, and Jan 1, 2023, were identified from the PubMed, EMBASE, Cochrane Library, and the ClinicalTrials.gov registry, using the search terms "Graves' disease treatment", "Graves' disease therapy", "Graves' disease management", "Graves' disease pathogenesis", "Graves' orbitopathy", "Graves' ophthalmopathy", "thyroid eye disease", "thyroid-associated ophthalmopathy", "thyroid ophthalmopathy", "endocrine ophthalmopathy". In vitro studies, preclinical studies, retrospective studies and randomized clinical trials performed in Europe and US were included. Only English language articles were included. Only peer-reviewed journal articles were included.

Epidemiology

The prevalence of GD is ~1.2% worldwide, with a female-to-male ratio of ~ 10:1. The incidence ranges from 20 to 40 new cases/100 000/year, with a lifetime risk of 3% for women and

0.5 % for men[191,20].Patients can be affected at any age, but the incidence peaks between 30 and 50 years[1,19,20].

GO is observed in ~25-30% of GH patients [21,22] with an incidence of 0.54-0.9 cases/100.000/year in men and 2.67-3.3 cases/100.000/year in women[22]. Mild forms are more common, whereas severe GO accounts for 5-6% of cases[18,22], the latter being more common in men [23].A recent prospective, observational study provided new trends in clinical presentation and management of GO[22]:patients were older in 2019 compared to 2012, with a reduced frequency of moderate-to-severe forms, suggesting improvement in management timing and treatment[22].

Pathogenesis and molecular mechanisms

Graves' disease

Genes that confer susceptibility have been identified[244,25]as well as hypermethylation of several genes, including those encoding TSH-R and proteins involved in T-cell signaling[25](Box 2). As an autoimmune condition, GD arises from the breakdown of self-tolerance towards autoantigens, with TSH-R being the most prominently recognized. Antigen-presenting cells (APCs), such as B-cells, macrophages, dendritic cells, and thyroid cells, identify TSH-R peptides. The interaction between the T-cell receptor (TCR) and major histocompatibility complex (MHC) triggers T-cell activation, subsequently activating B-cells through the interplay of TCR and MHC, along with the binding of cluster of differentiation 40 (CD40) to its ligand CD40L. Following activation, B-cells differentiate into plasma cells, producing TSH-R autoantibodies (TRAbs) that stimulate thyroid cells, leading to increased proliferation and hormone secretion, ultimately resulting in hyperthyroidism.

TSH-R autoantibodies

TSH-R is a G-protein coupled receptor (GPCR) with seven transmembrane helices. The signal transduction of the TSH-R is intricate and is currently being refined. It encompasses beta-arrestin signaling, IGF-1R crosstalk, and engagement with various G proteins. The receptor exists in multiple configurations, and there are diverse signal pathways that may or may not be activated as the receptor conformation changes upon ligand binding. This dynamic process can persist even after the receptors are internalized[26]. Recently, an elegant computer-based approach has been

introduced to gain insights into the hinge region, enabling the development of a comprehensive full-length model of the TSH-R[27].

IgG1 subclass autoantibodies binding TSH-R (TRAbs), originally referred to as long-acting thyroid stimulators[28-30], play a central role in GD [31]. Immunization against the TSH-R is essential for the development of GD in *in vivo* models (Box3). Oligoclonal production of TRAbs mostly arises from intrathyroidal B cells, reflecting the role of primary immune response[32]. TRAbs act as TSH-R agonists, promoting thyrocyte proliferation and activity[33], leading to increased thyroid hormone production. TRAbs recognize epitopes on the TSH-R-αsubunit, which can be secreted in a soluble form and may trigger and/or enhance the autoimmune reaction to the full-length receptor[34,35]. TSH-R also exists as multimers or high-order complexes[36], and multimeric forms of the α subunit may affect the affinity maturation of TRAbs[37]. Although stimulating TRAbs (TSAb) are the hallmark of GD, TSH-R-blocking (TSAb) and neutral antibodies rarely, have been detected[38]. In these patients, thyroid function can fluctuate between hyper- and hypothyroidism according to the serum levels of TBAb or TSAb[39].

Immune tolerance escape

Inflammatory reactions reflect delicate balance between T-helper (Th) and T-regolatory (Treg)cells. Tregs exert an immune-modulating action by reducing Th1 (involved in cytotoxic immune response) and Th2 cell activity (responsible for antibody-mediated response). A decrease in Treg-dependent immunosuppressive action has been reported in GD [40,41].

Thymic processing of immune cells provides deletion of autoreactive T-cells by clonal deletion and diversion[42]. T-cells selected to differentiate further, become Th1, Th2, or Th17, and each phenotype produces a unique pattern of cytokines[42]. Peripheral tolerance processing exists wherein self-reactive T-cells become unresponsive or are deleted after encountering self-antigens outside the thymus[42].Autoreactive T-cells against TSH-R escape both central and peripheral tolerance. Thus, they recognize TSH-R peptides presented by APCs (Figura1). Intrathyroidal T-cells mostly display a Th2 phenotype[43]. However, whether GD is driven by Th1 or Th2 response remains a matter of debate[44].

Both T and B-cells are involved in GD. In addition to acting as APCs, B cells differentiate into antibody-producing plasma cells through antigen-binding to B-cell receptor and a second signal provided by the costimulatory pathway CD40-CD40L (Figure 1).

An *in vivo* study in a mouse model suggested that the checkpoint regulator VISTA (T-type immunoglobulin domain-containing suppressor of T-cell activation) may play a role in autoimmunity development [45], and further investigations into this mechanism are warranted in GD.

The roleplayed by TEC in GD development is not well recognized. Their ability to release chemokines suggests a potential involvement in recruiting immune cells[46]. Furthermore, TEC in GD express MHC class II molecules and CD40, and may act as APCs.

Graves' orbitopathy

GO is an autoimmune condition characterized by immune-mediated inflammatory reactions against autoantigens shared by TEC and OFs[18] (Figure 2).OFs include a subset of $CD34^{+}$bone-marrow-derived fibrocytes that are the primary target of autoimmunity [1,47]. GO shows a multifactorial pathogenesis, with both genetic and environmental risk factors playing a role (Box 4).

TSH-R

After TSH-R expression in orbital tissue was first shown [48], immunohistochemical studies revealed TSH-R overexpression in GO orbital tissues[49]. The expansion of orbital adipose tissue is promoted by TSH-R activation, which triggers OFs and preadipocyte differentiation into adipocytes [50]. Mouse experimental models support the involvement of TSH-R[51-53]. High levels of circulating TRAbs are associated with GO activity[54,55] and TRAbs are an independent risk factor forGO[56].

