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Orthoptik

**Association between G protein polymorphisms
(GNAS1 T393C and GNB3 C825T) and course of Graves' disease
and Graves' orbitopathy**

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The T393C polymorphism of the Galphas gene (GNAS1) is associated with the course of Graves' disease.

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1. Introduction

1.1. Graves' orbitopathy (GO)

1.1.1. Overview

Clinically evident Graves' orbitopathy (GO) occurs in about half of the patients with Graves' disease (GD). It is one of the most difficult challenges in the clinical practice of ophthalmology. It is usually associated with Graves' hyperthyroidism, however it can occur in Hashimoto's thyroiditis (HT) and even when there is no evident thyroid dysfunction. Pathogenesis of GO, in contrast to Graves' hyperthyroidism, is not fully understood yet. There is a clear pathogenetic link between the stimulation of the G protein-coupled thyrotropin receptor (TSH-R) by the thyrotropin receptor antibody (TRAb) and hyperthyroidism. TSH-R is also considered the central antigen in GO but additional factors cannot be disregarded.

GO has variable clinical presentation. It may cause severe damage to vision and the orbital architecture. Potential sight-threatening complications include optic neuropathy and severe corneal exposure keratopathy.

1.1.2. Incidence and prevalence

The incidence rate of GD equals 40 cases in 100,000 people per year, and the prevalence is 0,5-2% (Weetman 2003). The prevalence of orbitopathy in GD is a matter of definition but clinically it amounts to 25-50% according to Jacobson et al. (Jacobson et al. 1985). However CT, MRI or ultrasonography identify orbital changes in up to 90% of GD patients (Wiersinga et al. 2002).

GO, like Graves' hyperthyroidism, is more common in women. In one study, the female: male ratio was 9,3:1 in patients with mild GO, 3,2:1 in those with moderate ophthalmopathy, and 1,4:1 in severe ophthalmopathy. Patients with GO are older than those with Graves' hyperthyroidism without GO.

GO is in 90-95% concomitant with GD. 3-5% of GO patients suffer from HT, further 0,5-5% demonstrate no thyroid affection. Severe forms affect 3-5% of GO patients. Eye disease tends to be more severe in older patients and in males (Daumerie 2007).

1.1.3. Pathogenesis

The pathogenesis of GO is not fully understood yet. Rather than being a complication of GD, GO is a concomitant expression of the same underlying pathological autoimmune process directed against cross-reactive autoantigens in the thyroid and retrobulbar tissues (Wiersinga et al. 2001). Like in GD, both genetic and environmental factors play an important role in the development of GO (Prabhakar et al. 2003).

1.1.3.1. Genetic susceptibility

Increased incidence of GD among members of a family indicates that genetics might play an important role in determining susceptibility to GD. Twin studies show far greater concordance in monozygotic (30-60%) than dizygotic (3-9%) twins (Brix et al. 2001). Three principal approaches to investigate the genetic complexity of AITD - genome-wide screens, linkage and candidate gene studies - contributed to the identification of possible genetic loci and particular genes. Several genome screens, using different methods of linkage analysis, showed varying degrees of evidence for the linkage between AITD inheritance and genetic markers. So far, twin studies concerning GO have not been performed and the results of family-based studies are conflicting. The candidate susceptibility genes in GD (with and without GO) include both, immune modifying genes (e.g. human leukocyte antigen (HLA), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), tumor necrosis factor (TNF), immunoglobulin heavy chain and interferon- γ (IFN- γ)) and thyroid specific genes (e.g. TSH-R). Most likely, these loci interact and their interactions may influence the disease phenotype and severity (reviewed by Prabhakar et al. in (Prabhakar et al. 2003)).

Up to 79% of the liability to develop GD is attributable to genetic factors, the remainder being presumably due to the environmental factors (Brix et al. 2001).

1.1.3.2. Possible antigens and antibodies

Both GD and HT can present with similar findings, including lymphocytic infiltration of the thyroid and anti-Tg and anti-TPO autoantibodies in the serum. The occurrence of anti-TSH-R autoantibodies (TRAb) is rather exclusively associated with the development of the GD phenotype. The clinical course may also oscillate between the two disorders. Moreover, both diseases may occur in different individuals of the same family, which can lead to the assumption that GD and HT have a partially shared pathogenesis (Manji et al. 2006). As the majority of patients in the present study have GD, it focuses on this disease.

The nature of the putative antigen(s) shared by the thyroid and the orbit remains elusive, but many elements support the idea that TSH-R represents the culprit antigen.

TSH-R:

TSH-R, expressed on the basolateral membranes of thyroid epithelial cells, is the primary regulator of the thyroid cell growth and thyroid hormone synthesis. From the time of cloning of the receptor complementary deoxyribonucleic acid (cDNA), significant progress has been made in clarifying the structure-function relation of the receptor. It is the 764 amino-acid glycoprotein G protein-coupled receptor. The heavily glycosylated and conformed ectodomain is the major site of the TSH binding, while seven transmembrane domains and a cytoplasmic tail are involved in signal transduction. TSH-R undergoes complex post-translational processing, involving dimerization, intramolecular cleavage and shedding of its ectodomain. Each of these processes may influence the antigenicity of TSH-R (reviewed by Prabhakar et al. in (Prabhakar et al. 2003)). TRAbs that mimic the TSH actions and stimulate thyroid cells are called TSH-R stimulating antibodies (TSAbs), whereas those that block the TSH action are called TSH-R blocking antibodies (TBAbs). Both TBAbs and TSAbs are seen in patients with GD (Sugawa et al. 1995).

The TSH-R transcripts have also been demonstrated in orbital fibroblasts and in a markedly enhanced number in preadipocytes (cells of the fibroblast lineage

that can, under certain culture conditions, differentiate into adipocytes). Consensus has now been reached that TSH-R is expressed at the messenger ribonucleic acid (mRNA) and protein level in orbital adipose/connective tissue specimens of GO patients, but scarcely in that of controls (reviewed by Prabhakar et al. in (Prabhakar et al. 2003)). The TSH-R expression is the greatest in an early disease and it decreases with time. It is positively correlated with the TRAb levels (Boschi et al. 2005). Differentiation into pre-adipocytes increases the expression of the TSH-receptor on orbital fibroblasts (Crisp et al. 1997; Crisp et al. 2000). This would serve as a beacon to T cells, specific for TSH-R, and cause homing to orbital tissues.

There is also clinical evidence that the TSH-R is a crucial autoantigen for GO (summarized in (Eckstein et al. 2009)). Higher TRAb levels are associated with a significantly higher activity and severity of GO. The persistence of TRAbs in patients with a therapy-resistant disease, in comparison to patients with an inactive disease, confirms the significance of TRAb in the pathogenesis of GO. Even after an anti-inflammatory therapy, TRAb levels and prevalence still correlate with the severity and activity of GO. Thus TRAbs seem to both trigger and maintain the autoimmune process in the orbits.

IGF-1R

The IGF-1 receptor is overexpressed by multiple cell types in patients with Graves' disease. Antibodies directed at it have been detected in those patients. The frequencies of IGF-IR+ B and T cells are substantially increased in patients with GD (Smith 2010).

Eye muscle autoantigens

Eye muscle autoantigens possibly involved in GO include: calsequestrin, collagen XIII, flavoprotein, sarcalumenin and a protein called G2s. Most of these antigens are localized intracellularly, so they might represent not the primary event, but a secondary response, and contribute to maintaining rather than triggering the ongoing autoimmune reactions in the orbit (Bednarczuk et al. 2007).

1.1.3.3. Cells and cytokines involved in immune response

Autoimmunity is a consequence of failure to differentiate self-antigens from non-self-antigens and it can turn into autoimmune disorders. Defects may appear at various stages of the immune response to self-antigens: central and peripheral tolerance mechanisms, apoptosis, T and B cells, regulatory T cells and chemokines. In the early phases, two immune processes take place in the thyroid. In the first stage, the accumulation of antigen presenting cells in the thyroid of susceptible individuals is the most prominent sign of the initiation of the autoimmune reaction. This may be due to the inflammatory signals following damage or necrosis of the target cells caused by specific environmental factors. Alterations in the metabolism or microenvironment of thyroid cells may also be the inducing factor. If the immune tolerance is lost, the antigen presenting cells present thyroid antigens, interact with and activate T helper lymphocytes. These in turn stimulate a cellular and/or humoral response via B lymphocytes (Bottazzo et al. 1983). Thyroid cells may also express the major histocompatibility complex II (MHCII) molecules and produce various cytokines (Ajjan et al. 1997).

The cell target within the orbit of the autoimmune response remains to be defined, but fibroblasts and adipocytes are most likely to be the first involved. A diffuse infiltration of lymphocytes, with sparse lymphoid aggregates, is present in the extraocular muscle interstitial tissue and in the orbital fatty connective tissues of patients with GO (Weetman et al. 1989). The majority of these cells are T lymphocytes and macrophages, with sparse B lymphocytes. T helper (Th), T suppressive (Ts) and T cytotoxic (Tc) lymphocytes are present, with a slight predominance of the latter (Heufelder et al. 1993). Cell-mediated (Th1-type) immune reactions may predominate in the orbit in the early, inflammatory changes of the disease. The proinflammatory cytokines (IFN- γ , interleukin-2 (IL-2), and TNF- α) produced by these cells, along with IL-1 derived primarily from macrophages and fibroblasts, may prove responsible for the stimulation of the glycosaminoglycan (GAG) synthesis in orbital fibroblasts, and may function as mediators of inflammation in the early disease. The GAGs are water-binding proteins and accumulate in both the muscle and connective tissues. The same cytokines stimulate the expression of immunomodulatory molecules (HLA-DR,

CD54, heat shock protein-72 and CD40) on orbital fibroblasts, potentially enhancing the propagation of autoimmune responses within the orbit. Another feature of the cytokines relevant to GO is their ability to stimulate the proliferation of orbital fibroblasts. In the recovery phase of GO, Th2-type lymphocytes (inducing IL-4, IL-5, and IL-10) may play an important role and result in the late-stage fibrosis of the extraocular muscles (Prabhakar et al. 2003).

1.1.3.4. Fibroblasts

As reviewed by Prabhakar in (Prabhakar et al. 2003), fibroblasts are important in the pathogenesis of GO. They are the precursor cells that give rise to more specialized components of the connective tissue - myofibroblasts and adipocytes. Orbital fibroblasts act as both the target and effector cells in the autoimmune process. When activated by the proinflammatory cytokines, orbital fibroblasts produce chemokines that initiate lymphocyte recruitment, and inducible cyclooxygenase that produces extremely high levels of prostaglandin E₂. Under such stimulation orbital fibroblasts synthesize high levels of GAGs. Orbital fibroblasts include a subpopulation of cells (preadipocytes) which differentiate into adipocytes. TSH-R is expressed on the orbital fibroblasts of GO patients and its expression increases parallel to adipogenesis (Valyasevi et al. 1999). Fibroblasts also display CD40, an important activation molecule. It links with CD154 present on T and mast cells, which activates them.

1.1.3.5. Non genetic risk factors

The risk factors of the occurrence or progression of GO, similar to those leading to GD, have been partially identified (Table 1.1.). The list is probably much longer and future research should aim at identifying most of the factors. Some of the already identified can be prevented but other cannot (Bartalena et al. 2002).

Factor	Notes
Non-preventable (endogenous)	
Age	Incidence peaks in 5 th and 7 th decades, more severe disease stages in older age
Gender	More severe disease in males
Preventable (exogenous)	
Cigarette smoking	Association with GO, its prevalence, severity and reduced response to treatment
Thyroid dysfunction	Both hyper- and hypothyroidism may negatively affect outcome of GO
Radioiodine therapy	GO progression/new occurrence in 15% of patients after RAI treatment
Stressful life events	Association with GO
Molecular mimicry to infectious agents	To be defined

Table 1.1. Risk factors of GO occurrence/progression (Bartalena et al. 2002), modified.

Cigarette smoking greatly increases the risk of GO. The relative risk of smoking is 7,7 for GO, but only 1,9 for GD without the eye disease (Prummel et al. 1993). Smokers tend to have more severe ocular involvement. The severity of GO is significantly linked to current rather than lifetime tobacco consumption (Prummel et al. 1993; Pfeilschifter et al. 1996). In addition, cigarette smoking has been documented to delay the response to the orbital radiotherapy and high-dose systemic glucocorticoids (Eckstein et al. 2003).

Dysthyroidism is also associated with the severity of GO (Prummel et al. 1990). GO progressively improves after the restoration of euthyroidism (Prummel et al. 1989).

Initial treatment of GD by RAI leads to death of thyroid cells and release of antigens into the circulation. There is a subsequent dramatic increase in the levels of TRAbs and in cell-mediated immunoreactivity to TSH-R. During this phase, GO exacerbates in 15% of patients (Bartalena et al. 1998). The risk

decreases later in the course of the disease (Perros et al. 2005). Smoking and stress are associated with an increased risk of developing GD and a reduced remission rate after the termination of an antithyroid drug treatment (ATD).

Molecular mimicry of infectious agents is also believed to break the self-tolerance and generate the autoimmunity to TSH-R (reviewed in (Prabhakar et al. 2003)).

1.1.4. Clinical symptoms and evaluation

The presentation of GO is highly variable. Usually it is bilateral but it can be unilateral too. The onset is rapid or insidious. Photos are used for evaluation of the GO development, progression and treatment results (Wiersinga et al. 2006).

1.1.4.1. Upper and lower lid retraction

Normally the upper lid is located 1-1,5 millimeters below the superior limbus, and the lower lid is located at the inferior limbus. Upper lid retraction (Dalrymple sign), often with temporal flare and scleral show, is the most common ocular sign of GO (Photo 1.1.a,b). Mechanisms for upper lid retraction include sympathetic drive of the Müller muscle, fibrosis of the levator muscle, upgaze restriction, and proptosis. Lid retraction occurs in both the upper and lower lids because of the sympathetically innervated tarsal muscle in both lids. Lid lag on downgaze (von Graefe sign) is another important feature of GO. While slowly moving the fixation object downwards, the examiner should observe if the eyelid lags behind the globe.

Dalrymple sign, von Graefe sign, lower lid retraction, the width of lid fissure and lid closure should be documented for future comparison and observation of the course of GO (Photo 1.2.a,b).



Photo 1.1.a,b Eyelid retraction.

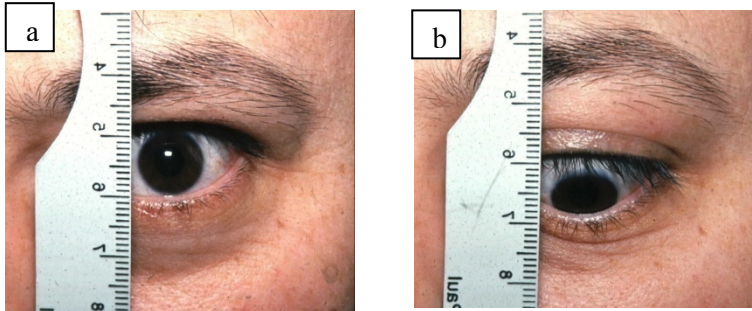


Photo 1.2. Lid assessment: a: width of lid fissure, b: lid lag on downgaze (von Graefe sign).

1.1.4.2. Soft tissue inflammation

Inflammatory changes may be assessed with the help of the Clinical Activity Score (CAS). Eyelid erythema, eyelid swelling, conjunctival redness, chemosis and inflammation of caruncle or plica are a part of it (Photo 1.3.a,b). Chemosis and periorbital edema are due to inflammation, and secondarily to decreased venous drainage from the orbit resulting from venous compression (Bahn et al. 1993).

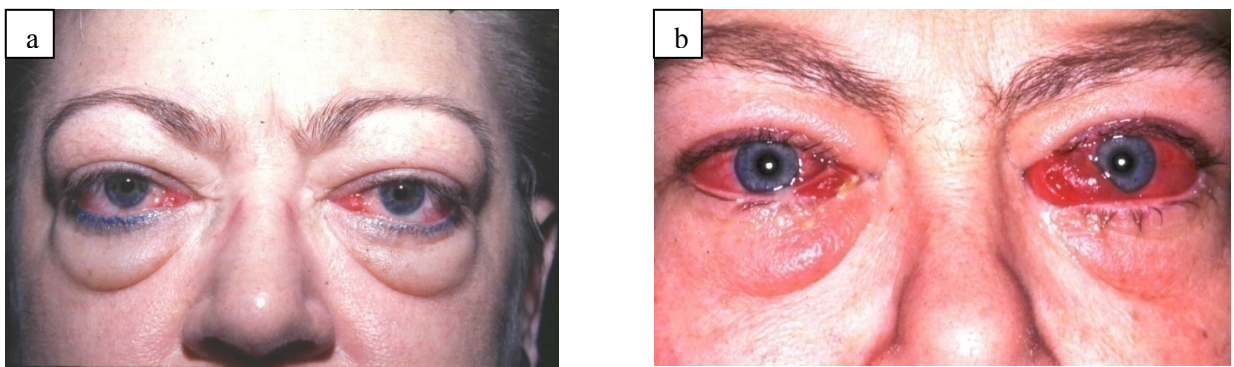


Photo 1.3.a,b. Inflammatory changes.

