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PROGNOSTIC FACTORS PREDICTING THE FAILURE OF MEDICAL TREATMENT IN GRAVES' DISEASE

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CONTENTS

ABBREVIATIONS

AIC – Akaike's information criterion
AITD – autoimmune thyroid diseases
APS – antigen presenting cells
ATD – antithyroid drugs
ATPO – antithyroid peroxidase antibodies
cAMP – cyclic adenosine monophosphate
CD40 – B-cell surface antigen CD40
CETP – cholesterol ester transfer protein
CYP7A – cholesterol 7 α-hydroxylase
CTLA-4 – cytotoxic T-lymphocyte-associated protein 4
DNA – deoxyribonucleic acid
EDTA – ethylenediaminetetraacetic acid
FCRL3 – Fc receptor-like 3
FH – family history of thyroid disorders
FT4 – free thyroxine
FT3 – free triiodothyronine
GD – Graves' disease
GO – Graves' ophthalmopathy
HCN2 – hyperpolarization-activated cyclic nucleotide-gated ion channel 2
HLA – Human Leukocyte Antigen
IFN – interferon
IL – interleukin
LDL – low-density lipoprotein
LYP – lymphoid tyrosine phosphatase
MHC – Major Histocompatibility Complex
MHCh – myosin heavy chain
mRNA – messenger Ribonucleic acid
PCR – polymerase chain reaction
PTPN22 – Protein tyrosine phosphatase-22
PTU – propylthiouracil
RAI – radioiodine
ROC – Receiver-operating characteristic
SERCA – sarcoplasmic reticulum calcium-activated ATPase
SNP – single nucleotide polymorphism
SREBP 2 – sterol regulatory element–binding protein 2
TG – thyroglobulin
TRAb – antibodies against thyroid stimulating hormone
TSH – thyroid stimulating hormone
TSHR – thyroid stimulating hormone receptor
VLDL – very-low-density lipoprotein
3'UTR – 3' untranslated region
XCI – X chromosome inactivation

INTRODUCTION

Autoimmune diseases are currently ranked as the third largest disease category in the world following heart diseases and cancer. Autoimmune thyroid diseases (AITD), including Hashimoto's thyroiditis and Graves' disease (GD), are the most common autoimmune endocrine disorders, affecting approximately 2–5% of the general population. Genetic susceptibility in combination with environmental and/or endogenous factors is believed to initiate both cellular and humoral immune responses against different antigens, expressed within the thyroid gland, and lead to the abnormalities in thyroid function. GD is the most common cause of hyperthyroidism. Susceptibility to GD includes a variety of factors. It has been suggested that genetic factors attribute to 79% of the susceptibility to develop GD; while non-genetic factors contribute 21% [43]. Epidemiological studies have shown that the development of GD depends on the iodine intake of the population. In Europe the prevalence of GD has increased due to the national iodine intake programs. Other non-genetic factors that may be involved in the development of GD are smoking, viral/bacterial infection, thyroid irradiation, chemicals and stress [249]. GD is 5 to 10 times more common in females and mostly occurs between 40 and 60 years of age. Although environmental and endogenous factors undoubtedly play an important role in the development of GD, the genetic factors have also been well established. Advances in genetic diagnostic methods in the past decade enabled the researchers to achieve significant progress. The strongest evidence for genetic susceptibility to GD comes from twin studies. Both immune modulating genes and thyroid-specific genes are involved in the genetic pathogenesis of AITD, including GD. The associations of CTLA-4 gene 49A/G and CT60 polymorphisms (SNPs) as well as thyroglobulin (TG) gene E33 C/T SNP with GD were studied by several groups of researchers in different populations, but studies have produced conflicting results. It was suggested, that these genes contribute to the genetic susceptibility to GD, as well as to the different phenotypes of the disease, disease severity, and, possibly, response to antithyroid drugs (ATD) therapy [249].

Autoimmunity against the thyroid-stimulating hormone (TSH) receptor is the main pathogenetic element of Graves' disease. Antibodies against TSH receptor (TRAb) stimulate the growth and the function of the thyroid follicular cells leading to the excessive production of thyroid hormones and goiter formation. GD is characterized by diffuse goiter, thyrotoxicosis and may be accompanied by ophthalmopathy, and occasionally dermopathy.

Thyroid function testing in GD reveals a suppressed serum TSH level and elevated levels of serum thyroxine and triiodothyronine.

Currently, the main treatment strategies for GD include antithyroid drugs, thyroid ablation with radioiodine (RAI), or surgery. The optimal treatment option is still under discussion. Initial treatment choice varies according to the geographic location. In Europe antithyroid drugs are the first line therapy. Antithyroid drugs are effective in controlling hyperthyroidism. Unfortunately, long-term remission is achieved in less than 30% of patients treated with antithyroid drugs. ATD therapy of about 12–18 months requires careful monitoring of patients for side effects, such as rash, arthralgias, urticaria, gastrointestinal symptoms and agranulocytosis.

Radioactive iodine is a safe and effective therapy, but sometimes it is associated with a difficult decision to give a high enough dose for the treatment to be effective and minimize the chance of recurrence and at the same time the risk of hypothyroidism. Approximately 10-30% of patients require more than one dose of ^{131}I. In patients with moderate to severe thyroid eye disease, radioiodine may worsen pre-existing ophthalmopathy. Pregnancy and lactation are the two absolute contraindications for the use of RAI therapy and pregnancy should be delayed at least 6 months after RAI treatment.

Thyroid surgery is related to high hospitalization costs and risk of surgery-related complications such as lesions of the parathyroid glands and recurrent laryngeal nerves.