IGF-1R

A key role of insulin-like growth factor 1 receptor (IGF-1R) in GO has been described[57,58]. IGF-1R is overexpressed in both B-[59]and T-cells [60] from GD patients.GD-IgG binding to IGF-1R triggers OF proliferation and secretion of hyaluronan and cytokines [61]. IGF-1R and TSH-R are colocalizedinOFs[62].Whether IGF-1R activation occurs through binding of stimulatory IGF-1R antibodies (IGF-1R-Abs) or exclusively via the established cross-talk between TSH-R and IGF-1R is not yet elucidated. Studies on IGF-1RAbs have yielded conflicting results[63,64]. A recent study suggests that IGF-1R Abs may protect against GO occurrence[65].

The orbital infiltration: T and B-cells

Orbital immune infiltration changes over the natural course of GO, which is characterized by an early cell-mediated immune reaction followed by humoral immunity[66]. In the first 18-24 months, Th1 cells are predominant and produce interleukin-2 (IL-2), IL-1, interferon-γ(IFN-γ), and tumor necrosis factor-α (TNF-α), which stimulate OFs to produce glycosaminoglycans. Later, the orbital infiltrate consists mainly of Th2 cells, which activate B-cells and antibody production. IL-4 produced by Th2 promotes collagen type 4 synthesis causing fibrosis. Th17and Treg cells have also been reported to play a role, by inducing expansion of extracellular matrix and development of fibrosis[67,68]. Besides T-cells, the orbital infiltrate in GO patients also consists of B-cells and both correlate with GO activity[69]. B-cells work as APCs, interact with T-cells through CD40-CD40L costimulatory pathway, and differentiate into plasmacells that release TRAbs (Fig.2)[1,70].

Orbital fibroblasts

The interaction between T-cells, OFs, and TRAbs contributes to OF activation (Fig.2). Once activated, OFs proliferate, secrete inflammatory molecules and glycosaminoglycans and differentiate into adipocytes, leading to the tissue remodeling typical of GO [1]. Since OFs are the target cells, their heterogeneity may affect tissue remodeling. In this regard, the surface marker CD90 has been reported to divide OFs into two subsets with different phenotypes and functions. Indeed, $CD90^{+}$OFs promote fibrosis whereas $CD90^{-}$ OFs promoteadipogenesis[70].

Epigenetics: new acquisitions

Epigenetics, a new research area in GO pathogenesis, includes histone modification, DNA methylation, and non-coding RNA (ncRNA). Hypermethylation or hypomethylation of certain genes may occur in OFs in the setting of autoimmunity[71], resulting in differential gene expression, which may affect cell proliferation and cell response to inflammation. NcRNA includes RNA transcripts lacking protein-coding functions, which can be classified into long non-coding RNA (lncRNA), circular RNA (circRNA), and microRNA (miRNA). The involvement of miRNA in GO is supported by several findings. miR-Let7-5p is higher in GD patients without GO compared to those with GO. Furthermore, miR-Let7-5p negatively correlates with the Clinical Activity Score (CAS) and the severity of GO[72]. Low serum miR-224-5p has been associated with reduced sensitivity to glucocorticoids[73]. Finally,miR-146a expression is higher in OFs compared to controls and is related to the production of TGF-β-induced fibrosis markers[74].

Treatment

Graves' hyperthyroidism

Conventional treatments

Current treatment options for GH include antithyroid drugs (ATD), surgery and RAI. ATD, (methimazole and propylthiouracil) are the first-line treatment to restore euthyroidism[75]. The most common side effects (reported in up to 13% of treated patients) are urticaria, pruritus, arthralgia and gastrointestinal symptoms [76]. Major adverse events are rare (0.1-0.3% of patients), but might be life-threatening, and include agranulocytosis, hepatotoxicity, aplastic anemia, thrombocytopenia and vasculitis[77]. Current guidelines recommend a course of ATD lasting 12–18 months. However, while a 12–18 month initial treatment period is reasonable for patients responding quickly to treatment and likely to achieve remission, the relapse rate after discontinuation of ATD is ~50%[7879], and a more personalized approach is recommended[80]. Patients with low chances of remission at the onset (e.g., high TRAb titres and smoking habit) or patients for whom relapse could be detrimental due to underlying conditions (e.g., patients with heart disease), may benefit from definitive therapy from the beginning or long-term ATD. RAI or surgery should be also considered in patients who experience GD relapse after ATD discontinuation, as a second ATD course rarely brings long-term remission.

RAI is a safe and well-tolerated option. Although it leads to hypothyroidism and lifelong replacement with levothyroxine, may be deemed necessary and can be open for discussion with patients, considering their perspectives and preferences. RAI is associated with *de novo* development or progression of GO[21], which is prevented by steroids given after radioiodine[21,81]. Rare RAI adverse events are sialodenitis and subacute thyroiditis, which may occur in ~1% of patients and can be treated with non-steroidal anti-inflammatory drugs or steroids[80,82].

Thyroidectomy can be considered in selected cases, namely large goiters, uncontrolled hyperthyroidism, severe GO, suspected thyroid cancer, patient preference[83].

Novel treatments

In patients with GH, the breakdown of immune tolerance triggers the recognition of self-antigens leading to a cascade of events that ultimately result in the production of TRAbs. None of the conventional treatments for GH currently available act on its pathogenesis. Recently, new

molecules that target different steps in the pathogenesis of GH, including early recognition of TSH-R peptides, T-cell activation, B-cell stimulation and survival, TRAbs production and TSH-R activation, have been tested with encouraging results (Figure 1) (Table 1)[84,85].

1-ATX-GD-59.

Antigen-specific immunotherapy with intact allergens has been successfully used in allergic disorders since 1911. Subsequently, the principle of tolerance induction has been applied to autoimmune diseases [86]. Progress has been made with the discovery of synthetic peptides termed "apitopes" (antigen-processing independent epitopes) that mimic naturally processed CD4+ T-cell epitopes. Apitopes can induce immune tolerance without the risk of an immune response to the administered antigen. ATX-GD-59, a TSH-R apitope, is a combination of two TSH-R synthetic peptides. ATX-GD-59has been shown to suppress CD4+ T-cell and T-dependent antibody responses to TSH-R in GD-associated HLA class II (*DRB1*0301*) transgenic mice [87].Immunotherapy with ATX-GD-59 has been tested in a Phase I Study, in GD patients with newly diagnosed and untreated hyperthyroidism[15].Tenpatients were treated with ATX-GD-59 in 10 i.d. injections over 18 weeks and followed for 12 weeks. In 50% of patients, normalization of free triiodothyronine was observed. Given the involvement of TSH-R, this treatment may also have a beneficial effect on GO[15]. ATX-GD-59 was well tolerated, with only mild local reactions in injection sites.