1.1.4.3. Exophthalmos (proptosis)

GO is the most frequent cause of uni- or bilateral proptosis in adults. The orbital fatty tissue and ocular muscle involvement are closely related to the degree of exophthalmos (Nishida et al. 2002) (Photo 1.4.a,b). The degree of proptosis can be estimated using the Hertel exophthalmometer.

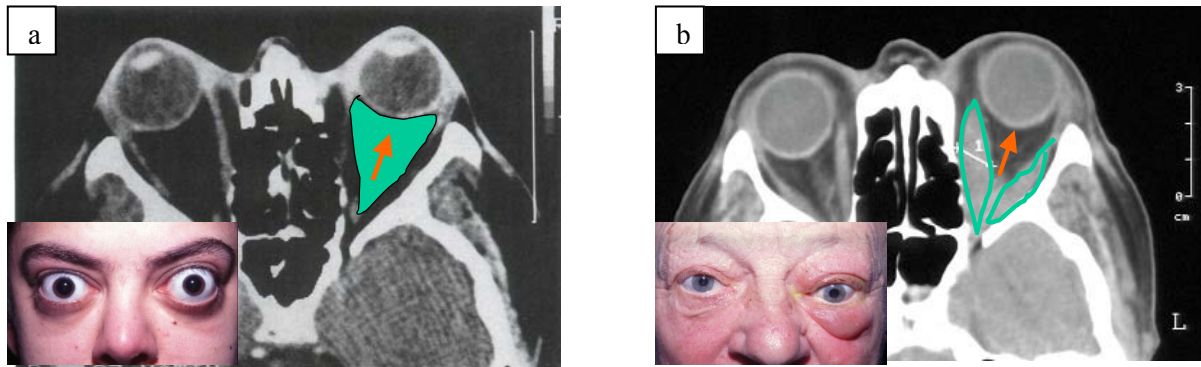


Photo 1.4. Proptosis mostly due to the increase in adipose (a) and muscle tissue volume (b).

1.1.4.4. Diplopia

Swelling and fibrosis, with consecutive contracture of extraocular muscles, may result in defective ocular motility in both orbits, even when the disease is clinically unilateral (Bahn 2001). Strabismus is common in GO, and it often presents as hypotropia or esotropia, since the inferior and medial rectus muscles are the most commonly involved extraocular muscles (Edsel et al. 2005). Some patients have no diplopia either due to symmetric involvement of both orbits, amblyopia or because the restriction affects the extremes of gaze irrelevant to daily life (Photo 1.5.a,b).

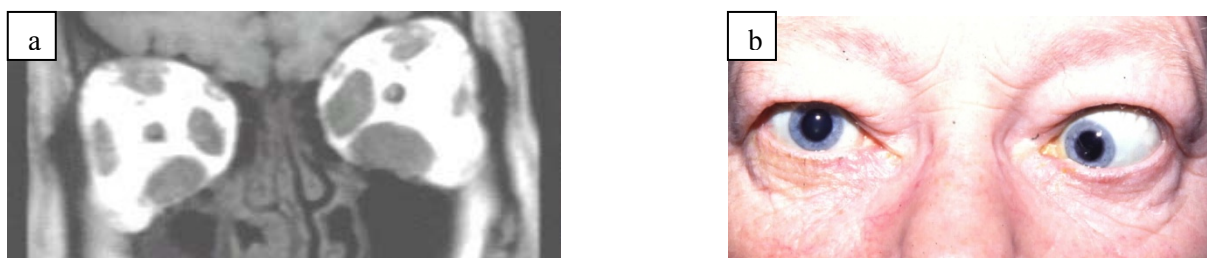


Photo 1.5.a,b Diplopia.

Certain tests performed in a sequence determine the presence of fusion, fusion vergences and deviations. These are established by cover tests and the prism cover test (Photo 1.6.a,b,c).

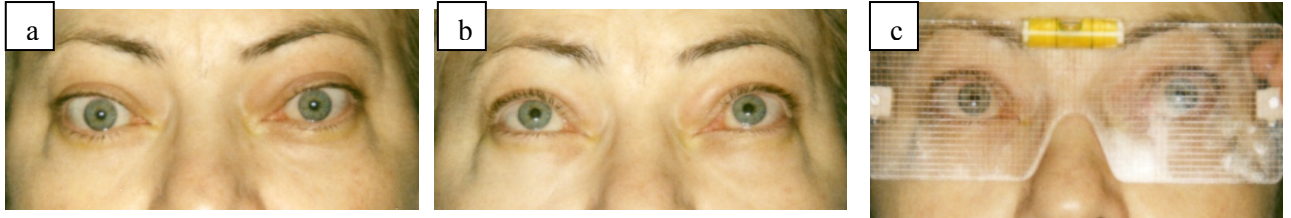


Photo 1.6.a,b,c Motility assessment: a: patient with upgaze deficiency and compensatory upward head position, b: ocular excursion measurement with light reflex method, c: ocular excursion measurement with Kestenbaum glasses.

The orthoptic assessment includes assessments of head tilt, squint angle if present, fusional amplitude, ocular ductions and the field of binocular single vision (Esser 1994).

1.1.4.5. Optic nerve compression

Extraocular muscle dysfunction and enlargement in the orbital apex may result from the GAG accumulation, edema and inflammation in the endomysial connective tissue, and may cause the optic nerve compression (3-5% of GO patients). It may occur with seemingly mild proptosis. For this reason it is important to record visual acuity, color vision, fundus assessment and the presence or absence of a relative afferent pupillary defect at each visit (Edsel et al. 2005). When suspecting the optic nerve compression, the visually evoked potentials, CT and MRI scans are helpful in making a diagnosis (Photo 1.7.a,b,c,d).

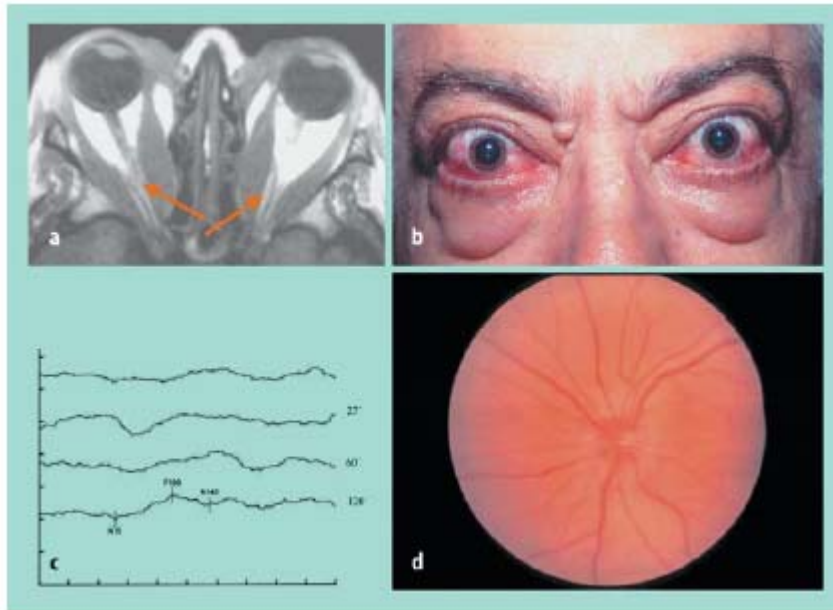


Photo 1.7. Optic nerve compression: a: MRI: extraocular muscle enlargement in orbital apex with straightened optic nerve, b: chemosis and periorbital edema caused primarily by decreased venous drainage from orbit, c: decreased visually evoked potentials, d: fundus: papilla edema.

1.1.4.6. Ocular surface damage

Due to the elevated lid aperture, impaired Bell's phenomenon, lagophthalmos, reduced tear production and tear film instability, the exposed cornea becomes dry and inflamed. The most severe cases may present corneal ulceration. The examination includes the basal tear secretion, Rose Bengal and fluorescein staining, impression cytology and break-up time (Eckstein et al. 2004; Heinz et al. 2004).

1.1.5. Activity and severity scores. Course of GO

A standard assessment of the activity and severity of the disease is a basis for a successful treatment. The activity of the disease, rather than its duration, is the prime determinant of the outcome of the immunosuppressive treatment in GO (Mourits et al. 1997; Terwee et al. 2005). The severity of the disease is the key indication for a therapy, while the therapeutic choice depends on the inflammatory activity. Therefore, Clinical Activity Score (CAS) (Mourits et al.

1997) (Table 1.2.) and the NOSPECS classification (Wiersinga et al. 1991; Gerding et al. 2000) (Table 1.3.) were developed - the former in 1997 by Mourits et al. and the latter in 1991 by Wiersinga et al. Both are widely used for their simplicity, reproducibility and descriptive character. At the first visit only the first 7 points of the CAS score can be used for evaluation.

Parameter	Score
Subjective activity signs (in the last 4 weeks)	
Spontaneous orbital pain	1
Gaze evoked orbital pain	1
Objective inflammation signs	
Eyelid erythema	1
Eyelid swelling considered as due to active GO (inflammatory phase)	1
Conjunctival redness in ≥ 1 quadrant considered as due to active GO (inflammatory phase)	1
Chemosis	1
Caruncle or plica inflammation	1
Progression signs (in the last 1-3 months)	
≥ 2 mm increase in proptosis	1
$\geq 8^\circ$ decrease in ocular excursion in any direction	1
Decrease of visual acuity equivalent to ≥ 1 Snellen line	1
Total score (max.)	10

Table 1.2. Clinical Activity Score (CAS) parameters.

Parameter\Score	0	1	2	3
Lid retraction	no	yes		
Soft tissue inflammation*	0	1-4	5-8	>8
Proptosis	<17mm	17-18mm	19-21mm	≥22mm
Site difference	<1mm	1-2mm	3-3.5mm	≥4mm
Extraocular muscle involvement	no		>20° upgaze >35° abduction	≤20° upgaze ≤35° abduction
Corneal defects	no	yes		
Optic nerve compression	no			yes

*upper lid edema 0-2, lower lid edema 0-2, conjunctival injection 1, conjunctival chemosis 1

Table 1.3. NOSPECS severity classification.

1.1.6. Natural history

In most cases the onset of ophthalmopathy is concomitant with the onset of hyperthyroidism, but the eye disease may precede or follow hyperthyroidism (Wiersinga et al. 2002).

The early phase of GO is the progressive deterioration (approximately 4-12 months) which, after reaching a plateau that lasts months or even years, is then followed by a slow but by no means complete improvement. In the Rundle's curve, the line depicting severity does not return to its baseline, in contrast to the activity line. The disease reaches inactivity (burnt-out stadium) after approximately 18-24 months (Hales et al. 1960).

A descriptive study by Perros et al. showed the natural course of GO. In 64,5% a spontaneous improvement was seen, in 22% the course of the disease was stable, while the progressive deterioration was seen in 13,5%. This has important implications for managing patients and is critical when assessing the effects of different treatments (Perros et al. 1995).

1.1.7. Prevention and treatment

1.1.7.1. Prevention of GO

Primary prevention (i.e. avoiding the occurrence of ophthalmopathy) is not feasible yet, but withdrawing from smoking by the GD patients' relatives might be important. In terms of secondary prevention (i.e. avoiding progression of a subclinical eye disease into overt and severe ophthalmopathy), apart from not smoking, early and accurate control of thyroid dysfunction (both hyperthyroidism and hypothyroidism), as well as an early diagnosis and treatment of a mild eye disease are also important. In terms of tertiary prevention (i.e. avoiding deterioration and complications of an overt disease), an early immunosuppressive treatment or orbital decompression as appropriate are essential. Withdrawal from smoking may increase the effectiveness of the immunosuppressive treatment (Wiersinga et al. 2002).

1.1.7.2. Treatment of GO

Mild forms of GO are sufficiently treated with symptomatic therapeutic interventions or local protective agents (lifting the head of the bed, eye drops, dark glasses).

The anti-inflammatory/immunosuppressive therapy is indicated in patients with moderate to severe active GO (CAS \geq 3 using 7 point score or CAS \geq 4 using 10 point score) or progressive GO (high or increasing NOSPECS score) without the tendency to spontaneous improvement. In uncertain cases the risk factors, i.e. the high/increasing TRAb values and smoking should also be included into the decision-making process.

Glucocorticoids, orbital radiotherapy or a combination of both are the most common immunosuppressants in active GO.

Glucocorticoids are effective on soft tissue, eye muscle involvement of recent onset and optic neuropathy. Orbital radiotherapy is particularly effective in

improving the eye muscle motility and decreasing the severity of diplopia. In very severe cases an immunosuppressive treatment should be considered (Bartalena et al. 2008). Very recently new drugs have been evaluated for their usefulness in the antiinflammatory treatment of GO. Most promising results have been achieved with rituximab (Salvi et al. 2007; Salvi et al. 2008).

Some GO patients require a surgical treatment. Orbital decompression, strabismus surgery and eyelid surgery, following this order, can improve their visual functions and appearance. If they do not require orbital decompression, then besides inactivity, an interval of 6 months of clinical stability is recommended (Eckstein 2010).

1.2. Autoimmune thyroid disease (AITD)

1.2.1. Overview

Autoimmune thyroid diseases (AITDs) are common polygenic multifactorial disorders. Environment contributes importantly to their emergence. The autoimmune thyroid diseases include Graves' disease (GD), Hashimoto's thyroiditis (HT), painless thyroiditis and atrophic thyroiditis. In the present study the AITD term refers to GD and HT. GD is a systemic condition which usually presents with hyperthyroidism and ophthalmopathy (GO), and occasionally, dermopathy and acropachy. GD is due to antibodies to the thyrotropin (TSH) receptor (TSH-R) activating the receptor that stimulates the growth of the thyroid and excretion of the thyroid hormones (Rapoport et al. 1998). GD is characterised by follicular hyperplasia and patchy lymphocytic infiltration of the thyroid (Armengol et al. 2001).

Thyroid autoantibodies against thyroid peroxidase (TPO) and thyroglobulin (Tg) in serum, plus varying degrees of thyroid dysfunction are characteristic for HT. It is considered to result from an immune response leading to aberrant infiltration of autoantigen specific lymphoid cells and destruction of thyroid follicles that results in hypothyroidism (Weetman 1996).

1.2.2. Diagnosis

The diagnosis of AITD is made on the basis of a physical examination and laboratory tests (thyroxine, triiodothyronine, TSH, TRAb, TPO Ab). In some cases imaging modalities are performed, e.g. thyroid ultrasonography or a radioactive iodine uptake test.

1.2.3. Treatment

Three main thyroid treatment options for patients with GD are the antithyroid drug (ATD) treatment, radioiodine therapy (RAI) and surgery. ATD is the first choice therapy in Europe. It is usually discontinued after 12-18 months. In case of relapse of hyperthyroidism, RAI, thyroidectomy or a different course of the ATD therapy are suggested, according to medical indications and the patient's wishes. The management of hyperthyroidism may play a role in the course of GO. The ATD therapy and thyroidectomy are associated with a gradual decrease in TRAb levels, whereas RAI increases TRAb levels within one year. While the ATD therapy and thyroidectomy are rarely associated with deterioration of the disease, RAI carries the risk of progression of ophthalmopathy in approximately 15% of patients, especially the high-risk patients who smoke, have severe hyperthyroidism, high levels of TRAb or a preexisting eye disease. However, the risk of the radioiodine-associated progression of the orbitopathy can be eliminated by a concomitant treatment with middle-dose glucocorticoids (Bartalena et al. 2008).

In case of hypothyroidism in the course of HT, a substitution therapy with levothyroxine is the method of choice.