The prevalence of GD in Lithuania is unknown. Krasauskas V. et al. performed a retrospective analysis of 7363 patients who had surgery due to benign thyroid disease between 1989 and 2000. Study results revealed, that 3.42% of all patients underwent surgery due to diffuse toxic goiter [146]. Veličkienė D. et al. study was aimed to identify clinical and laboratory parameters that would predict the outcome of treatment with radiotherapy in patients with endocrine ophthalmopathy [258]. Study results revealed that the pretreatment clinical activity score, total eye score and proptosis were higher in patients who successfully responded to retrobulbar irradiation.

As current treatment of GD is often unsatisfactory, we acknowledge the need to identify predisposing genetic and non-genetic factors associated with the development of GD. Some factors may determine response to antithyroid drugs therapy, thus identification of prognostic parameters may help select patients who have high recurrence risk after ATD therapy and recommend them an early thyroid ablation in order to avoid long, useless and potentially harmful ATD therapy.

The scientific novelty and practical significance of the work

Graves' disease is a polygenic and multifactorial autoimmune thyroid disease. Although the genetic factors undoubtedly play an important role in the development of GD, there are substantial ethnic differences in GD genetic predisposition between populations. There are not enough studies of European populations, that investigate associations of CTLA-4 49A/G and CT60 SNPs or TG E33 C/T SNP with GD and the influence of these SNPs on the remission of Graves' hyperthyroidism. Our study is the first study investigating the association of CTLA-4 49 A/G, CT60 and TG E33 C/T polymorphisms with GD in the Lithuanian population.

As the response to treatment for GD is unpredictable, it is often problematic to choose the optimal treatment approach. Endocrinologist must weigh the risks and benefits of each treatment option in many cases. The decisions to continue or stop antithyroid therapy in patients with GD and to choose surgery or radioactive iodine therapy at the right moment are the major challenges in the clinical practice. Reliable genetic and non-genetic predictors of relapse after ATD treatment would greatly improve patient management by facilitating the identification of patients, who require long-term ATD treatment, early surgery or radioiodine therapy. In our study we identify genetic and non-genetic risk factors that may affect disease presentation and predict response to ATD treatment in patients with Graves' disease.

1. THE AIM AND OBJECTIVES OF THE STUDY

The aim of the study:

To determine the value of demographic, clinical and laboratory features, thyroid ultrasound pattern and genetic factors as predictors of medical treatment failure in patients with Graves‘ disease.

The objectives of the study:

1. To determine the influence of gender, age and family history of thyroid disorders on medical treatment failure in patients with Graves' disease.
2. To evaluate the role of goiter size and thyroid echogenicity pattern in predicting the outcome of medical therapy.
3. To determine the prognostic value of thyroid stimulating hormone receptor antibodies for medical treatment failure in patients with Graves' disease.
4. To investigate genetic associations of CTLA-4 gene 49A/G and CT60 polymorphisms and thyroglobulin gene E33 C/T polymorphism with susceptibility to Graves' disease.
5. To determine the associations of CTLA-4 gene 49A/G and CT60 polymorphisms and thyroglobulin gene E33 C/T polymorphism with the outcome of medical treatment in Graves' disease.

2. LITERATURE REVIEW

2.1. Historical notes

The nature of the Graves' disease has remained unclear for many years. Initially, it was considered to be a cardiac, neurological or thyroid disease [213]. There was no agreement on the best treatment options for this condition. Surgery of the thyroid, orbit, autonomic nervous system, and pituitary gland has been employed. Medical treatment was introduced in early 1940s [162]. Graves' disease got its name from the Irish Doctor, Robert James Graves in 1835 [271]. This disease is also called Basedow's disease in Europe, from Doctor Karl Basedow, who reported the same symptoms in 1840 [162, 271]. It took some time to understand the patho-physiology of Graves' disease. In 1956 Adams and Purves discovered a long-acting substance in the serum of patients with GD, which stimulates the thyroid gland for a much longer period of time than the thyroid stimulating hormone (TSH). In 1965 this substance was called long-acting thyroid stimulator and was characterized as an antibody against a thyroid component [213]. In 1978 this thyroid component was identified as the thyroid stimulating hormone (TSH) receptor [82].

2.2. Epidemiology and risk factors for Graves' disease

Graves' disease affects approximately 0.2–1.6% of the general population [38, 53, 131] and is the underlying cause of 50–80% of cases of hyperthyroidism [65, 270]. GD is a polygenic and multifactorial disease that develops as a result of the interplay between genetic, environmental and/or endogenous factors [270]. Epidemiological studies have shown that development of GD depends to a great extent on the iodine intake of the population. In populations with sufficient iodine intake, hypothyroidism is more common than in iodine-deficient areas, whereas the prevalence of hyperthyroidism is higher in areas with mild to moderate iodine deficiency [156, 157]. In normal iodine intake areas GD accounts for 80% of hyperthyroidism cases, while in iodine-deficient areas only half of hyperthyroidism cases are due to GD [155]. In Europe the prevalence of GD has increased with national iodine intake programs [50], but the combined rate of ablative treatment for benign thyroid diseases has decreased during this period, probably due to conservative treatment of iodine-induced hyperthyroidism [49]. The prevalence of Graves' disease is lower in black population than in Caucasians or Asians (Table 2.2.1) [270].