K1-70

K1-70 is a human monoclonal antibody blocking TSH-R. It was isolated from a patient with thyroid autoimmune disease and high levels of serum TRAbs[88]. Among several TSH-R-blocking antibodies (i.e.: CS-17, M22, Mab-B2, 5C9), K1-70 is the most effective inhibitor of TSH-R *in vitro*[89] and it has been shown to reduce free and total T4 concentrations in rats[89]. The Rees Smith group, in 2022, published the results of a phase I clinical trial aimed at testing safety, tolerability, pharmacokinetics and pharmacodynamics[90]. Eighteen GD patients with hyperthyroidism controlled with ATD were treated with a single intramuscular or intravenous dose of K1-70. At the different doses tested, the drug was well tolerated with mild to moderate adverse events, among which fatigue, lethargy and diarrhea were the most common, and no serious adverse events. At the highest doses (25 mg im, 50 mg IV and 150 mg IV), evolution to hypothyroidism occurred in 100% of subjects. Improvement of GH was reported and an improvement in exophthalmos (>2

mm) was registered. These promising results make K1-70 a very interesting candidate among the new molecules to treat GH and GO[90].

Small molecule TSH-R antagonists

Small molecule agonists and antagonists can directly activate or inhibit TSH-R signaling. ANTAG-3, the best-studied inverse agonist, inhibits basal and agonist-induced signaling by decreasing TSH-R activity[91,92]. These compounds inhibited TSH-stimulated cAMP production *in vitro* and reduced thyroid hormone levels in mice treated with the thyroid-stimulating monoclonal antibody M22, suggesting they may prevent TRAb-induced GH. VA-K-14 and S37a, two additional TSH-R antagonists, reduce TSH- and TRAb-induced signaling *in vitro*[93,94]. These small molecules could lead to novel therapies for GD and GO.

Iscalimab

CD40 is a tumor necrosis factor receptor expressed on APCs. CD40 bindsCD40L on T cells and has a critical role in antigen presentation, by activation of a costimulatory pathway that leads to B-cell proliferation, Ig class switching and germinal centre formation[95]. Furthermore, CD40 is one of the major susceptibility genes for GD[3].Blocking CD40 activation in animal models suppresses experimental GD[96],whereasoverexpression of CD40 in the thyroid leads to increased antibody production and a more severe experimental GD[97].Iscalimab (CFZ533) is a fully human, non-depleting, monoclonal antibody that blocks CD154 (or CD40L) binding to CD40 and the subsequent activation pathway (Figure 1).A clinical phase two, open-label, single-arm study was conducted to test the effect of Iscalimab in GH[11]. Fifteen hyperthyroid patients were treated with five doses of Iscalimab(10 mg/kg) intravenously over 12 weeks and observed for 24 weeks. In 7/15 (47%) patients, free and total T3 and T4 normalized at week 20, and 2/15 (13.3%) patients had normalization of TSH as well. Six patients (40%) were considered non-responders, since they required ATD to normalize thyroid function during the follow-up. TRAb levels decreased across the study. Intriguingly, all patients with TSH-R-Ab < 20IU/L were responders. While the limited sample size precludes definitive conclusions, this evidence could be taken into consideration to assess the optimal population that stands to gain the most from the administration of Iscalimab. No serious adverse events were observed; however, 80% of patients had mild adverse events that did not require withdrawal of study medication (except one case of moderate cystitis). In conclusion, the efficacy of Iscalimab seems similar to that of a 12-month course of ATD with a

good safety profile. In order to understand why about half of the patients were non-responders, it was investigated whether a specific genetic background could interfere and predict response. Seven single nucleotide polymorphisms (SNP)of CD40 gene were analyzed in 13 patients treated with Iscalimab and three haplotype profiles were found. Two were associated with higher CD40 mRNA levels and response to treatment, and the third was related to low CD40 mRNA levels and no response to treatment[10]. These data suggest that certain genetic variants of the CD40 gene are associated with higher expression of CD40 and good response to Iscalimab.

Belimumab

Belimumab is a fully humanized IgG1 monoclonal antibody that targets B-cell activating factor (BAFF) and prevents its interaction with BAFF receptors on the B cell surface, ultimately resulting in B cell failure to mature, expand, and differentiate into antibodies-producing plasmacells. Increased circulating BAFF levels and a significant positive correlation between TRAbs and BAFF levels have been found in GH patients[98]. Genetic variants in *BAFF* seem to increase the susceptibility to GD[99]. The role of BAFF/BAFF-R interactions in GD pathogenesis is supported by their increased expression in infiltrating immune cells and thyrocytes from GH patients[100]and by the evidence that blocking BAFF by a BAFF-specific receptor-Fc in a GD mouse model reduced the resulting hyperthyroidism[101]. Further clinical studies are warranted to investigate the potential efficacy of Belimumab for treating GH.

Graves'orbitopathy

Conventional treatments

Intravenous glucocorticoids (ivGCs)have been used as the first-line treatment of moderate-to-severe, active GO since approximately 30 years. However, the recent European Group of Graves' Orbitopathy (EUGOGO) guidelines suggest the addition of mofetil mycophenolate to methylprednisolone[21]. Mycophenolate acts by inhibiting T and B-cells proliferation, and antibody and adhesion molecule production [102]. A multicenter randomized clinical trial has been conducted entailing the enrollment of 164 patients randomized to receive methylprednisolone (500 mg once/week for 6 weeks followed by 250 mg once/week for 6 weeks) or methylprednisolone plus oral mycophenolate (720 mg daily for 24 weeks). Even though there was no significant difference at 12 weeks (primary endpoint), the combination therapy provided a greater overall response rate

and improvement in the individual GO features compared to methylprednisolone monotherapy at 24 and 36 weeks[103].

Orbital radiotherapy is an effective second-line treatment for moderate-to-severe and active GO, in combination with glucocorticoids, mainly in patients with diplopia and/or restriction of extraocular motility[21]. Orbital decompression is used for ivGC-resistant sight-threatening GO or rehabilitative purposes. Squint and eyelid surgery can be used for rehabilitative and/or aesthetic purposes[21].

The conventional approach to treating mild Graves Orbitopathy (GO) primarily involved local measures, with major treatments reserved for cases significantly impacting quality of life. Given the role of oxidative stress in GO, the European Group on Graves' Orbitopathy (EUGOGO) conducted a randomized, placebo-controlled clinical trial in selenium-deficient European countries to evaluate the effects of selenium and pentoxifylline in mild GO[104]. The study included 159 patients with mild GO who were randomized to receive sodium selenite, pentoxifylline, or placebo for six months, followed by a six-month follow-up period. The study assessed the overall eye outcomes, including exophthalmometry, clinical activity score (CAS), eyelid aperture, diplopia, and visual acuity. Selenium treatment showed a significant outcome, with 61% of patients experiencing GO improvement compared to 36% in the placebo group. Notably, selenium-treated patients had better quality of life scores, including visual functioning and appearance, with persistent benefits even at the 12-month follow-up visit. Even though the study had limitations, such as the lack of baseline and end-of-treatment selenium concentration measurements, which leaves uncertainty about whether selenium deficiency correction contributed to the observed benefits, these promising results opened the possibility of the clinical use of selenium in patients with mild GO. Indeed, a 6-month selenium supplementation should be given to patients with mild and active GO of recent onset, according to the most recent EUGOGO guidelines[21].