1.2.4. Risk of relapse of hyperthyroidism

The recurrence rate of hyperthyroidism after termination of the ATD therapy amounts to 60% (Orgiazzi et al. 2002). But if the high TRAb levels persist in circulation during ATD therapy, discontinuation of the therapy almost invariably leads to the relapse of hyperthyroidism (Schott et al. 2004). Additionally, numerous controlled prospective studies performed in many parts of the world, with varying iodine intakes, have all confirmed the main initial features related to

the subsequent risk of relapse as: smoking, young age, male gender, a goiter larger than 40 millilitres, a hypoechogenic and hypervascular gland, presence of GO and the severity of hyperthyroidism (Orgiazzi et al. 2002).

1.3. G Protein and signal transduction

1.3.1. Structure and function of G protein, G protein cycle

Heterotrimeric G proteins (guanine nucleotide-binding proteins) consist of a large family of different α -, β - and γ -subunits. Coded by different genes, 5 β -, 13 γ - and more than 20 α -subunits are currently known. In addition, some of them have splice variants or are differentially modified. There are well over a thousand possible G protein heterotrimer combinations (Hildebrandt 1997). The $\beta\gamma$ -subunits should be regarded as functional monomers.

In a resting state, the guanosine diphosphate (GDP) bound α -subunit is associated with $\beta\gamma$ -dimer in the inner leaflet of the plasma membrane (Table 1.4.). After the activation of a coupling receptor, the α -subunit releases GDP in exchange for guanosine triphosphate (GTP), and the $\beta\gamma$ -subunit dissociates from the α -subunit. Both, the free α - and $\beta\gamma$ -subunits can direct the activity of a variety of effectors. TSH-R is coupled with G protein that activates the adenylyl cyclase. The α -subunit possesses intrinsic GTPase activity which hydrolyses the bound GTP to GDP after the activation. Then the $\beta\gamma$ -subunit reassociates with the α -subunit, thus ending the activation cycle (Bourne 1997).

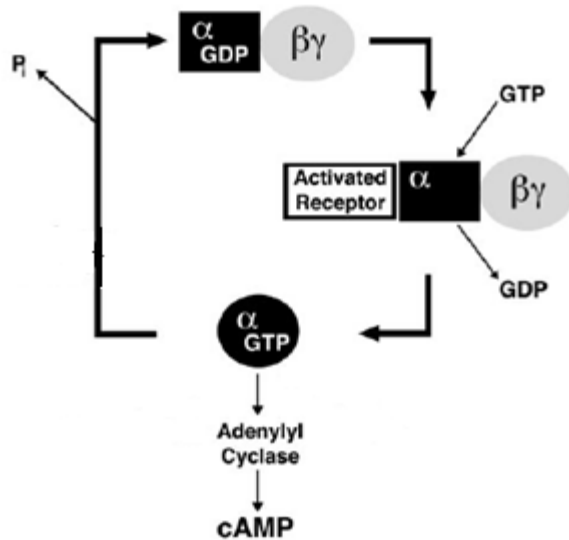


Table 1.4. G protein cycle discussed above (Weinstein et al. 2001).

1.3.2. Structure and function of $G\alpha$ -subunit

The α -subunit is present in all cells of a human body except mature spermatozoa. The effects of its stimulation by the seven-transmembrane receptor family or various tyrosine kinase receptors include the activation of adenylyl cyclase that boosts the intracellular cyclic adenosine monophosphate (cAMP) concentration. Consequently, the cAMP-dependent signal cascades can be either inhibited or activated (Frey 2006).

Types of G proteins:

- $G_{\alpha s}$ activates the cAMP-dependent pathway by stimulating the production of cAMP from ATP by the enzyme adenylyl cyclase.
- $G_{\alpha i}$ inhibits the production of cAMP from ATP.
- $G_{\alpha q/11}$ stimulates the membrane-bound phospholipase C beta, which then cleaves PIP_2 (a minor membrane phosphoinositol) into two second messengers, inositol trisphosphate and diacylglycerol.
- $G_{\alpha 12/13}$ is involved in the Rho family GTPase signaling and controls cell cytoskeleton remodeling, thus regulating cell migration.
- sometimes $G_{\beta\gamma}$ has active functions.

Many of the subunits are expressed ubiquitously, while some have temporal and/or differential expression.

TSH-R acts via Gas. The regulation of the thyroid cell proliferation in humans is dependent on TSH and a number of other growth factors, such as the epithelial growth factor, insulin-like growth factor-1, insulin and transforming growth factor α . TSH modulates the effects of these growth factors on thyrocytes (Siffert et al. 1995; Sheu et al. 2005).

Stimulation of Gas-coupled receptors leads to apoptosis in cells, which influences the course and response to the therapy of various tumors and inflammatory diseases.

In addition, different metabolic pathways are regulated by Gas. Inhibiting the expression of Gas in fibroblasts accelerates their differentiation into adipocytes (Wang et al. 1992).

1.3.3. Structure and function of G $\beta\gamma$ -dimers

G β -subunits, including G β 3, belong to the family of propeller proteins and consist of 7 regular domains defined by conserved aminoacid motifs, including tryptophan and aspartate (Roskopf et al. 2003).

The growth factors mentioned above and TSH act through three major signal transduction pathways, including the adenylyl cyclase, phospholipase C and tyrosine kinases, in order to induce phosphorylation and ultimately switch on the thyrocyte proliferation machinery. All the pathways are positively regulated by the $\beta\gamma$ -subunits of heterotrimeric G proteins (Clapham et al. 1993).

1.3.4. GNAS1 and GNB3 genes. GNAS1 T393C and GNB3 C825T polymorphisms

Polymorphisms are sequence variations in the human genome with the $\geq 1\%$ allele frequency. The exchange of a single nucleotide, commonly referred to as

the single nucleotide polymorphism (SNP), accounts for approximately 80% of all sequence variants (Sadee et al. 2001). The loss-of-function and gain-of-function mutations (as well as imprint defects) of the GNAS1 gene lead to diverse clinical phenotypes. Because of the fundamental significance of GNAS1 (GNB3 to lesser extend) for signal transduction, these genomic gene modifications are expected to lead to pleiotropic manifestations and are suited for prediction of the risks and courses of diseases, as well as responses to and side-effects of therapies (drugs, irradiation etc.). Somatic (sporadic) mutations in GNAS1 gene, either activating or inactivating, have been implicated in some tumors and rare genetic disorders. Mutations which produce constitutively active forms of G α can lead to endocrine tumors and excess hormone secretion (Weinstein et al. 2001).

Besides mutations in TSH-R, the somatic activating mutations in the G α gene may cause toxic thyroid adenomas, although less frequently (Duprez et al. 1998).

GNAS1 T393C

The GNAS1 human gene is localized on the 20q13.2 chromosome and consists of 13 exons, as well as 3 additional upstream promoters that generate alternative gene products. G α s is preferentially expressed from the maternal allele in human thyroid glands (genomic imprinting) (Liu et al. 2003).

A common C393T polymorphism is T to C substitution at the 393 nucleotide position. It is located in the exon 5 of the GNAS1 gene encoding the α -subunit of heterotrimeric G proteins (numbering according to the cDNA sequence) (Frey et al. 2006; Frey 2006). Although this nucleotide exchange is silent and does not modify the coded aminoacid (isoleucine), it changes the folding structure of the messenger ribonucleic acid (mRNA). Therefore, genotype-dependent differences in mRNA due to the altered secondary structure could finally cause differences in G α s mRNA expression (Sheu et al. 2005).

It can be deduced from ethnic distributions that from the evolutionary perspective (relative to Caucasians), “the original state” is the T393 allele. Such differences in genotype distribution in ethnic groups generally indicate that the associated phenotypes were important for evolution and brought their carriers certain advantages (Frey et al. 2006).

GNB3 C825T

The GNB3 human gene is localized on the 12q13 chromosome and is composed of 11 exons and 10 introns (Rosskopf et al. 2000). A common C825T polymorphism is the C to T change at the 825 nucleotide position. It is located in the exon 10 of the GNB3 gene encoding the $\beta 3$ -subunit of heterotrimeric G proteins (Siffert et al. 1998). The 825T allele associated with alternative splicing of the exon 9 gives rise to a truncated splice variant termed G $\beta 3$ -s. This splice variant lacking 123 nucleotides gives rise to a functionally active protein missing 41 aminoacids (Siffert et al. 1999). Another aberrant splicing process was identified. It affects the exon 10, giving rise to a splice variant referred to as G $\beta 3$ -s2 (Rosskopf et al. 2003).

The GNB3 825T allele is associated with enhanced G protein activation resulting in increased cell proliferation (Siffert et al. 1995). Overexpression of G $\beta 3$ -s and G $\beta 3$ -s2 enhances receptor-mediated GTP γ S binding that is measure for the G α GDP/GTP exchange. Therefore, the G $\beta 3$ -s γ or G $\beta 3$ -s2 γ dimers may actually interact with the G α proteins and for instance, destabilize the G α -GDP binding, which could facilitate the receptor-mediated G α -GDP/GTP exchange (Rosskopf et al. 2003). Additionally, G $\beta 3$ -s appears to be associated with the enhanced immune cell function in humans (Virchow et al. 1998). Lindemann et al. (2001) demonstrated that the GNB3 825T allele influenced cellular immune responses toward recall antigens and the interleukin-2 stimulation. Furthermore, lymphocyte chemotaxis and CD4⁺ T cell counts were significantly enhanced in individuals homozygous or heterozygous for the 825T allele. The conclusion was that the 825T allele status is predictive of immunocompetence and that GNB3 could be a candidate gene in disorders associated with an inadequate immune response (Lindemann et al. 2001).

The 825T allele is most frequently found in Blacks - 79%, East Asians show an intermediate frequency - 42%, while the lowest frequencies are found in Caucasians - 30%. In nonhuman primates, the C alleles are present at the GNB

825 locus, suggesting that C is the ancestral state at these positions (Roskopf et al. 2000).

1.3.5. Association between GNAS1 T393C and GNB3 C825T alleles and different diseases

G protein defects can cause a disease in several ways. They may result in the production of a G protein that cannot hydrolyze GTP and terminate the signal, which leads to increased activity of the downstream effector, even in the absence of extracellular stimuli. Decreased production of a normal G protein or production of an unstable G protein can reduce the normal response to hormonal stimulation. Abnormalities affecting the ability of a G protein to switch to the “on” state may result in an increase or decrease in the downstream signal. An increase occurs when the defective protein releases GDP and binds GTP more rapidly than normally, whereas a decrease, when the protein releases GDP more slowly or binds GTP less avidly (Farfel et al. 1999).

The GNAS1 T393C status is a predictive marker for the clinical outcome of colorectal, bladder and renal cell cancer (Frey et al. 2005; Frey et al. 2005; Frey et al. 2006).

In most studies, the GNB3 825T allele increased the risk for obesity (Siffert et al. 1999), hypertension (Schunkert et al. 1998), coronary heart disease (Naber et al. 2000), stroke and depression (Zill et al. 2000) in the white population of European descent. Furthermore, the GNB3 825T allele might serve as a pharmacogenetic marker to predict the responses to diuretics (Turner et al. 2001), antidepressants (Zill et al. 2000), endothelin-1 and angiotensin II (Wenzel et al. 2002), to name but a few.

In the thyroid, adenomas showed a statistically significantly lower frequency of the 825T allele, which however remains of unknown biological significance (Sheu et al. 2005).

1.4. Aim of the study

Till now there are only a few clinical and serological parameters useful for prognostic statements in the clinical routine. In the genetic field there are many candidate genes and chromosomal regions which underscore the complexity and genetic heterogeneity of AITD and GO. However until now no strong susceptibility gene which can identify the patients with a higher risk for the disease progression or a poor therapy response has been found.

TSH-R is the central autoantigen for both GD and GO. The TSH-R autoantibody levels are associated with the prevalence, severity and response to the treatment in the thyroid and orbit disease. However there is no strong genetic linkage to the TSH-R gene. Like many other seven-transmembrane receptors, TSH-R is G protein-coupled. The TSH binding causes a conformational change in TSHR which allows it to act as a guanine nucleotide exchange factor. TSH-R activates the G protein by exchanging its bound GDP for GTP. The G protein α -subunit, together with the bound GTP, can then dissociate from the β and γ subunits to further affect intracellular signaling proteins or target functional proteins directly, depending on the α subunit type.

Therefore, the G protein genetic modifications may have significant impact on the TSH-R signaling and may affect the course of Graves' disease.

The aim of the study was to assess the possible role of the GNAS1 T393C polymorphism genotypes of the G protein subunit G α , and the GNB3 C825T polymorphism genotypes of the G protein subunit G β 3 in the pathogenesis and course of GD and GO.

For that reason a group of patients with Graves' orbitopathy, with and without the associated thyroid disease, was evaluated for the following parameters: the course of orbitopathy (mild/severe), remission or relapse rates of hyperthyroidism, levels of the TSH-receptor autoantibodies and thyroid parameters. The GNAS1 T393C polymorphism genotypes of the G protein subunit G α and the GNB3 C825T polymorphism genotypes of the G protein subunit G β 3 were then analysed in these patients and in a control group in relation to the above mentioned parameters.

The final aim was to find out if these polymorphisms were suitable biomarkers, compared to the levels of the TSH-receptor autoantibodies which today are the only biomarkers for monitoring the GO/GD patients.

2. Patients, materials and methods

This is a case-control association study.

2.1. Patients

2.1.1. Database patients

Since November 2000 till January 2008, 916 patients agreed to be included in the GO serum sample bank and database in the Center for Ophthalmology of the University Hospital in Essen. Their blood samples were taken in different stages of the disease. The clinical examination (standard protocol) for the database and the blood collection were carried out the same day.

Patients with infectious diseases (hepatitis B, C, HIV), under an anticoagulant treatment or with severe chronic diseases (e.g. tumors) were excluded from the database.

2.1.2. DNA donors

Blood samples from 359 patients of the GO database of Caucasian origin were available.

2.1.3. Inclusion criteria

Our patients met the following inclusion criteria:

The diagnosis of GO and AITD type Graves' disease = with hyperthyroidism (n=325)

- typical symptoms of GO
- initially, or at least once over the course of the disease, pathological TRAb levels and suppressed TSH;

For classifying GO (n=359)

- initial presentation within 6 months after the GO onset
- an ophthalmologic assessment 11-14 months after the GO onset;

For classifying the course of hyperthyroidism (GD) (n=272)

- an initial treatment of hyperthyroidism with the ATD treatment for one year
- follow-up for at least 24 months.

The GD remission was defined as a period of at least 12 months of euthyroidism after the termination of the ATD therapy (TSH and thyroid hormones within a normal range). When GD relapsed, patients were referred for a radioiodine therapy, thyroidectomy or a second one-year cycle of the ATD therapy, according to the medical indications and a patient's individual preferences. A definitive treatment (thyroidectomy or radioiodine therapy) of the thyroid was performed in case of relapse of GD or uncontrolled hyperthyroidism under the high-dose ATD therapy.

The missing information regarding laboratory values and a prior treatment was obtained from either an endocrinologist or a general practitioner.

2.1.4. Standard protocol for GO assessment

1. History was taken using a standard form:
 - a. endocrinological history
 - first symptoms and first diagnosis of thyroid dysfunction
 - previous treatment of thyroid hyperfunction
 - present treatment of thyroid hyperfunction
 - name and address of endocrinologist or general practitioner;

- b. ophthalmological history
 - first symptoms and first diagnosis of GO
 - previous treatment of GO
 - present treatment of GO
 - name and address of ophthalmologist
 - present complaints (spontaneous and gaze evoked orbital pain; decrease in eye motility, visual acuity and proptosis increase in last 3 months);
 - c. general history
 - height and weight
 - other diseases and treatment
 - tobacco smoking.
2. A standard GO clinical assessment included the following:
- a. Visual acuity
 - b. A lid function (significant lid retraction was defined as 1mm or more scleral show or a side difference of ≥ 1 mm)
 - c. Intraocular pressure - measured with the Goldmann tonometer in the primary position and upgaze
 - d. Proptosis (significant proptosis was the proptosis of 17mm or more and the ≥ 1 mm side difference)
 - e. The eye position and motility (significant impairment was defined if monocular excursions were reduced by $\geq 5^\circ$)
 - f. Presence or absence of corneal defects
 - g. Presence or absence of the optic nerve compression
 - h. CAS
 - i. NOSPECS

Concerning GO, follow-up visits were performed every 3 months until the disease had been inactive for at least 6 months, or the surgical rehabilitation of the pathology was completed. Clinical activity was assessed according to the 10 point score (CAS) of Mourits et al. (Mourits et al. 1997).