Table 2.2.1. *Annual incidence of GD in various geographic areas*

Country	Cases/100.000 population per year
United States (1989–2001) [114, 172]	38.3
Sweden (2003–2005) [3]	21
Denmark (1997–2000) [48, 158, 172]	31.2
Poland (1987–1990) [131]	4
Slovenia (1999) [281]	28
New Zealand (1983–1985) [44]	15
United Kingdom (1972–1993) [172, 255]	50
Iceland (1980–1982) [108, 172]	19.3
South Africa (1974–1984) [129]	8.75
Northern China (1999–2004) [172, 243]	120

GD can occur at any age, but its peak incidence is between 40 and 60 years [152, 270]. About 1% to 5% of patients with GD are children, but the disease is rare in children under 5 years of age; the peak annual incidence occurs at the age of 11 to 14 years [233]. GD is 5 to 10 times more common in women than men. Female sex hormones have been proposed as obvious candidates to explain this sexual dimorphism. The presence of estrogen receptors on immune cells has been demonstrated [60] with the modulation of hormonal processes by cytokines [135]. It has been found that estrogens can stimulate the secretion of immunoglobulins (Ig); moreover, women presented with higher serum Ig concentrations than men [99]. Women present a predominant T helper 2 (Th2) lymphocytes that secrete interleukin 4 (IL-4), a cytokine which influences antibody production by B lymphocytes, and interleukin 10 (IL-10) [99]. As with other autoimmune diseases, GD activity decreases during pregnancy, whereas the onset or worsening of GD is commonly seen during the postpartum period [270]. Hormonal effects on the immune response may explain this fluctuation of disease activity during and after pregnancy. During pregnancy, the immune system is suppressed with a fall in the T helper/suppressor cell ratio; whereas in the first postpartum months T cell activation and thyroid autoantibody production occur [197].

Fetal microchimerism, the transfer of fetal cells into the maternal circulation during pregnancy, and triggering autoimmunity of the organ in which they live, has also been implicated in etiology of autoimmune diseases [95]. Several evidences have shown that females with GD frequently have microchimeric fetal cells residing within their thyroid glands [14, 232]. Intrathyroidal fetal microchimeric cells can persist for a long time [34] and

act as effectors cells or as targets of an immune response [95]. Recently, skewed X chromosome inactivation (XCI) has been suggested to partly explain the female preponderance in GD. In female cells, one of the two X chromosomes is inactivated in early embryonic life and this epigenetic mechanism ensures that men and women have equal expression of the genes from the X chromosome, despite the difference in X chromosome number. The X chromosome inactivation is a physiologic process, which leads to random inactivation of the maternal and paternal X chromosomes in 50% of cells [176]. However, this random process sometimes generates an unbalanced inactivation with one copy of the X chromosome preferentially expressed. Preferential use of either the paternal or maternal X-chromosome in 80% or more of the cells is termed skewing of XCI. Skewed XCI may lead to the escape of X-linked self-antigens from presentation in the thymus or in other peripheral sites that are involved in tolerance induction [176]. This phenomenon may result in the loss of immunological tolerance and play a significant role in the development of autoimmune thyroid diseases [42, 279]. A higher frequency of a skewed XCI was found in females with GD compared to healthy controls, indicating a possible role of XCI in the etiology of GD and in the female preponderance in GD [42]. Another theory that can explain the female preponderance in GD is the use of oral contraceptives. Some studies found association between the use of oral contraceptives and a lower frequency of GD [235, 259]. It has been speculated that the contraceptive hormones suppress the endogenous production of one or more female sex hormones modulating thyroid autoimmunity [259]. Another explanation could be that the use of contraceptives reduces the risk of pregnancy and abortion, which both may influence thyroid autoantibody production [149].

It has been calculated that 79% of the susceptibility to develop Graves' disease can be attributed to genetic factors, leaving 21% for environmental factors [43]. Environmental factors that may have an effect on the development of GD include iodine intake, cigarette smoking [197], stress [252], external [107] and internal [181] thyroid irradiation, drugs such as antiretrovirals [98], interferon-alpha (IFNα) [196], Campath-1H [61], viral and bacterial infections [197, 249], seasonal variation and allergy [197]. Excess iodine intake is associated with highly iodinated thyroglobulin, which is more immunogenic than poorly iodinated TG. Other mechanism includes a direct toxic effect of iodine on thyroid cells via free oxygen radical generation, and immune stimulation by iodine [39]. Cigarette smoking is associated with higher risk of GD and especially of Graves' ophthalmopathy (GO) and the risk increases with the intensity of smoking [27, 114]. The

mechanism by which smoking increases the risk of Graves' hyperthyroidism is unknown. Smokers have higher serum thiocyanate concentrations than nonsmokers. Thiocyanate inhibits iodide transport into thyroid cells, which may decrease thyroid hormone synthesis [94]. Benzpyrene, another component of cigarette smoke, may stimulate thyroid secretion by stimulating the sympathetic nervous system [33]. Smoking also has effects on the immune system. It increases the production of the proinflammatory cytokines from mononuclear cells [208]. Although smoking decreases serum immunoglobulin concentrations, smokers have higher serum concentrations of autoantibodies than nonsmokers [228]. Smoking increases the risk for a relapse of Graves' hyperthyroidism and the chances of an exacerbation of the eye disease after treatment with ^{131}J [27, 185]. It also reduces the efficacy of radiotherapy and corticosteroid treatment of the GO [80]. The reason for the strong association of smoking with GO is also unknown. Hypoxia may play a role, because fibroblasts show a significant increase in proliferation and glycosaminoglycan production when cultured under hypoxic conditions [174].

Some studies established an association between GD and negative life events in the year preceding the diagnosis of Graves' hyperthyroidism [252]. This may be explained by stress, which stimulates the immune system through neuroendocrine networks [234]. Stress might activate the sympathetic nervous system or be associated with enhanced secretion of stress hormones, such as glucocorticoids and catecholamines [197,200]. Stress hormones can induce T helper cell imbalance and production of cytokines by dendritic cells, expressing the TSH receptor [272]. One study showed that four personality traits (hypochondria, depression, paranoia and mental fatigue) were positively related to the relapse rate after ATD in Graves' disease, and that stressful life events correlated with the titer of thyroid stimulating hormone receptor antibodies [93].