Novel treatments

Teprotumumab

Teprotumumab is a humanized monoclonal antibody that blocks IGF-1R. In 2017, in a double-masked, multicenter, randomized trial, 88 patients with moderate-to-severe active GO were assigned to receive placebo or teprotumumab (eight intravenous infusions at 3-week intervals, with a starting dose of 10 mg/kg, which was later increased to 20 mg/kg)[105].The primary endpoint was

a composite evaluation which included reduction of CAS by at least 2 points and decrease in exophthalmos by at least 2 mm. At 24 weeks, a greater proportion of responders (69%)was observed in the teprotumumab group. The most notable effect was reduction of exophthalmos, with an average decrease of ~2.5 mm[105]. Subsequently, a phase 3 clinical trial was performed. The study included 41 patients treated with teprotumumab and 42 patients with placebo. The primary outcome was a decrease in exophthalmos by at least 2 mm[57]. The response rate to teprotumumab was 83%, compared to 10% in the placebo group. Additionally, secondary outcomes such as CAS, diplopia, and quality of life (QoL) were better in patients given teprotumumab[57]. The results led to the approval of teprotumumab by the US Food and Drug Administration in January 2020 for GO, regardless of activity and severity. A pooled data analysis of these two studies with an off-treatment follow-up has been performed to define long-term responses[106]. At 72 weeks following the discontinuation of Teprotumumab, 33% of patients observed a deterioration in exophthalmos, while 31% experienced diplopia relapse. Moreover, based on composite evaluation, 17% of patients were no longer considered responders in the long-term term[106]. In a continuation of the second clinical trial teprotumumab was administered to non-responders or those who experienced a flare-up[107]. The majority of them achieved a response to treatment[107].

Unfortunately, there are some limitations to using teprotumumab[108]. Costs of treatment are quite high and the safety profile suggests caution. Thus, although teprotumumab was found to be generally safe in clinical trials[57,105],it carries hyperglycemia risk, especially in patients with pre-existing diabetes[57,105,109], and real-world data indicate hearing impairment as a relatively frequent event (12%) [110]. Ulcerative colitis[111,112]and amyloid encephalopathy [113]have also been reported during the post-marketing period[108].

Rituximab

Rituximab is a chimeric human/mouse monoclonal antibody that binds CD-20 positive B-cells inducing their depletion[114]. In addition, rituximab reduces IGF1-R positive T-cells[115]. Following early uncontrolled studies indicating a potential benefit in moderate-to-severe, active GO[116,117], two randomized clinical trials were conducted resulting in conflicting results[118,119] that can be explained, at least in part, by the longer GO duration in one study[120]. According to a meta-analysis including 12 studies, rituximab may have a beneficial effect on GO activity, measured by CAS, and low efficacy in improvingproptosis[121]. Recently, a single 100 mg infusion of rituximab has

been reported to achieve GO inactivation in15/17 GC-resistant patients with moderate-to-severe active GO. Additionally, around 60% of patients showed improvement in GO severity, and no flares were seen[122]. For the time being, rituximab may be considered as a second-line treatment for moderate-to-severe active GO[21,108]. Despite its efficacy, the safety profile of rituximab still carries concerns. The most frequent adverse events include nose and throat itches, infusion reactions, nausea, and fever, but severe infections, progressive multifocal leukoencephalopathy, vasculitis and gastrointestinal complications have been described[21,118].The use of rituximab has been also tested in GH. A prospective study with a small cohort of patients suggested a beneficial role of rituximab in mild relapsing GH[123]. A prospective study by El Fassi et al. compared short-term methimazole treatment with or without rituximab in 20 GH patients[124]. Treatment with rituximab resulted in sustained remission in 4/10 patients after 24 months compared to 0/10 in the control group, and it was most successful in those with low TRAb levels at presentation (<5 IU/L) [124]. Intriguingly, rituximab was found to enhance the remission rate of GH in young patients aged 12-20 years when combined with a short course of ATD. In a recent multicenter, single-arm, phase 2 trial, 27 patients received a single 500-mg dose of rituximab and 12 months of ATD titrated according to thyroid function. ATDs were discontinued after 12 months, and the primary outcome was assessed at 24 months using A'Hern design to distinguish an encouraging remission rate (40%) from an unacceptable rate (20%). Thirteen out of 27 patients (48%) achieved GH remission, providing evidence of a promising remission rate. However, the lack of a control group underscores the need for a randomized clinical to validate the efficacy of rituximab[125].

Tocilizumab

Tocilizumab is a humanized monoclonal antibody that targets IL-6R, representing a valid second-line treatment for moderate-to-severe, active GO[21]. In a small clinical trial, 32 GC-resistant patients with moderate-to-severe, active GO were randomized to receive iv tocilizumab (8mg/kg, four doses with a 4-week interval) or placebo, showing a significantly greater rate of inactivation in patients given tocilizumab, with limited efficacy on exophthalmos and diplopia. However, placebo-treated patients also had a surprisingly high rate of inactivation, suggesting that the improvement was affected by the delayed effect of previous GC therapy or GO natural history[126]. Larger multicenter randomized clinical trials are warranted.

Batoclimab

Batoclimab is a fully human anti-FcRn monoclonal antibody that blocks FcRn-IgG interactions and accelerates autoantibody degradation. Its efficacy has been reported in other IgG-mediated autoimmune diseases[127].A proof-of-concept randomized trial in patients with GO was recently published[128]. The trial was prematurely interrupted because of an unexpected increase in serum LDL-cholesterol reflecting a reduction in albumin levels. However, the efficacy of batoclimab was evaluated in 65 of the 77 planned patients. All patients who received 6 weekly subcutaneous injections of batoclimab had a significant reduction in TRAbs and orbital muscle volume decreased compared to placebo group. The improvement in exophthalmos and CAS did not reach the statistical significance, probably because of the small number of patients[128].

Linsitinib

Linsitinib is an orally available dual small-molecule inhibiting IGF-1R. Encouraging results have been recently reported in an experimental GO mouse model[129].Gulbins et al. showed that linsitinib can prevent hyperthyroidism development when administered in the early phase of the disease. In the chronic phase, linsitinib reduced immune infiltration (T-cell and macrophages) of orbital tissues as well as orbital inflammation[129].*Invitro* studies have shown that linsitinib, in combination with a small molecule TSH-R inhibitor, ANTAG3, inhibits hyaluronan secretion by cultured OFs[130]. Taken together, these data suggest that linsitinib may be a very promising drug for GO.