During the follow-up, all patients with ≥ 4 CAS values (in cases of severe impairment of motility also when CAS=3) were offered a systemic steroid treatment. The regimen for a mild and moderate disease began with 1,5 milligrams of fluorocortolone orally per kilogram of body weight, tapered by 10 milligrams every 4 days. In severe cases, intravenous steroids were given every other day for 5 days, a single dose of 500 milligrams of prednisolone hemisuccinate per day. This scheme was followed by the oral regime as in the mild disease.

Orbital irradiation was performed if patients suffered from the impaired ocular motility or if the clinical activity increased after the steroid withdrawal (regimen: total dosage usually 12-20 Gy). The severity of orbitopathy was recorded applying the modified NOSPECS classification as described before (Eckstein et al. 2006). 11 to 14 months after the onset of GO, patients were grouped according to the severity of the disease. The mild course was defined as CAS < 4 (almost an inactive disease) and/or NOSPECS < 5 (mild signs of GO), whereas the severe course, as CAS > 4 (still an active disease) and/or NOSPECS > 5 (marked signs of GO) (Table 2.1.).

Course of GO	NOSPECS	CAS
Mild	0-4	0-3
Severe	5-14	4-10

Table 2.1. Classification of GO course in two groups according to CAS and NOSPECS scores (if either CAS or NOSPECS belonged to severe group, the course was classified as such).

2.1.5. Healthy control group

820 healthy blood donors for the GNAS1 T393C polymorphism and 1855 healthy blood donors for the GNB3 C825T polymorphism assay were recruited at the Department of Transfusion Medicine of the University Hospital in Essen. They were Caucasians of both genders, 18-65 years old, with weight of over 50 kilograms, and under no medication treatment or drug abuse. All samples were

collected randomly from the blood donors. These control groups were previously characterized in detail (Siffert et al. 1999).

2.2. Materials

The whole venous blood was obtained from each patient with the help of “Butterflies” (Dispomed Ecoflo), the brown serum and red EDTA tubes (Sarstedt). The serum was analyzed for TSH, free thyroxine, free triiodothyronine and the TRAb values. The whole blood was used for the DNA isolation. Afterwards, the samples were stored in -80°C.

2.3. Experimental procedures

2.3.1. DNA isolation

QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used for DNA purification from the whole blood EDTA samples. 20µl Proteinase K, 200µl blood sample and 200µl Buffer AL were added to a microcentrifuge tube, mixed by pulse-vortexing for 15 seconds and incubated at 56°C for 10 minutes. Then, 200µl 100% ethanol was added and the sample was again mixed by pulse-vortexing for 15 seconds. Afterwards, the mixture was carefully applied to the QIAamp Spin Column and centrifuged at 8000rpm for 1 minute. The spin column was in the next step placed in a clean collection tube, and the tube containing the filtrate was discarded. 500µl Buffer AW1 was added and centrifuged at 8000rpm for 1 min. The spin column was placed in a clean collection tube, and the tube with the filtrate was discarded. The same procedure was repeated with 500µl Buffer AW2 with centrifugation at 14000rpm for 3 minutes. Then 200µl Buffer AE was added, incubated at room temperature for 1 min and centrifuged at 8000rpm for 1 minute. The samples containing the isolated DNA were stored in -20°.

2.3.2. Polymerase Chain Reaction (PCR)

Appropriate volumes of the Taq PCR MasterMix (Eppendorf, Germany) (Table 2.2.), primers (Invitrogen, Germany) (Table 2.3.) and aqua pro iniectionae were dispensed into the PCR tube and mixed, avoiding formation of foam. Then, the template DNA was added (Table 2.4.). As PCR is a sensitive technique capable of amplifying even trace amounts of DNA, the components (excluding template DNA) were mixed in DNA-free environment using aerosol-resistant barrier tips, to avoid contamination. Template DNA was added afterwards in a separate room. In the next step, the PCR program was started on the thermal cycler. After 5-minute denaturation at 94°C, 38 cycles of DNA amplification were performed for 30 seconds at 94°C (denaturation), 40 seconds at 64°C (annealing) and 45 seconds at 72°C (extension).

Component	Concentration
Taq DNA Polymerase	1,25 U
KCl	50mM
Tris-HCl	30mM
Mg ²⁺	1,5mM
Igepal-CA630	0,1%
Each dNTP	200µM

Table 2.2. Components of Eppendorf MasterMix (2,5x) and their final concentrations in PCR.

	GNAS1 T393C	GNB3 C825T
Forward primer (sense)	FOK2 5'-TGTGGCCGCCATGAGCAA-3'	BSE1 5'-GCTGCCCCAGGTCTGATCCC-3'
Reverse primer (antisense)	FOK1 5'- TAAGGCCACACAAGTCGGGGT-3'	BSE2 5'- TGGGGAGGGTCCTTCCAGC-3'

Table 2.3. Names and sequences of oligonucleotide primers used for PCR.

Components	GNAS1 T393C	GNB3 C825T
Eppendorf MasterMix (2,5x)	13µl	12µl
Forward primer	1µl	2µl
Reverse primer	1µl	2µl
Aqua pro iniectionae	15µl	14µl
Template DNA	2µl	2µl

Table 2.4. Components used for PCR.

2.3.3. Restriction and visualisation of final products

In the presence of a buffer (NEB and MBI Fermentas, St.Leon-Rot) and aqua pro iniectionae, the PCR products were digested using a restriction enzyme (NEB and MBI Fermentas, St.Leon-Rot) (Tables 2.5. and 2.6.) at 56°C for 3 hours (GNAS1 T393C) and 1,5 hour (GNB3 C825T) respectively. The final products were separated by electrophoresis on a 2,5% agarose gel, and visualized by ethidium bromide staining (Sigma, Deisenhofen, Germany) under ultraviolet light. Photos were taken and analysed.

Components	GNAS1 T393C	GNB3 C825T
Restriction enzyme	0,1µl	0,2µl
Puffer Y Tango	2,5µl	3µl
Aqua pro iniectionae	17,4µl	16,8µl

Table 2.5. Restriction components.

	GNAS1 T393C	GNB3 C825T
Restriction enzyme	BSeGI 5'-NNCATCC-3'	BSED1 5'-CCNNGG-3'

Table 2.6. Names and sequences of restriction enzymes.

The CC sequence is recognized and cut by restriction enzymes. Therefore it is assumed that in GNAS1 T393 the unrestricted products (145bp) represent the TT genotype, and the completely restricted products (78 and 77bp) represent the CC genotype. The presence of both the unrestricted and restricted products indicates the heterozygous TC genotype (Photo 2.1.).

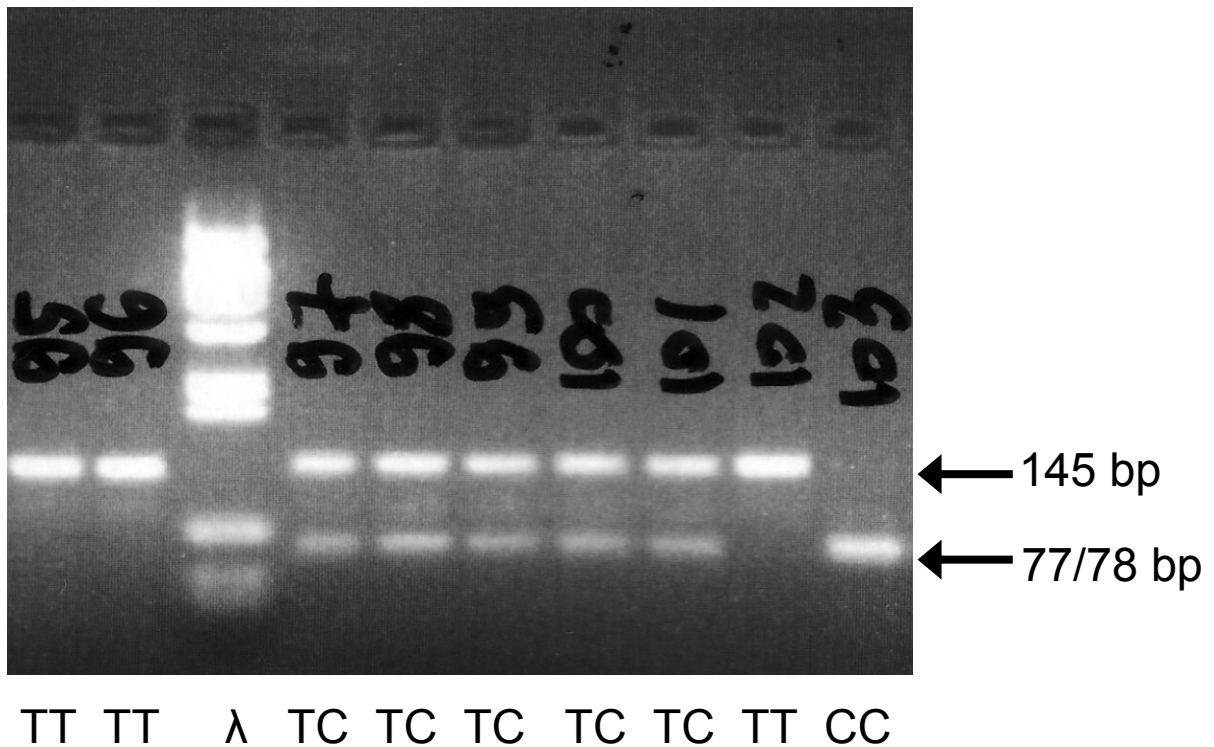


Photo 2.1. Photograph of agarose gel representing electrophoresis outcome of restricted and unrestricted PCR products of GNAS1 T393C gene. Handwritten numerals indicate ID numbers of patient samples.

Analogically, in GNB3 C825T, the unrestricted products (269bp) represent the TT genotype, and the completely restricted (153 and 116bp), the CC genotype. The presence of both unrestricted and restricted products indicates the heterozygous TC genotype (Photo 2.2.).

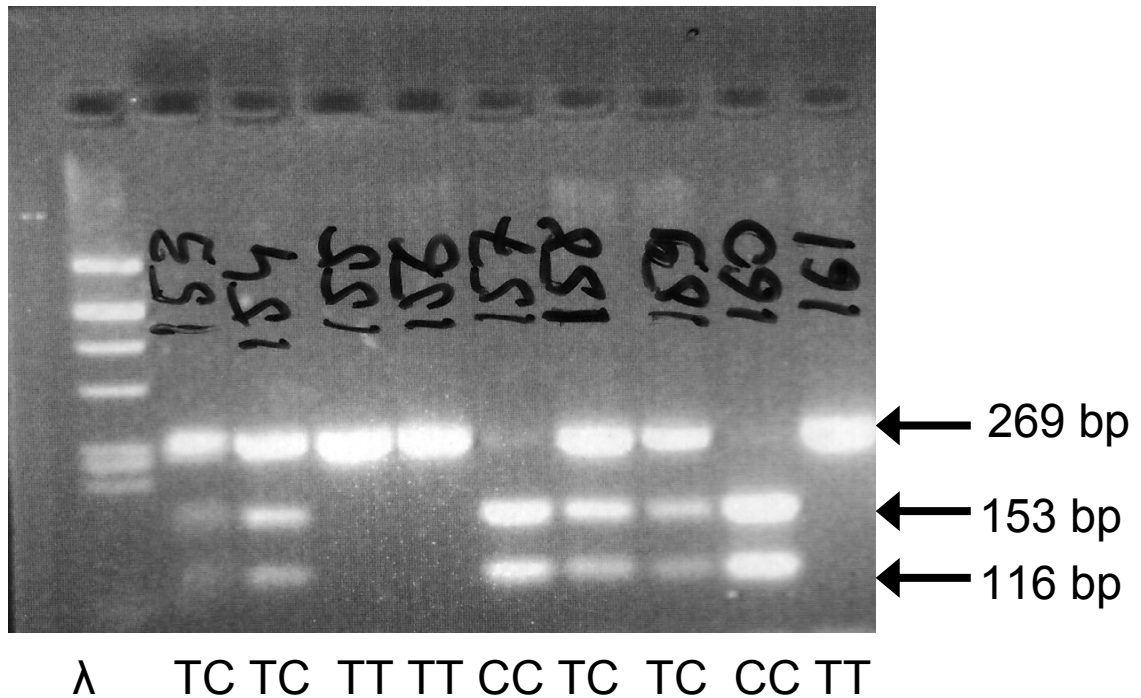


Photo 2.2. Photograph of agarose gel representing electrophoresis outcome of restricted and unrestricted PCR products of GNB3 C825T gene. Handwritten numerals indicate ID numbers of patient samples.

2.3.4. Thyrotropin receptor antibody (TRAb) assay

TRAb were measured using the second generation TSH binding inhibitory (TBII) assay based on the TSH-R human recombinant (Costagliola et al. 1999) (TRAK human LIA®, B.R.A.H.M.S AG, Hennigsdorf/Berlin, Germany). This assay is calibrated in international units (IU) based on the standard WHO reference, MRC 90/672. As described in more detail by Schott et al. the 50% inhibition of tracer binding corresponds to 7-8 IU/l. Values ≥ 1.5 IU/l (about 10% of tracer binding) were regarded as positive, values between 1 and 1.5 IU/l as borderline and values < 1.0 IU/l as negative (Schott et al. 2000). The TRAb values were evaluated at the GD onset, 6 months after the beginning of the ATD therapy, as well as 6 months after the GO onset.

2.4. Statistic analysis

Group comparisons were performed with the chi-square test for categorical variables, and with the t-test for normally distributed continuous variables. The Mann-Whitney or Kruskal-Wallis tests were used for non-normally distributed continuous variables. Stepwise logistic regression was carried out in order to establish whether and to what extent the course of hyperthyroidism was influenced by the following parameters: the GNAS1 genotype, TRAb levels 6 months after the beginning of the ATD treatment, the course of GO, age, gender, and smoking. The ROC plot analysis was performed in order to define the TRAb cut-off levels for the prediction of the GO and GD courses.

For all the tests, p values less than 0,05 were considered significant. Analyses were performed with the GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA), SPSS 15.0 (SPSS, Chicago, IL, USA) and SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

2.5. Medical Ethics Committee approval

Written consent was obtained from all the participants. The study was approved by the Medical Ethics Committee of the University of Essen.

3. Results

3.1. Patient characteristics

3.1.1. Age at GO onset

The incidence was the highest in 30-60 years old patients (Fig. 3.1.). The median age was 47,9 years (15,7-83,0).

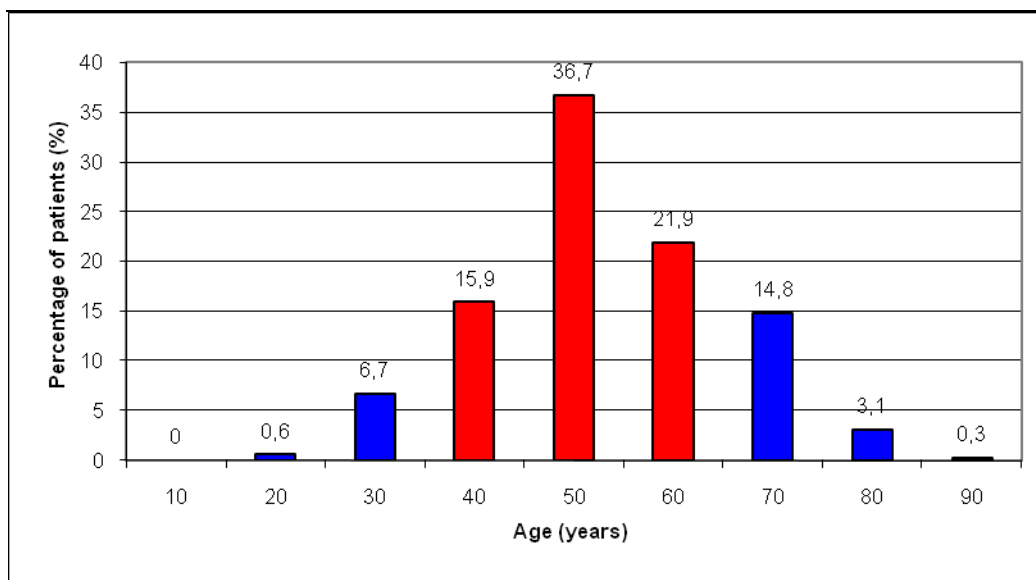


Fig. 3.1. Age of patients at GO onset.