Some studies suggested that thyroid external irradiation to the cervical region and internal irradiation by radioiodine (^{131}J) may increase the risk of GD. External neck irradiation also increases the risk of GO. Irradiation of the thyroid gland may expose thyroidal antigens to the immune system and thus induce autoimmunity by stimulation of dendritic cells [164,230].

For many years viral and bacterial infections have been attractive environmental triggers for autoimmunity, but there is no clear evidence that infection directly induces Graves' disease. Several infectious agents implicated in the pathogenesis of GD include Yersinia enterocolitica [41, 51, 67, 275], Coxsackie B virus [145], retroviruses [127], Helicobacter pylori [89]. However, the strongest association of autoimmune thyroid disease with an

infectious agent was found with hepatitis C virus [87, 250]. Two main theories have been proposed for the induction of autoimmunity by infectious agents: (1) the molecular mimicry theory suggests that sequence similarities between viral proteins and self-proteins can induce a cross-over immune response to self-antigens [183]; (2) the bystander activation theory proposes that viral infection of a certain tissue can induce local inflammation (e.g. by cytokine release), resulting in activation of autoreactive T-cells that were suppressed by peripheral regulatory mechanisms [90].

GD is more often diagnosed in the spring and summer [276]. Some reports suggest that the seasonality of thyrotoxicosis may not be related to the warmer temperatures [192], but it may be caused by seasonal changes in iodine intake [193]. Another factor responsible for seasonal differences may be the seasonal variation in viral infections or in allergen exposure (Table 2.2.2) [197].

Table 2.2.2. *Endogenous and environmental factors involved in the etiology of GD and the possible mechanisms. Adapted from [196].*

Factor	Risk	Suggested mechanism
Female sex		
Postpartum period	Increased	T cell activation and antibody production
Fetal microchimerism	Increased	Fetal cells in maternal thyroid tissue
Skewed XCI	Increased	Lack of exposure to self-antigens on one X chromosome in the thymus
Oral contraceptives	Decreased	Protective effect of estrogens
Cigarette smoking	Increased	Hypoxia; the SNS stimulation; effects on immunity
Stress	Increased	Stress hormones excess; up regulation HPA axis
Iodine intake	Increased	Prolonged thyroid hyperactivity
External and internal irradiation	Increased	Expose thyroidal antigens to the immune system
Drugs		
HAART	Increased	Changes in CD4+ cells
IFNα	Increased	Stimulation of Th1 cells
Campath-1H	Increased	Decrease in Th1/Th2 ratio
Infection	Increased	Molecular mimicry; bystander activation
Allergy	Increased	Unknown; high IgE levels

HAART – highly active antiretroviral therapy; IFN – interferon; SNS – sympathetic nervous system; HPA – hypothalamo-pituitary-adrenal; Campath-1H – alemtuzumab.

2.3. Genetic analyses of complex diseases

Today it is clear that AITDs occur as a result of complex interactions between multiple genetic and environmental predisposing factors. Detecting gene-gene and gene-environment interactions remains a challenge, despite the existence of a number of efficient methodological methods. The main methodological approaches used for studying genetic predisposition to complex diseases are based on linkage or association analysis [220].

2.3.1. Markers

The markers used in genetic studies include single nucleotide polymorphisms (SNPs), microsatellites and copy number variations [263]. A SNP is a DNA sequence variation where alternative bases are present in different individuals at one nucleotide position [1]. SNPs are the most common type of variation in the human genome and occur, on average, once per 300 nucleotides. For DNA sequence variation to be called a SNP it must be present in at least 1% of the general population; less frequent variations are described as mutations. SNPs may fall within coding sequences of genes, non-coding regions of genes, or in the regions between genes. A SNP in the coding region can result in the same amino acid (synonymous SNP) or in a different amino acid (non-synonymous SNP). SNPs in non-coding regions may have consequences for gene splicing or transcription [277]. Microsatellites, or simple sequence repeats, are DNA sequences that consist of tandem repeats of 1–6 nucleotides. One common example of a microsatellite is a (CA)n repeat, where n varies between alleles. Microsatellites are typically co-dominant. In contrast to SNPs, microsatellites can have multiple alleles and are hypervariable [211]. Copy-number variant is a segment of DNA that is 1 kb or larger and is present at a variable copy number in comparison with a reference genome. Classes of CNVs include insertions, deletions and duplications [84,88].

2.3.2. Linkage Analysis

Linkage analysis detects genes with major influences on the development of a disease without any prior assumptions on disease pathogenesis. It is based on the observation that if two markers are located near each other on a chromosome they remain linked during meiosis and will co-segregate in families because the likelihood that a recombination will occur between them is low. Therefore, if a marker is close to a disease susceptibility gene, its alleles will co-segregate with the disease in families and the marker will

show linkage with the disease. The locus where the marker is located can then be fine-mapped in search for the susceptibility gene [194, 244].

2.3.3. Association Analysis

Association analysis is more sensitive than linkage analysis in detecting weak genetic risk factors and currently it is the preferred method. Association analysis is based on comparing the frequency of a variant in patients and controls. If a statistically significant difference in the frequency of a variant is observed between cases and controls it is concluded that this variant is associated with the disease [246]. There are possible explanations for the existence of an association between an allele and a disease: (1) the associated allele itself is the genetic variant causing an increased risk of the disease, and (2) the associated allele does not cause the disease by itself but is in linkage disequilibrium (LD) with the causative variant [111, 249].

2.3.4. Candidate genes

In searches for complex disease susceptibility genes, the candidate gene approach or genome-wide screening can be applied using linkage or association analysis. Based on the knowledge of the gene's biological function, a candidate gene is believed to play a role in the genetic susceptibility to a complex disease. AITD susceptibility genes, identified by the candidate gene approach, are the human leukocyte antigen DR (HLA-DR) gene, the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) gene and the thyroid stimulating hormone receptor (TSHR) gene [236].