Sirolimus

Sirolimus, also known as rapamycin, is an antiproliferative, antifibrotic, immunosuppressive drug used to prevent organ rejection and treat lymphangioleiomyomatosis[131].Its molecular target is the serine-threonine kinase known as “mammalian target of rapamycin” (mTOR) which plays a role in regulating cell growth, proliferation, motility, and survival[131]. Through mTORC1 inhibition, sirolimus counteracts T cell activation, adipogenesis, and fibroblast migration and transition into myofibroblasts, which are important mechanisms involved in GO[131-133]. A retrospective investigation has been recently performed to assess the efficacy of sirolimus (2 mg orally on the first day, followed by 0.5 mg/day for 12 weeks) as a second-line therapy in patients with moderate-to-severe active GO compared to methylprednisolone [134]. Thirty patients (15 per group) previously treated with methylprednisolone with no beneficial effects were included. The primary endpoint was the overall outcome of GO at 24 weeks, based on a composite evaluation.

The proportion of GO responders, at 24 weeks was higher in patients treated with sirolimus (86.6%) compared with those given methylprednisolone (26.6%). The GO QoL and the proportion of proptosis (80% vs 13.3%) and CAS responders (86.6% vs 33.3%) were greater in the sirolimus group compared to methylprednisolone group[134]. Given the study design and the small sample size, further randomized clinical trials are needed.

Atorvastatin

Given the knowledge that statins counteract the occurrence of GO in GD[135,136], and elevated LDL-C is a risk factor for GO[137,138], it is reasonable that statins may improve the response rate in GO. The STAGO study was a phase II randomized clinical trial[139]. The purpose was to assess the efficacy of atorvastatin on the overall response of GO to ivGCs. Eighty-eight patients with moderate-to-severe, active GO and high LDL-C serum levels were included and randomly assigned to statin (ST) or no-statin (NST) treatment. Patients in both groups received ivGCs. Patients in the ST group were treated with atorvastatin 20 mg/daily for 24 weeks. The primary outcome was the overall response of GO at 24 weeks, assessed by a composite evaluation. A greater proportion of GO responders was observed in the ST compared to NST group both at 12 and 24 weeks. Moreover, the improvement in QoL was higher in patients treated with atorvastatin both at 12 and 24 weeks. The incidence of GO relapse was significantly higher in NST. Interestingly, there was no difference in LDL-C levels between responders and non-responders within the statin group at 12 and 24 weeks, suggesting that the beneficial effect of atorvastatin was likely due to its pleiotropic actions rather than to lowering cholesterol[139].

Conclusions

The evolving landscape of GD and GO demands a closer examination of potential treatment modalities, given the current absence of treatments addressing the underlying disease etiology. Despite advancements in delivery and safety, current treatment methods for GD carry significant limitations, such as high relapse rates upon discontinuation of ATD and the occurrence of hypothyroidism following RAI or surgery. The journey from laboratory discoveries to clinical applications holds promise for transforming the care paradigm. New potential treatments for GD have emerged, employing diverse mechanisms of action. These include the restoration of immune tolerance through immunomodulatory TSH-R peptides and the counteraction of TSH-R signaling using activation with rituximab, iscalimab, and belimumab. As in other autoimmune disease, the

inhibition of IgG recycling through neonatal Fc receptor blockade with rozanolixizumab and efgartigimod may result in a new therapeutic approach in GD as well. Clinical studies on these treatments are predominantly open-label phase 1 and 2 trials. Further randomized controlled studies are warranted to confirm the efficacy and safety of the new compounds.

The occurrence of GO further complicates the treatment landscape, necessitating a nuanced approach. Major advances in the pathogenesis of GO have been made over the last few years, unlocking novel avenues for patient management. Although the FDA has approved teprotumumab as first-line therapy for the treatment of moderate-to-severe GO, its use has not been sanctioned in other countries, where the most commonly used treatment is still methylprednisolone. This discrepancy generates disparities in the guidelines among different scientific societies and in day-to-day clinical practice across the world, making patient management even more complicated.

The increasing understanding of GO pathogenesis has given rise to the suggestion of additional novel medications, such as linsitinib and tocilizumab. The eagerly awaited results from large, randomized multicenter trials will be crucial to confirming the promising yet preliminary findings.

Competing interests

Michele Marinò is a member of the Advisory Board of Horizon Pharma.

'The remaining authors declare no competing interests'

Figure legends

Figure 1

Pathogenesis of Graves' hyperthyroidism and mechanisms of action of the most promising recently proposed therapies

In loss of self-tolerance, thyroid stimulating hormone receptor (TSH-R) peptides are recognized by antigen-presenting cells (APCs), namely B-cells, macrophages, dendritic cells, and thyroid cells. The interaction between T-cell receptor (TCR) and major histocompatibility complex (MHC) promotes activation of T-cells, which in turn activate B-cells through interaction between TCR and MHC and binding of cluster of differentiation 40 (CD40) to its ligand CD40L. Once activated, B-cells differentiate into plasma cells, producing TSH-R autoantibodies (TRAbs), which stimulate thyroid cells to proliferate and secrete hormones, resulting in hyperthyroidism. Five mechanisms and sites of action of novel treatments for Graves' hyperthyroidism are represented. (a) K1-70 is a human monoclonal antibody which binds and blocks TSH-R. (b) Iscalimab is an anti-CD40 monoclonal antibody that blocks CD40-CD40L costimulatory signaling. (c) ATX-GD-59 is an apitope that can prevent TRAbsproduction and restore immune tolerance. (d) Belimumab is a recombinant, fully human, monoclonal antibody against B-cell activating factor (BAFF), which prevents BAFF from interacting with its receptors. Belimumab inhibits primary humoral immune responses by depleting naïve B-cells that are dependent on BAFF for their survival; (e) Small molecules bind the transmembrane domain of the TSH-R, blocking its activation. Created with BioRender.com

Figure 2

Pathogenesis of Graves' orbitopathy (GO) and mechanisms of action of the most promising recently proposed therapies

The autoimmune reaction against thyroid stimulating hormone receptor (TSH-R) leads to autoreactive T- and B-lymphocytes that infiltrate orbital tissues, triggering fibroblast proliferation and differentiation into adipocytes. Activated CD34+ fibroblasts and the autoreactive T-and B-cells promote the release of pro-inflammatory cytokines and glycosaminoglycans, especially hyaluronic acid, contributing the typical connective tissue remodeling. Four mechanisms and sites of action of the novel treatment procedures for GO are represented. (a) Mycophenolate has antiproliferative activity on both T- and B-cells; (b) Rituximab is a chimeric mouse-human monoclonal antibody that causes B-cell depletion by binding cluster of differentiation 20 (CD20),

a cell-surface antigen expressed on their surface; (c) Teprotumumab is a monoclonal antibody against insulin-like growth factor 1 receptor (IGF-1R) that disrupts activation of orbital fibroblasts; (d) Tocilizumab is an anti-interleukin 6 receptor (IL-6) humanized monoclonal antibody. IL-6 is a proinflammatory cytokine overexpressed in orbital fibroblasts and capable, among other actions, of stimulating TSH-R expression in orbital fibroblasts. Created with BioRender.com

Box 1. Graves' disease and Graves' orbitopathy: clinical presentation.