3.1.2. Gender distribution

There were 307 (85,5%) females and 52 (14,5%) males. The female: male ratio equaled 5,9:1.

3.1.3. Smoking

There were slightly more smokers than nonsmokers (188 vs. 171; 52,4% vs. 47,6%).

3.1.4. Duration of hyperthyroidism at GO onset

On average Graves' hyperthyroidism was diagnosed 2 months (median) before the onset of GO. However, this relation varied ranging from 30,4 years before to 3 years after the onset of GO. The majority (67,2%) of patients manifested GO simultaneously or within 6 months before or after the onset of the thyroid disease. GO was rarely present (1,5%) earlier than 6 months before GD onset. In contrary, the GO onset later than 6 months after the GD onset was more frequent (31,3%) (Fig. 3.2.).

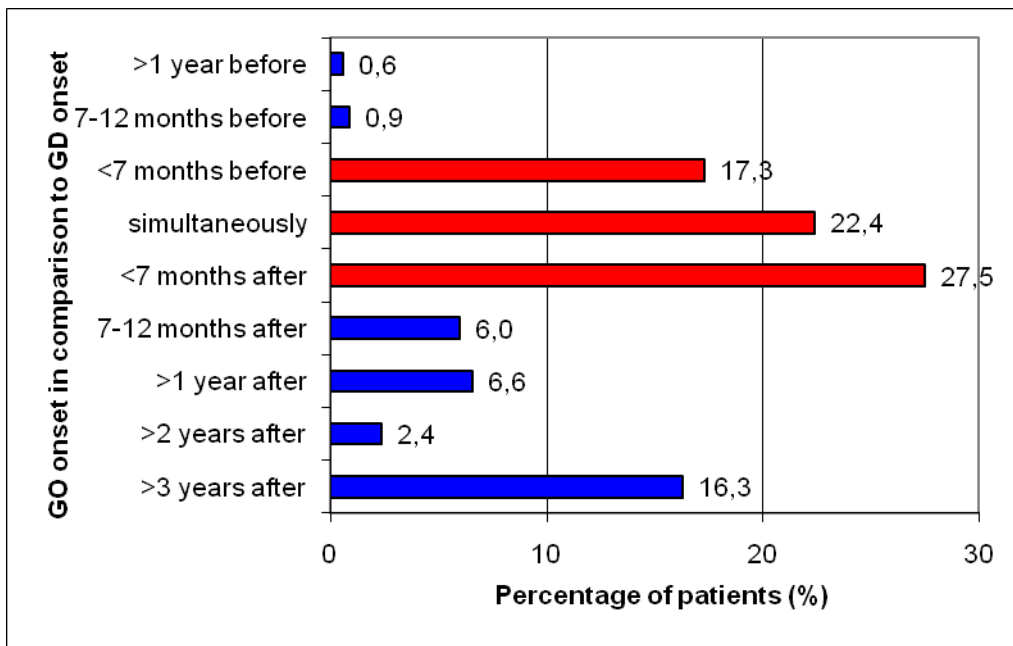


Fig. 3.2. Time relation between GO and GD onsets.

3.1.5. Frequency of GO signs

When classifying the course of GO, 11 to 14 months after its onset, the prevalence of the signs of GO was evaluated. The most common GO sign was

the soft tissue inflammation, followed by proptosis. The least frequent sign was the optic nerve compression (Fig. 3.3.). The mean (SD) CAS and NOSPECS values equaled 3,1 (2,3) and 5,0 (2,9).

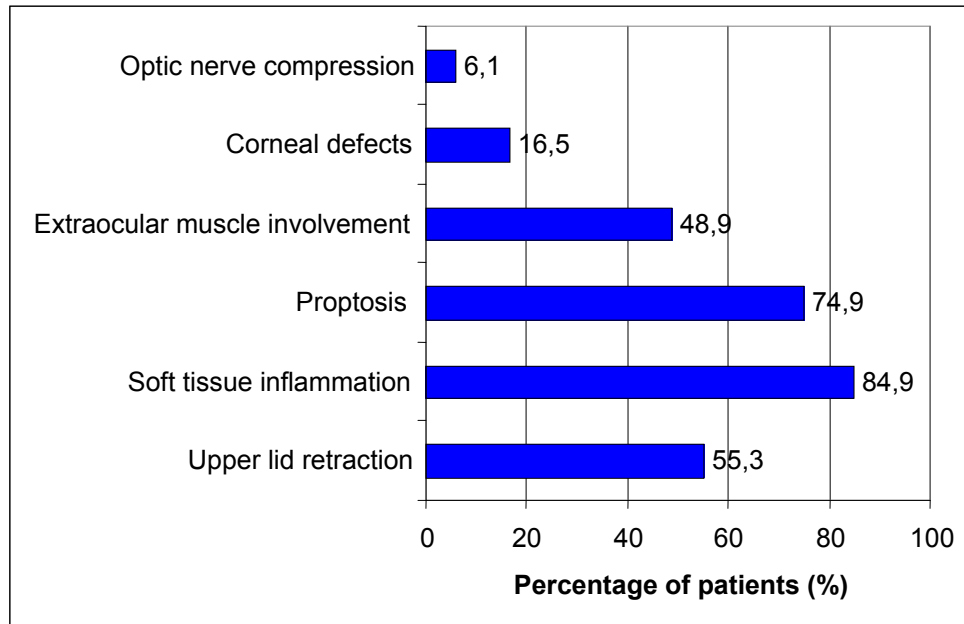


Fig. 3.3. Frequency of particular GO signs 11 to 14 months after GO onset.

3.1.6. Thyroid status at GO onset

The majority of 359 patients, 152 (42,3%), presented the thyroid hyperfunction simultaneously with GO, or up to 6 months prior or after GO. Out of 144 (40,1%) patients in whom the thyroid hyperfunction preceded the eye pathology more than 6 months, 60 patients had undergone the definitive thyroid treatment before the GO manifestation. In 29 (8,1%) patients, the thyroid disease occurred more than 6 months after the onset of GO. 14 (3,9%) patients presented Hashimoto's thyroiditis and 20 (5,6%) were euthyroid during the whole follow-up.

3.1.7. TRAb levels 6 months after onset of GO in relevance to thyroid function

The TRAb values 6 months after the GO onset differed in relevance to the type of the thyroid involvement ($p < 0,0001$): GD vs. euthyroidism ($p < 0,001$) and GD vs. HT ($p < 0,05$). There was no significant difference of the TRAb values in the groups with euthyroidism and HT ($p > 0,05$) (Fig. 3.4.).

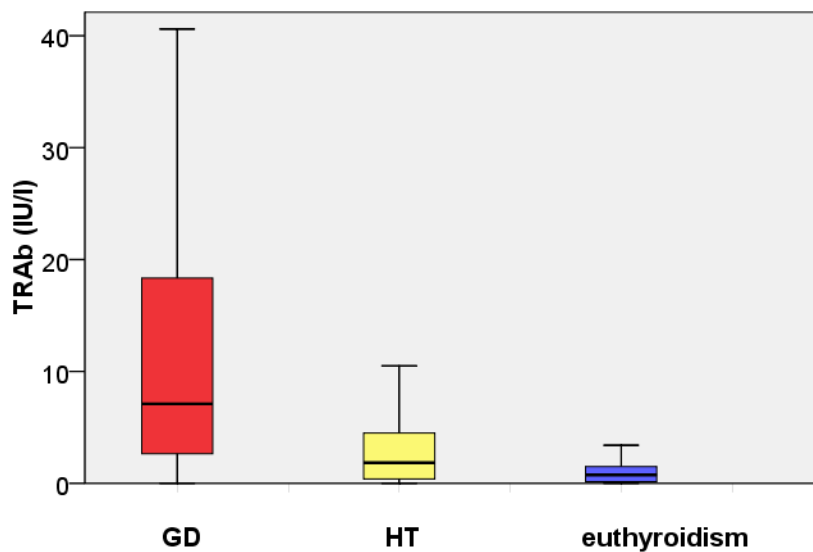


Fig. 3.4. TRAb levels (IU/l) in relevance to thyroid function.

The TRAb values in relevance to the course of GO and hyperthyroidism are presented in the chapters 3.4.5. and 3.5.8.

3.1.8. Family history of AITD and other autoimmune diseases

13,1% of GO patients confirmed other autoimmune diseases (diabetes mellitus type 1, vitiligo, rheumatoid arthritis, neurodermitis, Crohn disease, myasthenia gravis, and autoimmune gastritis). The family history of AITD was positive in 29,5% of the patients (Fig. 3.5.).

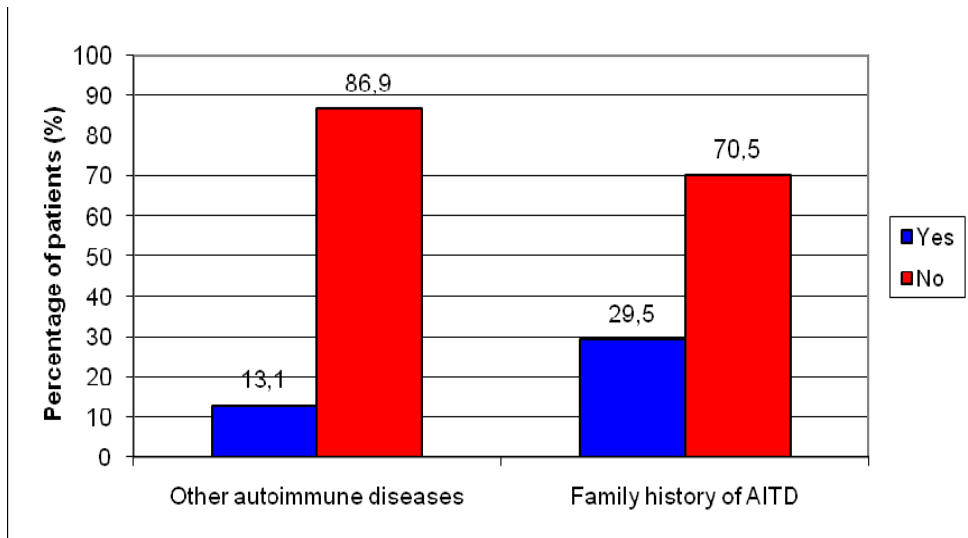


Fig. 3.5. Frequency of family history of AITD and concomitant autoimmune diseases.

3.2. GNAS1 and GNB3 genotype GD versus controls

Genotype distribution was compatible with the Hardy-Weinberg equilibrium.

There was no significant difference of the GNAS1 and GNB3 SNP genotypes ($p=0,86$ and $0,67$) and allele distribution ($p=0,94$ and $0,41$) between the control group and patients with hyperthyroidism (Tables 3.1. and 3.2.).

Sample	n*	CC n (%)	CT n (%)	TT n (%)	Cn	Tn	fT (%)
Controls	820	235 (28.6)	403 (49.2)	182 (22.2)	869	771	47.0
GD patients	268	78 (29,1)	127 (47,4)	63 (23,5)	283	253	47,2

Table 3.1. GNAS1 T393C genotype and T allele frequency (fT) in patients with GD and controls. n=4 patients could not be genotyped due to technical difficulties.

Sample	n	CC n (%)	CT n (%)	TT n (%)	Cn	Tn	fT (%)
Controls	1859	906 (48.7)	791 (42.6)	162 (8.7)	2603	1115	30.0
GD patients	269	139 (51,7)	108 (40,1)	22 (8,2)	286	152	28,3

Table 3.2. GNB3 C825T genotype and T allele frequency (fT) in patients with GD and controls. n=3 patients could not be genotyped due to technical difficulties.

3.3. GNAS1 and GNB3 genotype GO versus controls

Genotype distribution was compatible with the Hardy-Weinberg equilibrium.

There was no significant difference of the GNAS1 T393C and GNB3 C825T genotypes ($p=1,0$ and $0,95$) and allele distribution ($p=0,84$ and $0,95$) between the controls and patients with GO (Tables 3.3. and 3.4. and Fig. 3.6.).

Sample	n	CC n (%)	CT n (%)	TT n (%)	Cn	Tn	fT (%)
Controls	820	235 (28,6)	403 (49,2)	182 (22,2)	869	771	47,0
GO patients	354	100 (28,2)	172 (48,6)	82 (23,2)	372	336	47,5

Table 3.3. GNAS1 T393C genotype and T allele frequency (fT) in patients with GO and controls. n=5 samples could not be genotyped due to technical difficulties.

Sample	n	CC n (%)	CT n (%)	TT n (%)	Cn	Tn	fT (%)
Controls	1859	906 (48,7)	791 (42,6)	162 (8,7)	2603	1115	30,0
GO patients	355	175 (49,3)	148 (41,7)	32 (9,0)	498	212	29,9

Table 3.4. GNB3 C825T genotype and T allele frequency (fT) in patients with GO and controls. n=4 patients could not be genotyped due to technical difficulties.

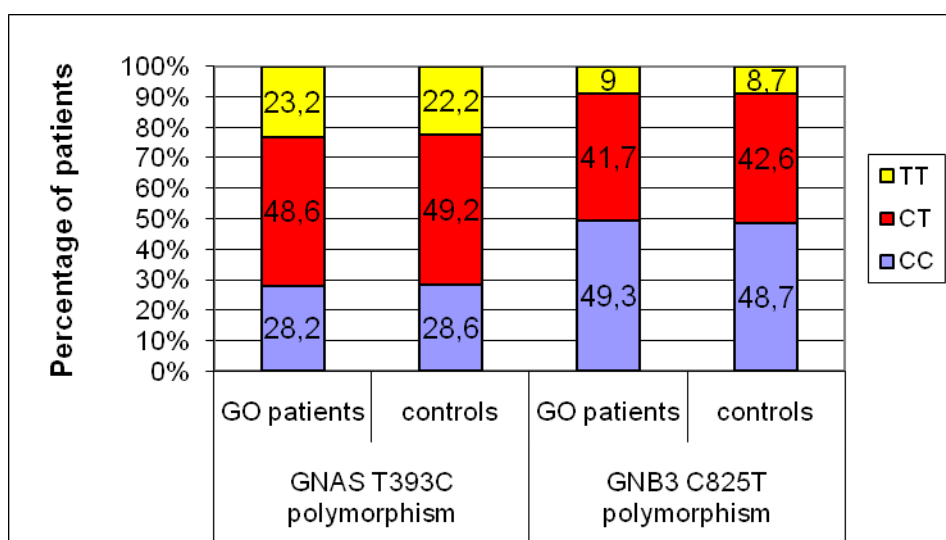


Fig. 3.6. Genotype distribution of GNAS1 T393C and GNB3 C825T SNPs in patients with GO and controls.

3.4. GNAS1 and GNB3 genotype, nongenetic factors and course of GO

3.4.1. Course of GO

359 patients were followed up for median (range) 25 months (11 to 235). After a 12-month follow-up (range 11 to 14 months), the clinical course was classified as mild in 152 patients (42,3%), while 207 patients (57,7%) showed a severe course. Oral or intravenous steroids were administered to 300 patients (83,6%), while 190 (53,0%) received a retrobulbar irradiation therapy.

3.4.2. Overview of all factors with possible influence on course of GO

Table 3.5. shows that the GNAS1 T393C and GNB3 C825T genotypes have no influence on the course of GO. Many clinical parameters were identified as risk factors for the course of GO: age, gender, thyroid volume and antibodies, as well as smoking.

Parameter	Course of GO			P
	All	mild	severe	
n(%)	359	152(42,3)	207(57,7)	
GNAS1 T393C genotypes CC/TC/TT	100/172/82	37/82/30	63/90/52	0,118
GNAS1 T393C fT*	47,5	47,6	47,3	0,930
GNB3 C825T genotypes CC/TC/TT	175/148/32	73/63/13	102/85/19	0,975
GNB3 C825T fT	29,9	29,9	29,9	1,0
Gender (female/male)	307/52	136/16	171/36	0,068
Age at GO onset (years)	47,9(11,9)	44,2(11,7)	50,7(11,2)	<0,0001
GD duration at GO onset (months)	2,0(-36 to 364)	1,0(-26 to 334)	3,0(-36 to 364)	0,091
Smoking (smoker/nonsmoker)	188/171	60/92	128/79	<0,0001
Thyroid volume (ml)	20,0(2,1-135,0)	17,0(6,5-135,0)	26,0(2,1-105,0)	<0,0001
TRAb at GD onset (IU/l)	9,7(0,0-40,0)	6,5(0,4-40,0)	14,1(0,0-40,0)	0,013
TRAb 6 months after GD onset (IU/l)	5,0(0,0-40,0)	2,6(0,0-40,0)	6,8(0,0-40,0)	0,001
TRAb 6 months after GO onset (IU/l)	5,7(0,0-40,0)	3,2(0,0-40,0)	7,5(0,0-40,0)	<0,0001
Thyroid status at last control (remission/relapse)	62/210	36/62	26/148	<0,0001

*fT – T allele frequency

Table 3.5. Descriptive statistics of study patients sorted by course of GO. Categorical variables are given as numbers, continuous variables as means (SD) and medians (min-max). Due to technical problems n=5 samples could not be genotyped for GNAS1 T393C SNP and n=4 samples could not be genotyped for GNB3 C825T SNP.