2.3.4. Genome-wide screening

Genome-wide screening is a powerful tool, as it enables scanning of the whole genome for susceptibility genes without any prior assumptions on disease pathogenesis. It is performed by testing a panel of markers spanning the entire human genome for linkage or association (genome-wide association studies) with the disease [249]. Genetic markers located close to each other are inherited together more frequently than would occur by chance. This phenomenon is referred to as linkage disequilibrium. The International HapMap Project has demonstrated that the human genome is highly organized into blocks of high linkage disequilibrium, interspersed with hot spots, or areas at which recombinations are likely to occur [1]. This inheritance pattern of SNPs can be used to create haplotypes, a combination of alleles on the same chromosome that are transmitted together. Tag SNPs are specific SNPs that identify the particular haplotype.

2.4. The genetic component in Graves' disease

The identification of genes in individuals with increased risk for the development of AITD has been a slow process. The strongest evidence for a genetic contribution to the etiology of GD comes from twin studies where the concordance in monozygotic twins was 35%, compared to 3–7% in dizygotic twins, and in 79% of cases the development of susceptibility to GD was predicted by genetic factors [43]. Significant progress has been made in the past decade in mapping the AITD susceptibility genes and understanding the mechanisms by which they confer the risk for disease. The AITD susceptibility genes can be divided into two large groups: (1) immune modulating genes and (2) thyroid specific genes. The first group includes the HLA-DR gene, the CTLA-4 gene, the B-cell surface antigen CD40 (CD40) gene, the Fc receptor-like 3 (FCRL3) gene, the protein tyrosine phosphatase-22 (PTPN22) gene and others, while the second group includes the TG gene and TSHR gene [104, 246]. These genes contribute to the genetic susceptibility to AITD, as well as to the different phenotypes of AITD, disease severity and, possibly, response to therapy [249].

2.4.1. Immune-modifying genes

The first discovered AITD gene was Human Leukocyte Antigen (HLA) /Major Histocompatibility Complex (MHC) [20]. HLA region on chromosome 6p21.31 is highly polymorphic and contains many immune response genes [53]. HLA region encodes genes that are grouped into three classes: class I genes (HLA-A, HLA-B and HLA-C), class II genes (HLA-DR, HLA-DP and HLA-DQ) and class III genes [160]. Of the three identified HLA classes, the focus usually goes to class I and class II. HLA are essential molecules for the function of the immune system. HLA class I molecules interact with CD8+ lymphocytes which are the main cytotoxic effector cells. HLA class II molecules present antigens to CD4+ lymphocytes that initiate and regulate specific immune response. HLA class II molecules are permanently expressed on the surface of dendritic cells, macrophages, B cells, whereas expression in other cells may be induced by inflammation [194]. A large number of studies have been performed to assess HLA allelic associations with GD; however results were inconsistent among different ethnic populations. For example, in Caucasian populations HLA-DRB1*03, C*03, C*07, C*16 and the DRB1*03-DQB1*02-DQA1*05:01 haplotype was found to be associated with a high risk of GD [53, 222, 223, 246], while DRB1*07:01–DQA1*02:01 haplotype showed a protective effect against GD [282]. However, these GD associated HLA alleles were

presented at very low frequencies in Asian populations. HLA-B*46, HLA-DRB1*08:03, HLA-DRB1*09:01 and HLA-DRB1*14:03 alleles were reported as risk factors of GD in Asian populations [53, 77, 117, 184].

The cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) gene is located on chromosome 2q33 and has been found to be associated with risk for AITD by the candidate gene approach [21]. CTLA-4 consists of four exons that encode different functional domains [133]: exon 1 encodes a leader peptide, exon 2- the ligand-binding domain, exon 3- the transmembrane domain, and exon 4- the cytoplasmic tail [188]. The CTLA-4 gene is translated into 2 protein isoforms: a full-length protein (flCTLA-4) and a soluble protein (sCTLA-4), which lacks exon 3 due to alternative splicing [189]. On resting T cells, CTLA-4 protein levels are low or undetectable [188]. Human CTLA-4 is expressed on activated CD4+ and CD8+ T cells and constitutively on CD4+ CD25+ T regulatory cells, few non-lymphoid normal cells [242]. T cell activation leads to the increased expression of CTLA-4 [210]. Its peak membrane expression is observed after 48–72 h of stimulation by antigen [188]. Under normal circumstances, the CTLA-4 interacts with the B7 molecule on the surface of antigen presenting cells (APS) and suppresses T-cell activation in order to prevent T-cell overactivity [136,144]. The CTLA-4 transmits an inhibitory signal to T cells by inducing cell-cycle arrest; suppresses inflammatory cytokine production [56,78]. In addition, CTLA-4 mediates antigen-specific apoptosis of T cells and suppresses autoreactive proliferation of T lymphocytes [103]. Little is known about sCTLA-4 function. Although sCTLA-4 was thought to be expressed on nonstimulated human T cells, no evidence of this soluble isoform has been found in the serum of healthy subjects [182,189]. Decreased or absent CTLA-4 activity permits uninhibited T-cell activity and a prolonged, unregulated immune response, making CTLA-4 an attractive candidate gene for autoimmunity [21]. The CTLA-4 gene has been found to be associated with all AITD, including GD, and also with other autoimmune disorders such as type 1 diabetes mellitus, Addison's disease, Sjögren's syndrome, systemic lupus erythematosus, and myasthenia gravis [126]. The CTLA-4 gene is a highly polymorphic gene. Several CTLA-4 polymorphisms have been found to be associated with GD, most notably SNP at position 49 (49A/G SNP), a 3' untranslated region (3'UTR) AT dinucleotide repeat (microsatellite), a SNP downstream from the 3'UTR, designated CT60 (6230 G/A) and (-318) T/C in the promoter region (Fig. 2.4.1.1) [194].