Patients with GH may exhibit various clinical features ranging from subclinical presentations to overt hyperthyroidism. Tachycardia, shortness of breath, tremor, and heat intolerance are the most common symptoms. Atrial fibrillation occurs in ~10% of patients, and elderly patients can experience heart failure. Neurogenic symptoms predominate in patients < 50 years old. GH patients may experience weight loss, and bone turnover can be affected, resulting in osteopenia and osteoporosis. Menstrual bleeding may be light, decreased in frequency, or absent[140].

Although GD primarily involves the thyroid, patients may also experience extrathyroidal manifestations, including GO, acropachy and pretibial myxedema (PTM). PTM occurs in 0.5-4.3 % of patients[141] and is a localized skin thickening due to hyaluronan deposition [17,142]. PTM typically occurs on the lateral or anterior side of the legs, but it can involve other skin surfaces. Clinical manifestations range from nonpitting edema to nodular, plaque, and polypoid forms. About 1% of GD patients are affected by thyroid acropachy, with clubbing of toes and fingers, periosteal proliferation of the phalanges shafts and soft tissue swelling [17,142].

The natural history of GO is biphasic, with an active phase lasting 6-18 months followed by an inactive phase[143].Clinical manifestations of GO can be ascribed to three features: inflammation of soft tissues, glycosaminoglycans overproduction, and increase in adipose tissue. Inflammation and exophthalmos are typical of the early phase, whereas diplopia occurs when inflammation becomes organized resulting in fibrosis[143,144]. Extraocular muscle enlargement results in impaired ocular motility and diplopia and, in the most severe cases, dysthyroid optic neuropathy (DON) due to compression of the optic nerves at the orbital apex[144,145]. GO can be classified into active or non-active, based on inflammation extent, commonly assessed by CAS[21,146]. Severity is established by the extent of proptosis, lid retraction, diplopia, and corneal and optic nerve involvement[21]. Mild GO rarely progresses to more severe forms [147].Sight-threatening GO is quite

rare, due to corneal breakdown (because of severe exophthalmos and/or lagophthalmos) or DON, and requires urgent treatment[21].

Box 2. Genetic susceptibility to Graves' disease, and environmental risk factors.

The Human leukocyte antigens (HLA)complex increases the risk of GD occurrence by 2-4 fold, contributing 5% of the estimated genetic susceptibility to GD[148].An association between TSH-R gene polymorphism and the risk of GD has been described [149]. Several elements involved in the immune checkpoint and immune response regulation, have been also reported to play a role. Forkhead box P3gene (FOXP3), alternatively referred to as Scurfin, encodes a crucial transcription factor involved in the development of regulatory T (Treg) cells, thereby affecting the etiopathogenesis of autoimmune thyroid diseases. Polymorphisms and abnormal acetylation of FOXP3 gene are associated with the risk of developing GD [150-152]. Fc receptor-like protein 3 (FCRL3) protein is involved in the regulation of the immune system by affecting B cell signaling. FCRL3 polymorphisms may increase the susceptibility to GD[5-8]. Other immune-regulatory genes, including cluster of differentiation 40 (CD-40), protein tyrosine phosphatase non-receptor type 22 (PTPN22), and cytotoxic T lymphocyte antigen-4 (CTLA-4),have been linked to GD[9], but their impact varies, depending on several factors, especially ethnicity [153]. Through the interaction with CD40 ligand (CD40L), which is expressed by activated $CD4^+$ T cells, CD40 is essential to trigger B cells. The CD40-CD40L co-stimulatory pathway plays an important role in the pathogenesis of GD by promoting autoreactive B-cell activation. The CD40 gene is a key susceptibility gene for GD[10]. Recently, Bufalo et al. published a case-control study that confirms the crucial role of CTLA4 polymorphisms in GD susceptibility and demonstrates the role of PTPN22 polymorphisms in patients' clinical features, suggesting that these genes may influence the severity of the disease[12].

Sex, psychological stress, iodine, irradiation, infections, post-partum period, radioiodine (RI) treatment for other conditions are recognized risk factors[145]. Immune-modulating agents, especially alemtuzumab, have also been reported as potential triggers. Infections, including SARS-CoV-2[154,155]can induce GD through several mechanisms[156].

Box 3. Animal models of Graves' disease.

TSH-R is extensively preserved within the species, making the immune system highly tolerant. Over the past few decades, several attempts have been made to overcome tolerance and create an appropriate animal model. The most successful have been achieved by injecting TSH-R-expressing adenoviruses or plasmid encoding TSH-R to introduce the human TSH-R extracellular domain into the body. However, these models require repeated immunization thereby producing inconsistent results[35,157-159].

In 2023 Bao and colleagues proposed a new Cre-loxP system-based mouse model able to induce the expression of the human THS-R-A subunit mimicking GD with an 81.25% frequency[160]. The Cre-loxP system-based mouse was established by inserting the CAG-lox-P-STOP-loxP-hTSH-R A subunit cassette into the Rosa 26 locus of the mouse genome. Conditional expression of the hTSH-R-A subunit was successfully achieved by a single intramuscular injection of the transactivator of transcription-Cre recombinase. Twenty-four (8 control and 16 GD) mice were investigated. The development of hyperthyroidism was investigated by measuring thyroid hormone and TSH-R Ab levels which were significantly higher in GD mice compared to controls at weeks 8 and 12. Histological analysis showed thyroid hypertrophy and diffuse enlargement in GD group. Before euthanasia, mice were subjected to MRI to investigate GO occurrence. Even though there was no significant proptosis, GD mice had a significant increase in retroorbital tissue volume compared to control mice. These findings were confirmed by histology. In addition, F4/80+ staining and Masson's trichrome staining of orbital tissues showed macrophage infiltration and muscle fibrosis in the GD group. This novel mouse model opens new perspectives for investigation in GD and GO animal models[160].

Recently, new immunomodulatory therapies have been also assessed and small cyclic peptides from the antigenic region of the extracellular subunit of the TSH-R were tested to induce immune tolerance and prevent GD occurrence[161,162].

Box 4. Genetic susceptibility to Graves' orbitopathy, and environmental risk factors.