3.4.3. Course of GO and GNAS1 and GNB3 genotype

There were no significant differences of the genotype and allele distributions in the GNAS1 T393C and GNB3 C825T SNPs ($p=0,12$; $0,93$; $0,98$ and $1,0$, respectively) between the patients with the mild and severe GO (Fig. 3.7.).

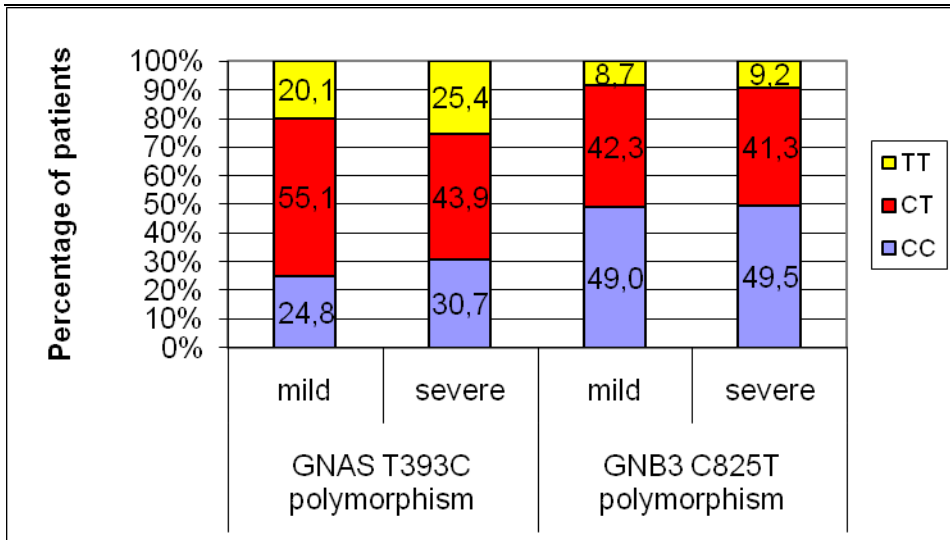


Fig. 3.7. Genotype distribution of GNAS1 T393C and GNB3 C825T SNPs sorted by course of GO.

3.4.4. Relation between course of GO and gender

307 females (136 with a mild course, 171 with a severe course) and 52 males (16 with a mild course, 36 with a severe course) were included in the study. The female: male ratio equaled 8,5:1 in the patients with the mild course and 4,75:1 in the patients with the severe course of GO. A tendency to develop more severe GO symptoms was found in men ($p=0,068$) (Table 3.5.).

3.4.5. Relation between course of GO and age at GO onset

The course of GO depended on the median age of the patients at the disease onset ($p<0,0001$) (Fig. 3.8.). The older the patient, the more severe the GO symptoms. The CAS and NOSPECS values illustrate the associations: for CAS $p<0,0001$, $r=0,239$; for NOSPECS $p<0,0001$, $r=0,334$.

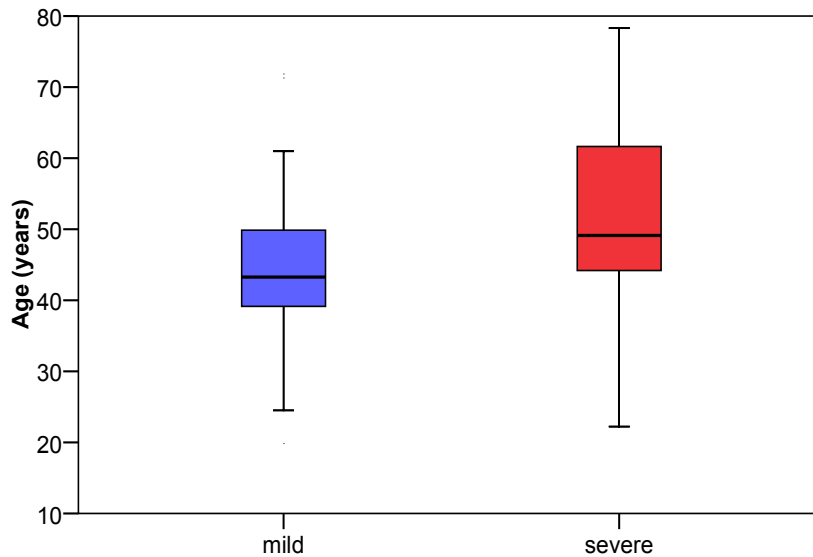


Fig. 3.8. Relation between age of patients at GO onset and disease severity.

As far as particular NOSPECS symptoms are concerned, there was a significant difference in the mean age of the patients relative to the presence of: the soft tissue inflammation ($44,1 \pm 11,6$ and $48,6 \pm 11,8$ respectively; $p=0,009$), extraocular muscle involvement ($44,0 \pm 11,2$ and $52,0 \pm 11,2$ respectively; $p < 0,0001$), corneal defects ($47,2 \pm 11,7$ and $51,4 \pm 12,0$ respectively; $p=0,013$) and the optic nerve compression ($47,5 \pm 8,5$ and $53,5 \pm 10,4$ respectively; $p < 0,0001$). No significant difference regarding the upper lid retraction was found.

3.4.6. Relation between course of GO and GD duration

There was no significant difference in the median duration of the thyroid disease at the GO onset in relation to the course of GO ($p=0,091$).

3.4.7. Relation between course of GO and smoking

There were more smokers (60 with the mild and 128 with severe course, 39,5% and 61,8% respectively) than nonsmokers (92 with the mild and 79 with severe course, 60,5% and 38,2% respectively) ($p < 0,0001$).

Smoking increased the risk for the severe course of GO (OR=2,49 [1,62-3,82]; $p<0,0001$).

No significant differences in TRAb levels between smokers and nonsmokers were found at the GD onset, 6 months after the GD onset and 6 months after the GO onset ($p=0,307$; 0,181 and 0,092, respectively).

3.4.8. Relation between course of GO and thyroid volume

As shown in the Table 3.5. and Fig. 3.9., there was a significant difference of the thyroid volume in the patients with the mild and severe courses of GO ($p<0,0001$). The thyroid volume was also related to the TRAb values 6 months after the onset of GO ($p<0,0001$, $r=0,302$).

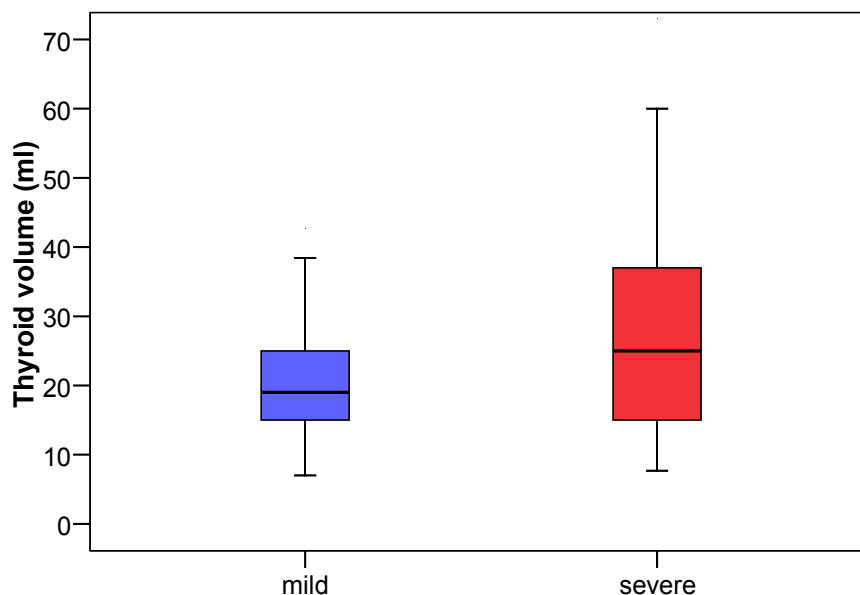


Fig. 3.9. Thyroid volume and course of GO.

3.4.9. Relation between course of GO and TRAb levels

There was a significant difference of the median TRAb values 6 months after the GO onset in the patients with the mild and severe courses of GO, $p<0,0001$ (Fig. 3.10.).

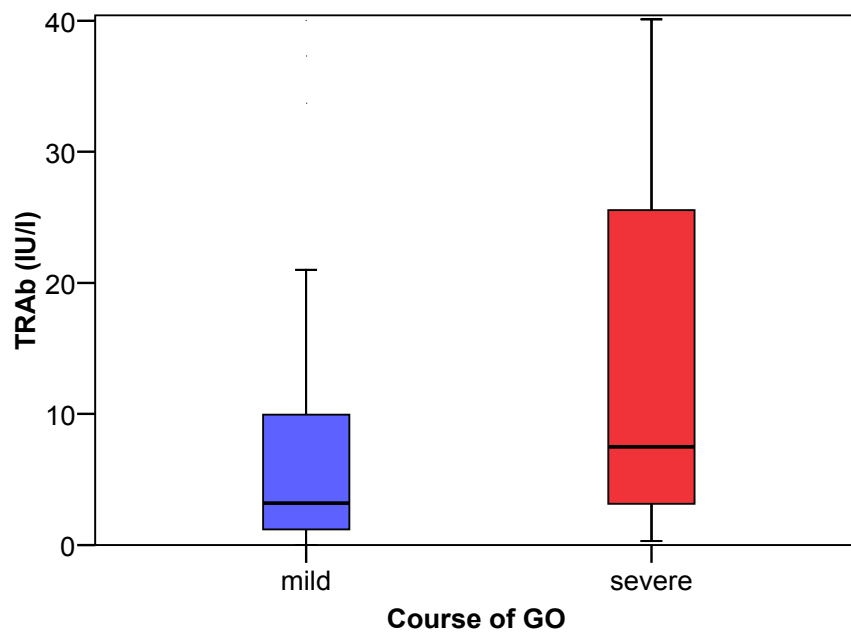


Fig. 3.10. TRAb levels 6 months after GO onset and course of GO.

In order to predict the course of GO the ROC plot analysis was performed which defined the cut-off levels of the TRAb values 6 months after the GO onset (Table 3.6. and Fig. 3.11.).

	TRAb cut-off (IU/l)	Sensitivity (%)	Positive predictive value	Odds ratio(95%CI)
mild GO	<3,0	48	0,65	5,4(3,4-8,8)
severe GO	>11,2	51	0,75	3,5(2,1-5,8)

Table 3.6. TRAb cut-off levels for prediction of course of GO at specificity level of 90%.

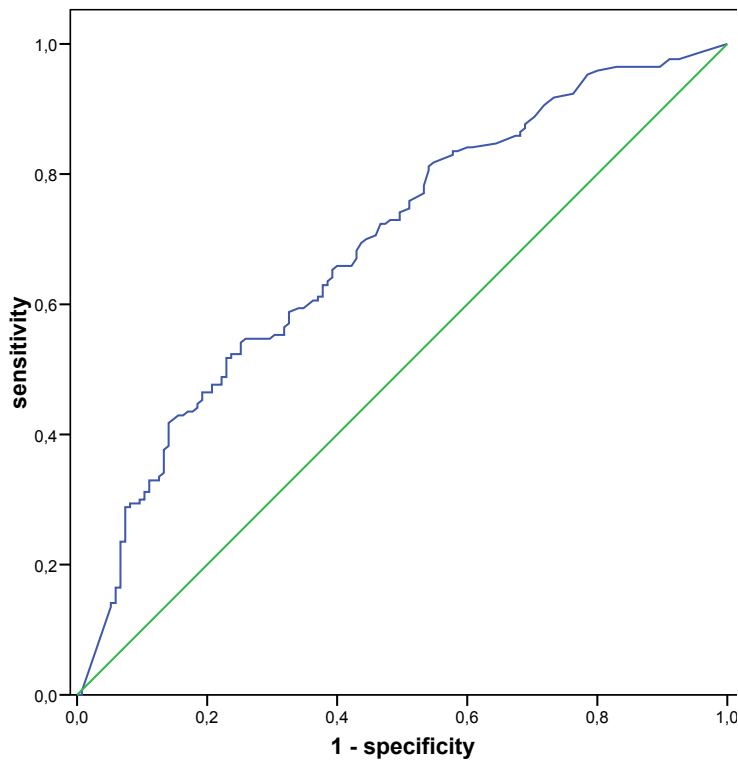


Fig. 3.11. ROC curve for TRAb levels 6 months after GO onset in patients with severe course of GO.

3.4.10. Relation between course of GO and course of thyroid disease

This topic will be dealt with in the chapter 3.5.9.

3.5. GNAS1 and GNB3 genotype, nongenetic factors and course of hyperthyroidism

3.5.1. Course of thyroid disease

The course of hyperthyroidism could be determined in 272 patients. They were followed up for median 35 months (24-559). By the end of the observation period the patients had either received a definitive thyroid treatment or completed a follow-up period of 12 months after the termination of the ATD

therapy. Among the relapsing patients, 120 (59,4%) underwent thyroidectomy, 51 (25,3%) received a radioiodine treatment and 31 (15,3%) both the treatments. Further 8 patients who relapsed were not assigned the definitive thyroid treatment by then. 62 (23,5%) patients remained in remission.

The course of hyperthyroidism could not be classified in 53 patients, as they were still under the ATD therapy or had not completed the follow-up period of 12 months after the cessation of ATD.

3.5.2. Overview of all factors with possible influence on course of thyroid disease

Table 3.7. presents an overview of the candidate factors that could influence the course of hyperthyroidism. The strongest association was found to the level of the TSH-R autoantibodies. The higher the antibody levels, the significantly poorer the prognosis for the remission of hyperthyroidism. The GNAS1 genotype was also associated with the course of hyperthyroidism. The TT genotype carries a higher risk of relapse. The presence of at least one C allele means a better prognosis. Whereas older age and higher thyroid volume were associated with a poorer prognosis. Smoking and gender were not associated with a higher risk of the hyperthyroidism relapse.

Parameter	All	Thyroid status at last control		P
		remission	relapse	
n(%)	272	62(22,8)	210(77,2)	
GNAS1 T393C genotypes CC/TC/TT	78/127/63	15/39/8	63/88/55	0,013
GNAS1 T393C genotypes CC+CT/TT	205/63	54/8	151/55	0,025
GNAS1 T393C fT*	47,2	44,4	48,1	0,47
GNB3 C825T genotypes CC/TC/TT	139/108/22	34/24/4	105/84/18	0,782
GNB3 C825T fT	28,3	25,8	29,0	0,490
Gender (female/male)	238/34	57/5	181/29	0,229
Smoking (smoker/nonsmoker)	146/126	29/33	117/93	0,215
Age at GO onset (years)	48,5(19,8-78,3)	45,9(24,1-78,3)	49,1(19,8-74,1)	0,023
Thyroid volume (ml)	25,0(8,0-135,0)	19,5(8,5-57,0)	28,5(8,0-135,0)	0,031
TRAb at GD onset (IU/l)	12,9(0,0-40,0)	5,4(1,0-32,8)	20,5(0-40,0)	<0,0001
TRAb 6 months after GD onset (IU/l)	8,0(0,01-40,0)	3,4(0,01-24,0)	11,4(0,5-40,0)	<0,0001
Course of GO (mild/severe)	272	36/26	62/148	<0,0001

*fT – T allele frequency

Table 3.7. Descriptive statistics of study patients sorted by thyroid status at last control. Categorical variables are given as numbers, continuous variables as means (SD) and medians (min-max). Due to technical problems n=4 samples could not be genotyped for GNAS1 T393C SNP, n=3 samples could not be genotyped for GNB3 C825T SNP.

3.5.3. Course of hyperthyroidism and GNAS1 and GNB3 genotype

Genotype distribution was compatible with the Hardy-Weinberg equilibrium.