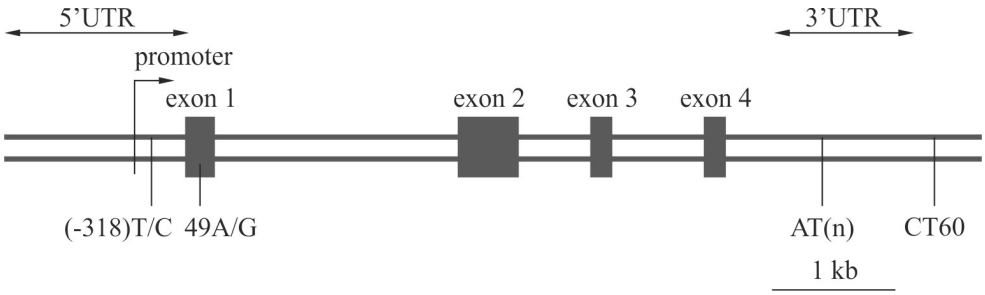

Fig. 2.4.1.1. *Location of the polymorphic markers within the CTLA-4 gene. UTR, untranslated region. Adapted from [55]*

A polymorphism that changes CTLA-4 functionality or reduces its cell surface expression may result in heightened T-cell activation, and potentially, lead to the development of autoimmunity. Several CTLA-4 SNPs and the 3'UTR microsatellite have been analyzed in detail for their effect on CTLA-4 function and expression [125]. The 49A/G polymorphism on exon 1 of the CTLA-4 gene results in amino acid change from threonine to alanine in the signal peptide of CTLA-4 receptor and influences post-translational processing leading to inefficient glycosylation [16, 22, 55, 194]. Some studies have shown association between the G allele at this position and reduced control of T cell proliferation [144]. This association could be due to a direct effect of the 49A/G SNP or due to another polymorphism in linkage disequilibrium with the 49A/G SNP [125]. It has been suggested that long AT dinucleotide repeat alleles decrease the stability of CTLA4 messenger ribonucleic acid (mRNA) and impair the inhibitory function of the protein leading to reduced control of T cell proliferation [238]. Non-coding CT60 SNP located in the 3'UTR is potentially important because it may be associated with an alteration in the ratio of splice forms of the CTLA-4 gene. This ratio may affect disease susceptibility [253]. Some studies suggest that 49A/G and CT60 SNPs are associated with lower levels of sCTLA-4 transcripts [253]. The exact mechanisms of this phenomenon are still unknown. It can be suggested that in patients with autoimmune disease abnormal translation of both CTLA-4 transcripts (soluble CTLA-4 and full-length CTLA-4) and/or intracellular trafficking and release of sCTLA-4 may occur [188]. Analysis of the (-318) promoter SNP, resulting in amino acid cytosine substitution to thymine, has revealed that T allele, in comparison to the C allele, causes an 18% higher promoter activity [266]. Individuals carrying the T allele of the (-318) polymorphism have been shown to have significantly elevated expression of CTLA-4 on the surface of stimulated cells, and significantly increased concentration of CTLA-4

mRNA in resting cells [161]. The (-318) SNP may affect CTLA-4 levels by altering the binding of a transcription factor, LEF-1, whose binding site encompasses the C/T polymorphism [55]. An association between the CTLA-4 gene and AITD, including GD, was demonstrated in population-based studies. These associations have been consistent across populations of different ethnic backgrounds, such as Caucasians [278], Japanese [24,141], and Koreans [138]. Some studies suggest that CTLA-4 may also influence the severity of the AITD phenotype [125, 241, 264].

CD40 is a costimulatory protein, expressed primarily on B cells and other antigen presenting cells, and plays a major role in inducing B cell activation and proliferation [113, 151], immunoglobulin class switching [121], antibody secretion [45], and generation of memory cells [17]. The CD40 gene is located on chromosome 20q and whole genome linkage scanning has identified strong linkage of CD40 to GD [245]. The causative variant predisposing to GD is a C/T polymorphism in the Kozak sequence, a nucleotide sequence that is essential for the initiation of translation of the CD40 molecule. The CC genotype of this SNP has been shown to be associated with GD [25]. The CD40 Kozak SNP influences CD40 translational efficiency. The C allele of this polymorphism increases the translational efficiency of CD40 mRNA transcripts, resulting in the increase in CD40 protein production by 20–30% when compared to the cases with the T allele [123]. Therefore, it is possible that increased CD40 expression driven by the C allele contributes to the disease etiology by lowering the threshold of activation to thyroid antigens in autoreactive B cells. Another possibility is that the C allele enhances CD40 expression on B cells and thyrocytes [123, 173], resulting in cytokine secretion (e. g. IL-6) and activation of resident T-cells in the thyroid by bystander mechanisms [124, 173].

FCRL3 gene, one of the most important loci for susceptibility to autoimmune diseases, is located on chromosome 1q21–q23 [54]. Protein FCRL3 is mainly expressed in secondary lymphoid organs and particularly in B lymphocytes and may play a role in the regulation of the immune response [79]. Certain polymorphisms in FCRL3 promoter have been associated with some autoimmune diseases. Most notably SNP, present at position (-169) within the promoter, can cause elevated FCRL3 expression on B cells and the differentiation of B cells into autoreactive cells [54,143]. A four marker haplotype within FCRL3 gene (fcrl3_3 to fcrl3_6) shows highly significant associations with a number of autoimmune diseases, including rheumatoid arthritis [119], GD [143,221] and systemic lupus erythematosus [209].