Twin and family studies and family clustering indicate that inheritance predisposes to GD[163]. Several genes have been identified and twin studies show a 30% concordance rate, indicating modest penetrance[4,163]. However, the genetic profile of GD is unclear[9,164]. Epigenetic factors, namely histone modification, DNA methylation, and noncoding RNAs may contribute GO

pathogenesis[71].In addition, the gut microbiota is believed to play a crucial role in the development GD and GO. Changes in gut microbiota have been reported in GD patients with or without GO[13] and perturbation of gut microbiota in GO patients compared to healthy controls, with increased levels of Lactobacillus, Prevotella, and Veillonella often found in GD patients, has opened an intriguing topic[14,165]. Treatment with the antibiotic vancomycin was shown to be promising in reducing disease severity, whereas fecal material transfer (FMT) from GD/GO patients has been observed to exacerbate the condition in mouse models. Common therapeutic agents for GD and GO can also impact the gut microbiota. Dysbiosis in GD and GO is primarily attributed to antigenic mimicry and an imbalance of T helper 17 cells (Th17) and regulatory T cells (Tregs). Several interventions, such as antibiotics, probiotics, and dietary modifications, have been actively explored in preclinical models and, to some extent, in clinical settings[165]. Future research is expected to unveil molecular pathways linking gut and thyroid functions and their influence on orbital autoimmunity. Microbiota-targeting therapeutics are likely to emerge as a crucial strategy in the management of GD/GO in the coming years.

Risk factors associated with GO include sex, age, smoking, radioiodine, and inadequate control of thyroid dysfunction[145]. A role of oxidative stress and selenium deficiency has been also reported[166,167]. A predictive score for the occurrence and progression of GO in GD has been proposed, with determinants such as smoking, duration of hyperthyroidism, the GO CAS and TSH-binding inhibitor immunoglobulins (TBII)[168].

An association between diabetes mellitus (DM) and GO has been recently described. DM enhances systemic inflammation, thereby promoting molecular mechanisms responsible for GO[169]. Since 2015, the role of low-density lipoprotein cholesterol (LDL-C) has been investigated. Two large retrospective studies[135,136] found a lower incidence of GO in GD patients treated with statins. In a cross-sectional investigation, high cholesterol correlated with the occurrence of GO [137], CAS was higher in patients with high cholesterol, with a correlation between CAS, total and LDL-C[137]. A retrospective analysis confirmed these results[138]. A retrospective investigation demonstrated that high LDL-C was associated with poor response to intravenous glucocorticoid (ivGC)[170]. Accordingly, the EUGOGO guidelines suggest correction hypercholesterolemia[21].

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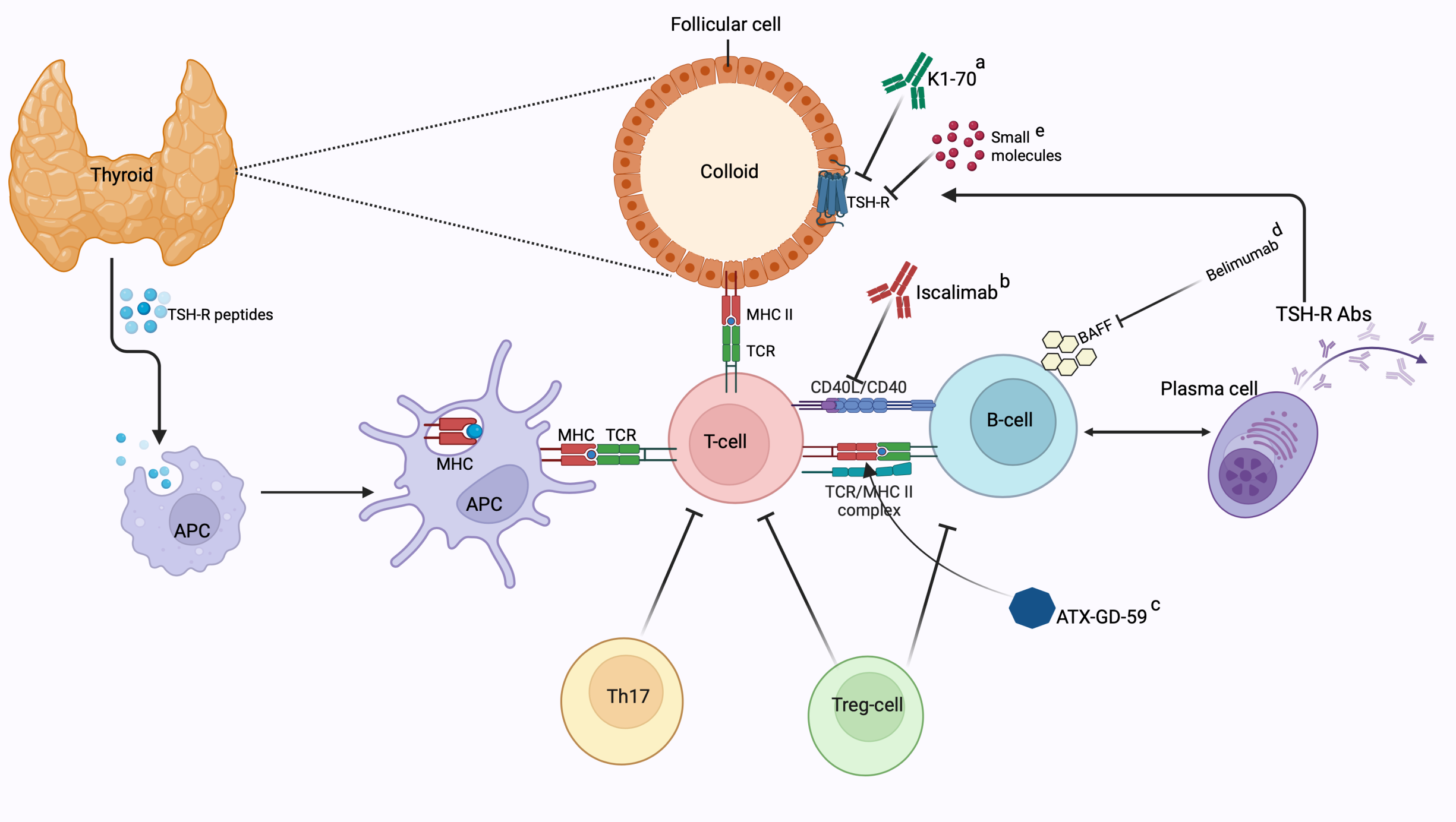
Treatment	*Mechanism of action*	*Dose and treatment protocol*	*Treatment indication*
ATX-GD-59	Inducing immune tolerance.	10 i.d. injections over a period of 18 weeks.	Phase 1 clinical trial[15].
K1-70	Human monoclonal antibody blocking thyroid stimulating hormone receptor.	Single i.m or i.v injection at different dosage (from 0.2 mg to 150 mg) have been tested.	Phase 1 clinical trial[90].
Small molecule TSH-R antagonists	Direct inhibition of thyroid stimulating hormone receptor activity.	N/A	Mouse model[92]; *In vitro*.[91].
Iscalimab	Inhibition of antigen presentation by blocking CD40.	5 intravenous injection (10 mg/kg) over 12 weeks.	Phase 2 clinical trial[11].
Belimumab	Prevetion of B cell maturation, clone expansion and differentiation into plasmacells	N/A	Mouse model[101]
Teprotumumab	Human monoclonal antibody blocking growth factor 1- receptor.	8 intravenous infusions over 24 weeks (once every 3 weeks; 10 mg/kg for the first infusion, then 20 mg/kg).	First-line treatment for GO regardless severity and activity in USA. Second-line treatment for moderate-to-severe active GO in Europe, according to availability and affordability [57,105].
Rituximab	Induction of B cells depletion by binding CD20 on B cell surface.	Two infusions of 1000 mg 2 weeks apart or a single dose of 500 mg.	Second-line treatment for moderate-to-severe and active GO of recent onset (<12 months), as long as dysthyroid optic neuropathy is excluded[21].
Tocilizumab	Target blocking of interleukin 6 receptor.	8 mg/kg every four weeks for 12 weeks.	Second-line treatment for moderate-to-severe and active GO[21].
Linsitinib	Target inhibition of the insulin growth factor 1-receptor.	75-150 mg BID for 24 weeks	Mouse model[129] In vitro[130] A multicenter randomized clinical trial is ongoing to define the potential use (NCT05276063).
Batoclimab	Target inhibition of the neonatal crystallizable fragment receptor (FcRn).	Subcutaneous administration of 680 mg once a week for 12 weeks followed by 340 mg once a week for 12 weeks.	Proof-of-concept randomized trial[128] A phase 3, multicenter, randomized, placebo-controlled, clinical trial is ongoing to define the potential use (NCT05517421).
Sirolimus	Systemic immunosuppression and anti-inflammation, and anti-fibrosis properties through the inhibition of the mTOR pathway.	2 mg as initial dose, followed by 0.5 mg daily for 12 weeks.	Off-label therapy[134].
Atorvastatin	Systemic immunomodulation and anti-inflammation.	20 mg once per day for 24 weeks, plus methylprednisolone 500 mg once a week for 6 weeks followed by 250 mg once a week for an additional six weeks.	Off-label therapy[139]