A statistical difference was found in the GNAS1 genotype between the remission and relapse groups ($p=0,013$) (Fig. 3.12.). However, the allele distribution ($p=0,47$) did not reach the significance level.

The homozygous TT carriers showed a significantly increased risk ($p=0,025$) of the hyperthyroidism relapse (odds ratio 2,5; 95% confidence interval 1,1-5,5).

The relapse rate in the homozygous TT carriers reached 87,3%, as opposed to 73,7% in the carriers of at least one C allele (CT/CC). The absence of the C allele was also significantly ($p=0,02$) associated with the higher TRAb levels 6 months after the initiation of the ATD therapy (10,0 IU/l in homozygous TT carriers vs 5,6 IU/l in CC/CT carriers) (Fig. 3.13.). The GNAS1 status was not related to the thyroid volume (CT/CC allele vs TT allele carriers $p=0,106$).

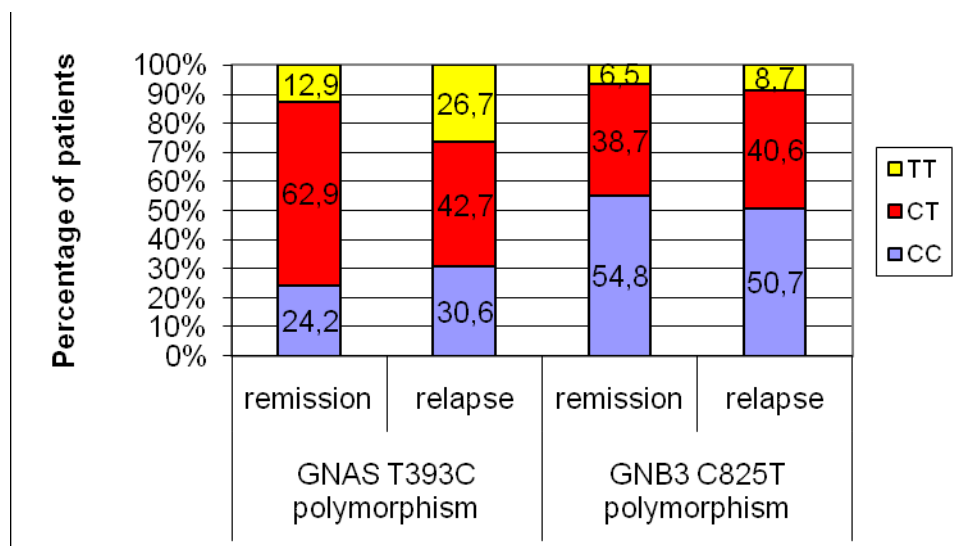


Fig. 3.12. Genotype distribution of GNAS1 T393C and GNB3 C825T SNPs sorted by course of GD.

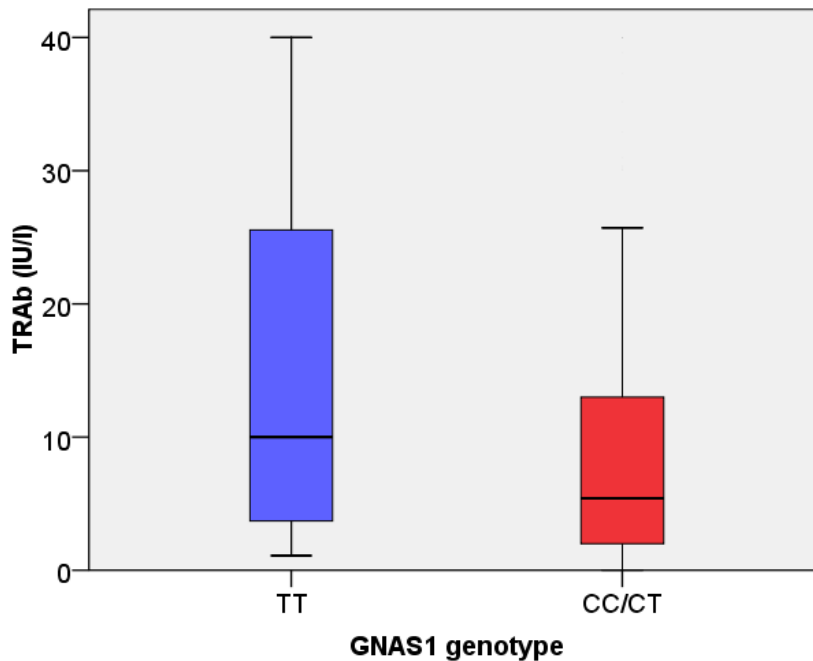


Fig. 3.13. TRAb levels (IU/l) 6 months after initiation of ATD therapy sorted by C allele of GNAS1 T393C genotype.

There was no statistical difference in the GNB3 C825T genotype between the remission and relapse group for either the genotype ($p=0,782$) or allele distribution ($p=0,49$).

3.5.4. Relation between course of hyperthyroidism and gender

238 females (57 in the remission and 181 in the relapse group) and 34 males (5 in the remission, 29 in the relapse group) were included in the study. The female: male ratio equaled 11,4:1 in the remission and 6,2:1 in the relapse patients. No significant connection between their gender and course of hyperthyroidism was found ($p=0,229$).

3.5.5. Relation between course of hyperthyroidism and age

There was a significant difference ($p=0,023$) in the median age of the patients in relevance to the course of hyperthyroidism. The older the patient, the higher the risk of the hyperthyroidism relapse (median age 49,1 vs 45,9 years in the remission group).

3.5.6. Relation between course of hyperthyroidism and smoking

There were 46,8% smokers in the remission and 55,7% in the relapse group. No significant connection between smoking and the course of hyperthyroidism was demonstrated ($p=0,215$).

3.5.7. Relation between course of hyperthyroidism and thyroid volume

There was a significant difference in the thyroid volume relative to the course of hyperthyroidism ($p=0,031$). The thyroid volume was also associated with the TRAb values at the diagnosis of Graves' hyperthyroidism ($p<0,0001$, $r=0,318$) and 6 months afterwards ($p<0,0001$, $r=0,380$).

3.5.8. Relation between course of hyperthyroidism and TRAb levels

The TRAb levels at the onset of hyperthyroidism and 6 months afterwards differed significantly between the remission and relapse groups (Fig. 3.14).

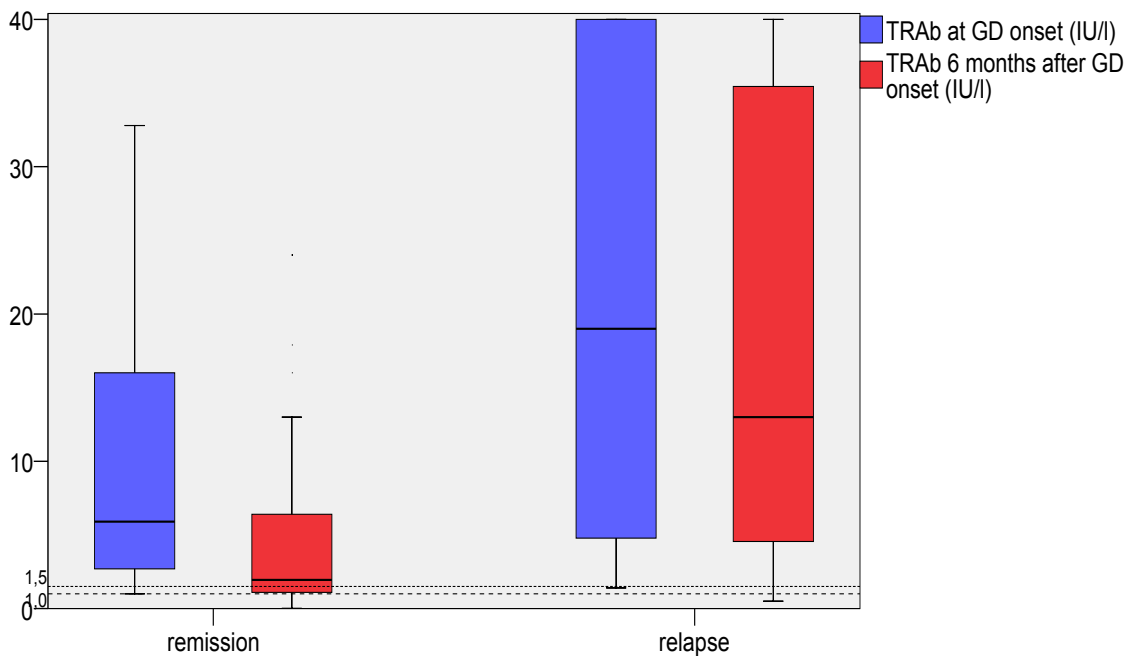


Fig. 3.14. TRAb levels (IU/l) at GD onset and 6 months afterwards, sorted by course of GD, for each $p<0,0001$. Dotted line indicates positive (>1,5 IU/l) TRAb levels, grey zone ranges between 1,0 IU/l - 1,5 IU/l.

In order to predict the course of hyperthyroidism the ROC plot analysis was performed which defined the cut-off levels of the TRAb values 6 months after the GD onset (Table 3.8. and Fig. 3.15.).

	TRAb cut-off (IU/l)	Sensitivity (%)	Positive predictive value	Odds ratio(95%CI)
remission	<2,0	36	0,58	16,7(6,8-41,0)
relapse	>9.95	53,6	0,88	4,9(2,1-11,6)

Table 3.8. TRAb cut-off levels for prediction of GD course at specificity level of 90% (remission), 81% (relapse).

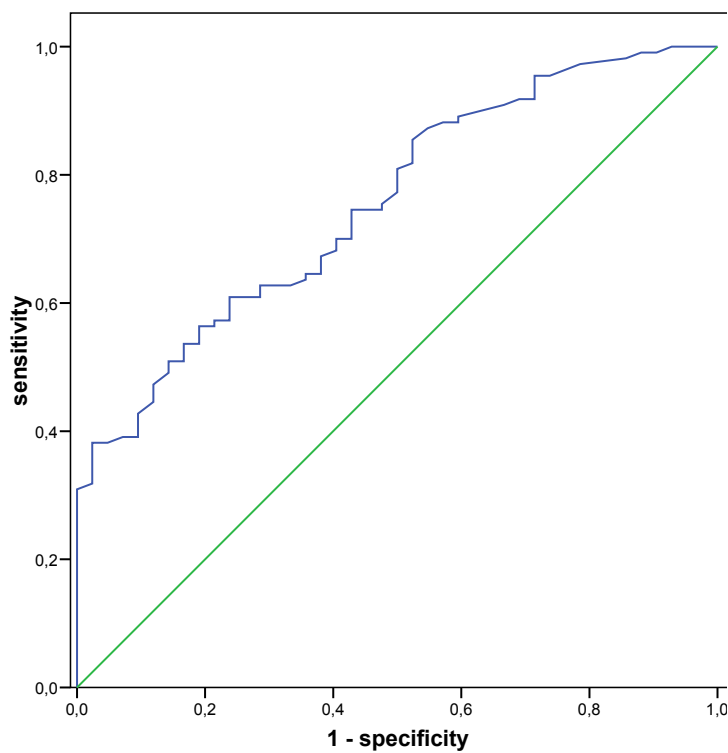


Fig. 3.15. ROC curve for TRAb levels 6 months after onset of hyperthyroidism in relapse group.

3.5.9. Relation between course of GO and course of hyperthyroidism

98 patients with the mild course of GO and 174 with the severe course were classified according to their thyroid status at the end of the study (Fig. 3.16. and 3.17.). The mild course was related to euthyroidism (OR=5,46 [1,93-15,46]; $p=0,0004$) and a higher GD remission rate (OR=3,48 [1,93-6,25]; $p<0,0001$). The course of hyperthyroidism was related to both the CAS and NOSPECS values ($p=0,006$ and $p<0,0001$ respectively). HT was not related to the course of GO ($p=0,783$).

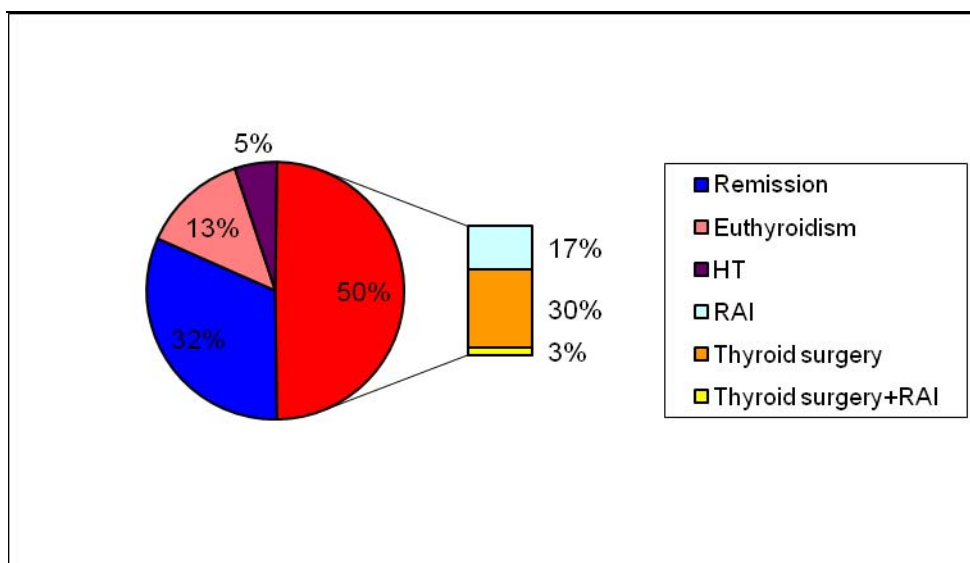


Fig. 3.16. Thyroid status at end of study in patients with mild course of GO. In red percentage of patients that underwent ablative therapy of thyroid (n=114).

RAI – Radioiodine treatment of the thyroid

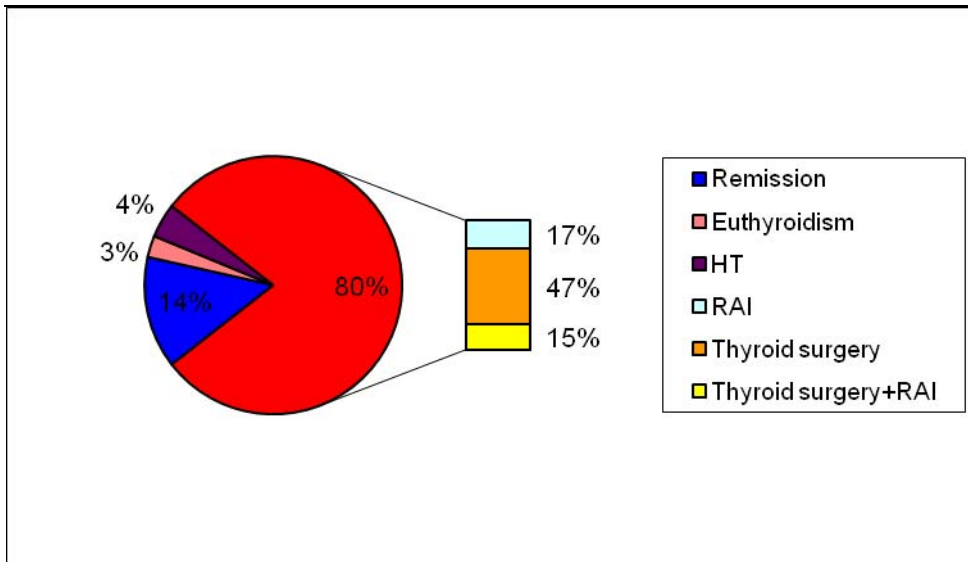


Fig. 3.17. Thyroid status at end of study in patients with severe course of GO. In red percentage of patients that underwent ablative therapy of thyroid (n=184).

RAI – Radioiodine treatment of thyroid

3.6. Stepwise logistic regression on risk factors that influence course of hyperthyroidism

A stepwise logistic regression was performed in order to test the association between the relapse rate of hyperthyroidism and the GNAS1 T393C SNP genotypes, the TRAb levels 6 months after the initiation of the ATD treatment, the age at the GD onset and smoking. A significance level of 0,1 was demanded for a given parameter to enter the model, and 0,2 to remain in it.

The GNAS1 genotype marginally failed the significance ($p=0,055$); the odds ratio for having the C allele vs. having no C allele was 0,116 (95%CI [0,013-1,051]) as long as the TRAb values 6 months after the beginning of the ATD therapy remained in the model (for TRAb: $p=0,0024$, odds ratio for one unit increase was 1,132 (95%CI [1,045-1,226])). Without TRAb in the model, the GNAS1 status (CC+CT vs. TT) and smoking became significant. The age stayed in the model, but did not reach the significance level at any point (Table 3.9.). The gender did not stay in the model.