The protein tyrosine phosphatase-22 (PTPN22) gene encodes the lymphoid tyrosine phosphatase (LYP), a molecule that, similarly to CTLA-4,

plays an important role in modulating T cell signal transduction and inhibiting antigen receptor induced T cell activation A nonsynonymous SNP in the PTPN22 gene (R620W), resulting in a substitution of tryptophan for arginine, was found to be associated with autoimmune diseases including rheumatoid arthritis [30], systemic lupus erythematosus [150], type 1 diabetes mellitus [224], GD [257] and Hashimoto's thyroiditis [68]. This substitution changes LYP protein function, causing T cell activation [125]. Although the PTPN22 gene R620W polymorphism showed a significant association with GD in Caucasians [257], this SNP was not found in the Japanese GD population [118].

2.4.2. Thyroid specific genes

Thyroglobulin is one of the major autoantigens for AITD, including both GD and Hashimoto's thyroiditis. Whole genome linkage studies identified a locus containing the TG gene on chromosome 8q24 [245]. Autoantibodies against TG are not disease specific but they are found in 50-70% of GD patients and in other AITD forms [194]. TG is a homodimeric glycoprotein synthesized and secreted by the thyroid cells into the follicular lumen. Synthesis of triiodothyronine (T3) and thyroxine (T4) follows a metabolic pathway that depends on the TG structure [106]. The TG protein molecule undergoes several important post-translational modifications, including iodination, glycosylation, sulfonation, and phosphorylation [63,101,231]. The varying degree of the posttranslational modifications of thyroglobulin, particularly iodination, gives a heterogeneous character to the thyroglobulin molecule. It has been suggested that these posttranslational modifications of TG may lead to changes in antigenicity and binding to HLA [125]. Case-control studies have reported that several SNPs in exons 10–12 and in exon 33 are significantly associated with AITD [248] or with the relapses of GD [116]. The E10 SNP does not change amino acids. The E12 SNP has been identified as causing an amino acid substitution from non-polar amino acid (valine) to non-polar amino acid (methionine) without change in protein structure in this region. The E33 SNP, causing a change from a hydrophobic amino acid tryptophan to a hydrophilic amino acid arginine, changes the structure of TG protein. There is evidence of the interaction between E33 TG variants and HLA-DRB1*03, which increases the risk of GD [23, 112]. These results suggest that interaction between an immunoregulatory gene (HLA-DR) and a thyroid-specific autoantigen gene (TG) may play a role in susceptibility to AITD.

The thyroid hormone receptor, expressed on the surface of thyroid epithelial cells, binds thyroid stimulating hormone (TSH) and, through

direct activation of adenylate cyclase, stimulates the production of thyroid hormones [72]. As thyroid stimulating hormone receptor (TSHR) antibodies in serum are the main serological markers of GD, TSHR gene was the obvious candidate for genetic studies [125]. Initial studies were focused on the three common non synonymous SNPs in the TSHR gene, which is located on chromosome 14q31 [251]. Two of these SNPs are located in the extracellular domain of the TSHR gene: D36H, which results in an aspartic amino acid substitution to histidine at position 36, and P52T, which changes amino acid proline to threonine at position 52. The third D727E SNP lies within the intracellular domain of the receptor and changes glutamic amino acid to aspartic acid. [246]. Most studies on the contribution of the TSHR gene to the genetic susceptibility to GD have focused on the SNPs in the extracellular domain of the TSHR [57, 128] because this domain is responsible for TSH and TSHR antibody binding. Amino acid changes in this region could theoretically change the amino acid sequence of TSHR T cell epitopes [201]. Initial studies suggested that the P52T SNP was associated with GD in females [69]. However, other authors were unable to confirm the association between the P52T SNP and GD in Caucasians [9, 57, 128]. Among Japanese large scale analyses of SNPs showed evidence of three haplotypes within TSHR intron 7 that were strongly associated with GD [110].

2.5. Pathogenesis

In Graves' disease B and T lymphocyte-mediated autoimmune process is directed against four well known thyroid antigens: thyroglobulin, thyroid peroxidase, sodium iodide symporter, and the thyroid stimulating hormone receptor [177]. TSHR, the primary autoantigen of Graves' disease, is responsible for the manifestation of hyperthyroidism. Thyroid stimulating hormone regulates thyroid cell growth and thyroid hormone production and secretion via thyroid stimulating hormone receptor (TSHR). TSHR holoreceptor is expressed on the surface of thyrocytes plasma membrane and variety of other cells (adipocytes, fibroblasts, osteoblasts, osteoclasts, bone marrow cells, cardiomyocytes) [175]. It is important to know the molecular structure of TSHR in order to understand the particular role of TSHR as a target for the autoimmunity in GD. The TSHR is a G protein coupled receptor synthesized as a single polypeptide with a large extracellular domain, seven transmembrane region and a small intracellular tail [6]. The single chain TSHR undergoes complex posttranslational processing involving dimerization and intramolecular cleavage. This could modulate its signal

transduction and result in the generation of self-antigens [154]. The TSHR is cleaved into two covalently linked A (α) and B (β) subunits (Fig. 2.5.1).