Table 1. Novel treatments for Graves' disease and Graves' orbitopathy
i.d., intradermal; i.m, intramuscular; i.v, intravenous; TSH-R, thyroid-stimulating hormone receptor, CD40, Cluster of differentiation 40; GO, Graves' orbitopathy.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Colloid
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TSH-R
TSH-R peptides
MHC II
TCR
Iscalimab b
Belimumab d
BAFF
TSH-R Abs
CD40L/CD40
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B-cell
MHC
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APC
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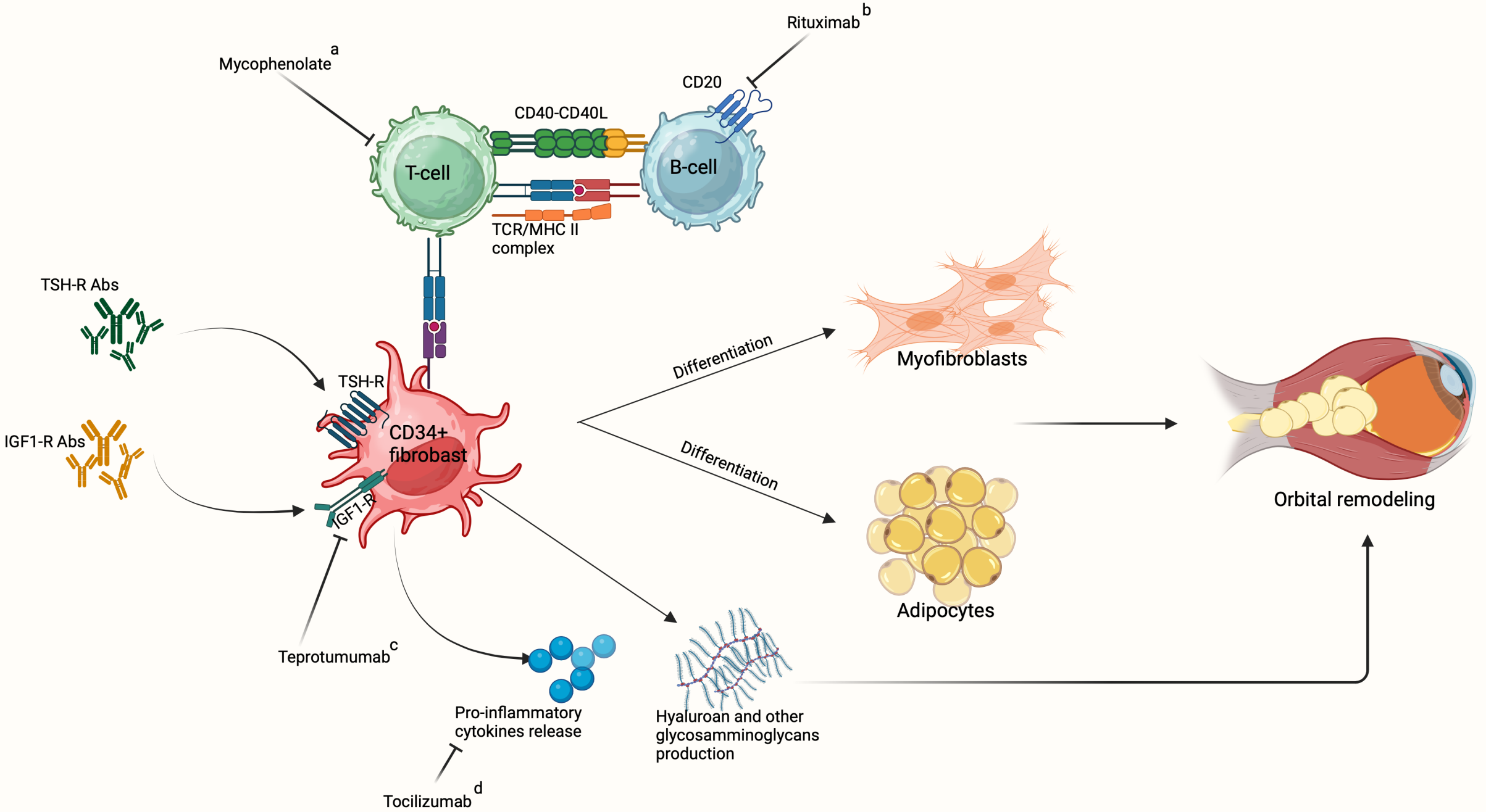
ATX-GD-59 c
Th17
Treg-cell


Mycophenolate a
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CD40-CD40L
T-cell
B-cell
TCR/MHC II complex
TSH-R Abs
IGF1-R Abs
TSH-R
CD34+ fibrobast
IGF1-R
Differentiation
Differentiation
Myofibroblasts
Adipocytes
Orbital remodeling
Teprotumumab c
Pro-inflammatory cytokines release
Hyaluroan and other glycosamminoglycans production
Tocilizumab d