Factors that influence the course of hyperthyroidism	p	Odds ratio (95%CI)
GNAS1 status CC+CT vs. TT	0,019	0,209 (0,056-0,775)
Smoking (yes/no)	0,013	2,961 (1,263-6,942)
Age (years)	0,059	1,108 (0,996-1,233) for every 3 years

Table 3.9. Results of logistic regression on risk factors that influence course of hyperthyroidism.

4. Discussion

4.1. Patient characteristics

The epidemiological parameters of our study group were compatible with those found in the literature. Our patients were recruited from a tertiary referral ophthalmology clinic, and thus included a high proportion of severe cases of GO, which have somewhat limited our research.

According to Perros et al. the mean age at the onset is 46 years for GD with GO and 40 with GD alone (Perros et al. 1998). In our patients, the median age was slightly higher (47,9 years), which was related to the bigger number of severe cases. 52% of smokers in our group, as compared to 40% reported in the recent EUGOGO study (Prummel et al. 2003), can be explained in a similar way.

Concerning the frequency of the eye symptoms, there was no difference in comparison to other tertiary referral centres (Bartley et al. 1996). The presence of GO can be interpreted as a more severe GD stage. Hence it is not surprising that the rate of hyperthyroidism remission was lower in our cohort, compared to a group of GD patients with less prevalent GO. The overall remission rate of such group is about 50% (Orgiazzi et al. 2002). With the presence of GO, the rate decreases to 21.6% (Vitti et al. 1997), similarly as in our cohort.

The frequency of concomitant autoimmune diseases was comparable to others (Szyper-Kravitz et al. 2005). The family history was slightly less frequent, comparing to a recent study done in the UK (29% vs 47.4% of GD females, 40.0% of GD males) (Manji et al. 2006).

4.2. Genetic susceptibility of Graves' disease (GD) and Graves' orbitopathy (GO)

There was no significant association between the development of GD or GO and the tested polymorphisms.

4.3. Influence of G protein genotypes and nongenetic parameters on course of GO

4.3.1. G protein polymorphisms and course of GO

The GNAS1 T393C and GNB3 C825T polymorphisms, and allele frequencies were related neither to the occurrence nor the course of GO. However, a significant relation between the more severe disease and absence of the C allele in the GNAS1 T393C gene was found in the thyroid disease. An analogical relation was expected in GO, since the severe stages of both GO and GD were significantly associated with the higher TRAb levels. According to Pritchard et al., IgGs of GO patients cause significant proliferation and inflammation in the orbital fibroblast cell cultures (Pritchard et al. 2002). One possible explanation could be the presence of GO in all patients. Unfortunately no patients with Graves' disease without GO were available for further comparison.

4.3.2. Nongenetic factors that influence course of GO

According to Bartalena et al., gender, age, smoking and thyroid dysfunction are all related to GO (Bartalena et al. 1998). The last three were confirmed in our study, while the association with gender failed the significance marginally ($p=0,068$). Moreover, the thyroid volume and TRAb levels 6 months after the GO onset were identified as the risk factors for the severe course of GO (comparable to (Eckstein et al. 2006)). The cut-off levels for TRAb in association with the mild or severe course of GO could nearly be confirmed: 75% of the patients with TRAb $\geq 11,2$ IU/l presented severe GO, while 65% of the patients with TRAb $< 3,0$ IU/l manifested the mild clinical course of the disease.

Smoking generally increases the incidence and severity of GO and is the strongest modifiable risk factor for GO (Prummel et al. 1993). In the present study, 2,5-fold higher risk of severe GO was noted for smokers. According to Cawood et al. (2007), superoxide radicals, hypoxia, nicotine and tar influence

the orbital fibroblasts proliferation, GAGs production, and HLA II molecules expression (Cawood et al. 2007).

4.4. Influence of G protein genotypes and nongenetic parameters on course of hyperthyroidism

4.4.1. G protein polymorphisms and course of hyperthyroidism

The genotype distribution of GNAS1 T393C SNP differed significantly in the remission and relapse GD patients ($p=0,013$). Particularly the homozygous TT carriers showed a much higher risk of relapse.

The results gave rise to several questions:

1. Are these random results?

Random results are unlikely because the genotype distribution in our group was compatible with the Hardy-Weinberg equilibrium. It was also similar to the genotype distribution in the controls.

2. What are the genetic and molecular mechanisms underlying these results?

The C393T polymorphism, the T to C substitution at nucleotide position 393, is silent and does not change the coded aminoacid. However this polymorphism does not seem neutral. Firstly, it can change the folding structure of the messenger ribonucleic acid (mRNA), and consequently its expression. Secondly, the T393C SNP could be linked to another SNP, which would modify the gene splicing and binding of the transcription factors. Bachmann (2006) examined the splice variants of different genotypes in the GNAS1 gene. He did not find any differences among the genotypes (Bachmann 2006) but he observed genotype dependent differences of the Gas-mRNA expression in adipose tissue. It could be shown in vitro that quantitative variations of the Gas expression influenced the efficacy of the signal transduction to the adenylyl cyclase (Krumins et al. 1997). Thus linked SNP in the promoter region of the

gene was suggested. Sequencing the promoter region revealed two new SNPs, although not linked to T393C SNP.

We assume there was increased signalling to TRAb in the patients with TT genotypes. The patients with the TT polymorphism of GNAS1 T393C had significantly higher TRAb levels and were more likely to relapse. In the stepwise logistic regression on the factors influencing the course of GD, GNAS1 T393C SNP lacked significance as long as the TRAb levels were included. The question whether only the increased levels of TRAb are responsible for the higher relapse rate, or the effect is significantly amplified by the TT genotype can therefore not be answered yet.

Besides TSH-R, much of the recent interest centers on IGF-1-R. It is a widely expressed tyrosine kinase receptor abundantly found in connective tissue of GD and GO patients (Douglas et al. 2007). Its downstream signaling pathway results in the IL-16 and RANTES (Regulated on Activation Normal T-lymphocyte Expressed and Secreted) expressions and consequently, leads to the T cell migration and lymphocyte infiltration of the thyroid gland as well as of the orbital connective tissue (Pritchard et al. 2003). The effects of RANTES are mediated by at least 4 G protein-coupled receptors (Pritchard et al. 2002), the signal transduction of which could be modulated by GNAS1 T393C SNP. However, the relevance of G α s to the downstream signaling pathway of IGF-1-R is indirect and not as clear as of TSH-R.

Natural mutations in G proteins frequently result in human diseases. Defects in G proteins almost always lead to endocrine disorders. Nonetheless, so far only GNAS1 has been identified as unequivocally causing such disorders. The observation that Albright's hereditary osteodystrophy (another disorder caused by a mutation of GNAS1) displays different phenotypes depending on the transmitting parent, leads to suspect that GNAS1 is an under tissue-specific imprinting control (Liu et al. 2000; Liu et al. 2005). Subsequently, evidence was provided that GNAS1 transcripts in the thyroid (as in other endocrine tissues, such as ovary and pituitary) are predominantly of a maternal origin (Liu et al. 2005). It may be speculated that the (partial) maternal imprinting of the GNAS1

gene in the thyroid is related to the relatively favourable clinical course of GD in patients with the heterozygous TC genotype of T393C SNP.

3. What is the clinical impact of the result?

The GNAS1 genomic gene modifications are generally suited for prediction of risks and courses of diseases, including endocrine disorders (Weinstein et al. 2001). Our research was the first to investigate the relevance of these polymorphisms to GD and GO. We found that GNAS1 T393C SNP was a minor disease modifying factor, and the TT allele, an independent risk factor for the hyperthyroidism relapse.

It is tempting to presume that the influence of GNAS1 T393C SNP is stronger in average GD patients than in those from a tertiary referral centre. An ideal study sample should include an adequate number of patients in remission, ones with a mild course of GO, and also ones with no orbital involvement.

4.4.2. Nongenetic factors that influence course of hyperthyroidism

The present study confirms that the age of patients and thyroid volume influence the course of hyperthyroidism in GD as suggested by Orgiazzi J et al (Orgiazzi et al. 2002). However, the association with gender and smoking was not found. The inconsistency regarding the influence of smoking on the course of GD might be attributed to the insufficient number of remission patients in our group.

The relapse of hyperthyroidism after the discontinuation of the ATD therapy was related to high TRAb levels. Cut-off levels of TRAb 6 months after the beginning of the antithyroid drug therapy were comparable to the earlier studies (Schott et al. 2004). In our study, 81% of the patients with TRAb levels $\geq 9,95$ IU/l 6 months after the beginning of the ATD therapy relapsed. The relapse rate was slightly lower in comparison to the study of Schott et al., where 97% of the patients relapsed by the TRAb cut-off levels of $\geq 10,0$ IU/l (Schott et al. 2004).

4.5. Relation between course of GO and GD

The presence of GO is considered the risk factor for the relapse of hyperthyroidism after the cessation of ATD (reviewed in: (Orgiazzi et al. 2002)). In a large retrospective trial, Vitti et al. (1997) reported the higher risk of the hyperthyroidism relapse in patients with GO (71.9%) than in those without GO (57.8%) (Vitti et al. 1997). Furthermore, the more severe course of GO, the higher rate of hyperthyroidism relapse. In our cohort, only 14% of the patients with a severe course remained in stable hyperthyroidism remission, as opposed to 37% with a mild GO. Many studies have shown that the severity of both GO and GD is closely related to the TRAb levels. In addition both diseases (GO and GD) share such risk factors as smoking, male gender, large goiter, hypoechogenic and hypervascular gland (Perros et al. 1993; Vitti et al. 1997; Bartalena et al. 1998; Orgiazzi et al. 2002)

Hence, the severity of GO indicates a certain risk for the severity of hyperthyroidism and vice versa (summarized in: (Eckstein et al. 2009)). In practical terms, the patients with severe GO and high TRAb levels face a much higher risk for relapse of hyperthyroidism than those with low TRAb levels and mild GO. In a previous study we reported, based on the stepwise logistic regression analysis, on the following parameters influencing the course of hyperthyroidism: course of GO, TRAb levels, age, gender and smoking (Eckstein et al. 2007). Most of the factors were confirmed in this study. Additionally, increased thyroid volume was related to both the severe course of GO and relapse of hyperthyroidism.

In clinical practice, the present study should encourage a faster decision for the definitive treatment of the thyroid, at least in those countries where prolonged and repeated ATD is still the first choice therapy (e.g. in Europe and Japan). Decisions based on the TRAb levels are possible as early as 6 months after the beginning of the ATD treatment. 12 months after the GO onset the decisions can be made according to the activity and severity scores of GO and again, the TRAb levels.

Future studies should prove whether the early definitive treatment of the thyroid dysfunction in high risk patients can improve the course of GO. The early definitive treatment of the thyroid in the patients most affected by the disease may at least shorten the period of fluctuation in the thyroid function and allow earlier surgical rehabilitation of the orbit.

5. Zusammenfassung

Die Biologie der Initiation und Progression von Morbus Basedow-Krankheit und der Endokriner Orbitopathie ist komplex. Für eine individuell angepasste Therapie der Schilddrüsenüberfunktion und der Orbitopathie ist die Kenntnis von Risikofaktoren die mit einem milden bzw. schweren Verlauf der Schilddrüsen- bzw. Augenerkrankung assoziiert sind wichtig. Hohe Spiegel der Anti-TSH-Rezeptor-Antikörper gemessen zu bestimmten Zeitpunkten im Verlauf der Schilddrüsen- und Augenerkrankung erlauben eine Risikobeurteilung.

Die Signaltransduktion des TSH-Rezeptors erfolgt über das G-Protein. Ziel der Arbeit war herauszufinden ob zwei Einzelnukleotid-Polymorphismen in der G α s und G β 3 Untereinheit des G-Proteins den Verlauf der Basedow Erkrankung beeinflussen.

In einer Serie von 359 Patienten haben wir Genotyp und Allelfrequenzen der Einzelnukleotid-Polymorphismen GNAS1 T393C und GNB3 C825T untersucht. Sie tragen nicht zur individuellen Anfälligkeit gegenüber der Morbus Basedow-Krankheit und der Endokrinen Orbitopathie bei. Doch der TT-Genotyp des GNAS1 T393C Gens ist mit einem signifikant erhöhten Risiko eines Rezidivs der Hyperthyreose verbunden (Odds Ratio 2,5; 95%-Konfidenzintervalle: 1,1; 5,5; $p=0,025$). Die zugrunde liegenden molekularen Mechanismen sind nicht leicht zu erklären. Eine der Hypothesen ist eine Änderung in der Sekundärstruktur der mRNA durch den Polymorphismus, die die Stabilität beeinflussen könnte. Klinisch waren die Anti-TSH-Rezeptor-Antikörper Spiegel in der Patientengruppe mit dem TT-Genotyp am höchsten. Aus diesem Grund kann die Frage, ob nur erhöhte TSH-Rezeptor-Antikörper bei Patienten mit dem TT-Genotyp für die höheren Rezidivraten verantwortlich sind oder ob dieser Effekt signifikant vom TT-Genotyp verstärkt wird, anhand der untersuchten Patientenkohorte nicht beantwortet werden. Aus diesem Grund muss die Kohorte noch erweitert werden, um herauszufinden ob die Genotypisierung des T393C Einzelnukleotid-Polymorphismus des GNAS1 Gens eine nützliche zusätzliche Untersuchung ist, um den klinischen Verlauf der Basedow-Krankheit vorherzusagen.

6. Summary

The biology of initiation and progression of Graves' hyperthyroidism and Graves' orbitopathy is complex. For the individually tailored therapy of the diseases it is very important to identify the risk factors. Research of the last century revealed that measuring the thyrotropin receptor antibody levels enables the prognostic statements at certain points in the course of hyperthyroidism and orbitopathy. Signal transduction of the thyrotropin receptor is coupled with G protein. The aim of the present study was to find out if two single nucleotide polymorphisms that encode the G α s and G β 3 subunits of G protein were related to the course of Graves' disease.

In a series of 359 patients, the genotype and allele frequencies of the GNAS1 T393C and GNB3 C825T single nucleotide polymorphisms were determined. They did not contribute to the individual susceptibility to Graves' hyperthyroidism and orbitopathy. However, the TT genotype of the GNAS1 T393C gene was associated with a significantly increased risk of relapse of hyperthyroidism (odds ratio 2,5; 95% confidence interval 1,1-5,5; $p=0,025$).

The underlying molecular mechanisms are not easy to explain. According to one of the hypotheses, changing a secondary structure of the mRNA influences its stability. Clinically, the thyrotropin receptor antibody levels are the highest in patients with the TT genotype. The question whether only the elevated antibody levels are responsible for the more severe course of hyperthyroidism or if this relation is supported by the TT genotype cannot be answered based on our study group. The studied cohort should be extended in order to find out if genotyping the T393C single nucleotide polymorphism of the GNAS1 gene is relevant for clinical decisions in the treatment of Graves' hyperthyroidism.

7. References

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8. Abbreviations

cAMP	cyclic adenosine monophosphate
AITD	autoimmune thyroid disease
ATD	antithyroid drug treatment
CAS	Clinical Activity Score
cDNA	complementary deoxyribonucleic acid
CT	computer tomography
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DNA	deoxyribonucleic acid
GAG	glycosaminoglycans
GD	Graves' disease
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GO	Graves' orbitopathy
HLA	human leukocyte antigen
HT	Hashimoto's thyroiditis
IFN- γ	interferon- γ
IGF-1-R	insulin-like growth factor-1 receptor
IL-1	interleukin-1
IU/l	international units per liter
MHCII	major histocompatibility complex II
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
OR	odds ratio
PCR	polymerase chain reaction
RAI	radioiodine
	Regulated on Activation Normal T-lymphocyte Expressed and
RANTES	Secreted
SD	standard deviation
SNP	single nucleotide polymorphism
TBAb	TSH-R blocking antibodies
TBII	thyrotropin-binding inhibitory immunoglobulin
Tc	T cytotoxic lymphocyte

Tg	thyroglobulin
Th	T helper lymphocyte
TNF	tumor necrosis factor
TPO	thyroid peroxidase
Ts	T suppressive lymphocyte
TSAb	TSH-R stimulating antibodies
TSH	thyrotropin
TSH-R	thyrotropin receptor

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10. Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.