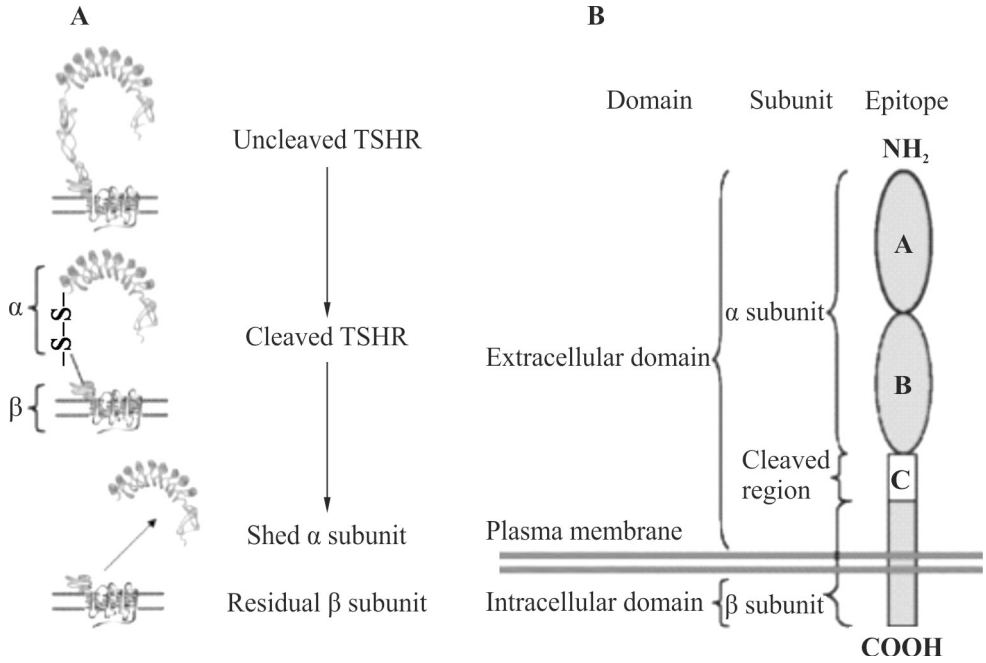

Fig. 2.5.1. *TSHR structure, posttranslational processing*

(A) Three forms of the TSHR: the single chain TSHR undergoes cleavage and becomes a two-subunit structure (α/A and ß/B) connected by disulfide bonds. Upon reduction, the α/A subunit is shed from the cell surface and leaves the ß/B subunits on the membrane. (B) Schematic representation of the structure of the TSHR. Adapted from [154].

Both subunits are stabilized by disulfide bonds [15]. A large water soluble A subunit is a ligand-binding (TSH and autoantibody) region, while a small water insoluble B subunit is responsible for transmission of the signal to G proteins [216]. In subsequent steps after reduction of the disulfide bonds, holding the subunits together, the TSHR may shed its A subunit from the cell surface, leaving an 2.5–3-fold excess of B subunits on the cell membrane [154]. These posttranslational events may influence the antigenicity of the receptor and contribute to the break of self-tolerance to TSHR. Antibodies against TSHR are directed almost exclusively against the A subunit; that suggests their immune processing outside the thyroid gland [52]. An interesting fact is that no fragment of the shed A subunit has ever been detected in the peripheral circulation, most likely because it accumulates in the draining lymph nodes or is rapidly degraded [15]. The TSHR cleavage

and shedding are dynamic changes regulated by TSH [153]. In thyroid tissue, the TSHR cleavage is almost complete [262].

The hyperthyroidism in GD is caused by the circulating immunoglobulin G (IgG) antibodies against TSHR (TRAb), produced outside and within the thyroid. These antibodies bind to and activate the receptor, leading to an increased concentration of intracellular cyclic adenosine monophosphate (cAMP) [74]. However, not all TRAb observed in GD are thyroid stimulators. According to their ability to induce the generation of intracellular thyrocyte cAMP, TRAb have been classified as stimulating (increasing cAMP concentrations), blocking (reducing cAMP concentrations), and neutral (with no effect on TSH binding and no effect on cAMP levels) [175]. The different biological effects of these TRAb on thyroid cell function and growth result from different binding sites on TSHR [106]. Activation of TSHR stimulates hypertrophy and hyperplasia of the thyroid follicules, causing thyroid enlargement, visible as goiter, and increased thyroid hormone production [270]. Some authors found that the level of TRAb correlates with the severity of the disease (degree of hyperthyroidism), and with the severity of Graves' ophthalmopathy and may predict the outcome of the disease [81]. The degree of thyroid stimulation in patients depends on the concentration and bioactivity of the different types of autoantibodies. Both stimulating and blocking antibodies can coexist in patient's serum and their concentrations can fluctuate over time, resulting in changes in disease activity. After treatment of GD TSHR blocking autoantibodies may become the prevalent type of antibodies and cause hypothyroidism [239]. Moreover, one of the possible explanations for the remission of thyrotoxicosis during pregnancy includes changes of antibodies from stimulatory to blocking type [148]. The clinical significance of neutral TRAb, which have no such actions, is uncertain [15].

The anti-sodium-iodide symporter, antithyroglobulin and antithyroid peroxidase antibodies (ATPO) appear to have little influence on the pathogenesis of hyperthyroidism in GD. However, they are markers of autoimmune disease against the thyroid. Lymphocytic infiltration of thyroid in GD reflects the immune response to thyroid antigens and can be correlated with the titer of thyroid antibodies [171,177]. Current assays detect antibodies against TSHR in 95–96% of untreated hyperthyroid patients with Graves' disease, although only sensitive assays can demonstrate their functional characteristics [287]. Up to 90% of patients with Graves' disease have antibodies directed against the thyroid peroxidase and a lower proportion, approximately 50–70%, have antibodies against thyroglobulin [265].

Activated T cells, directed against thyroid and nonthyroid antigens, are present in peripheral blood of patients with Graves' disease [18, 170]. Thyroid cells play very important role in the initiation of Graves' disease. They express HLA class II molecules and can act as APC, that present thyroid specific autoantigens to T helper lymphocytes [106]. Lymphocytic infiltration of the thyroid consists predominantly of Th2 lymphocytes subtypes, with smaller numbers of B lymphocytes, dendritic cells and monocytes/macrophages [170, 195]. Many activated T lymphocytes release IFN-γ, a strong stimulator of the inflammatory process and immunological response, and various other cytokines and growth factors which stimulate cell proliferation [18, 170, 270] (Fig. 2.5.2).

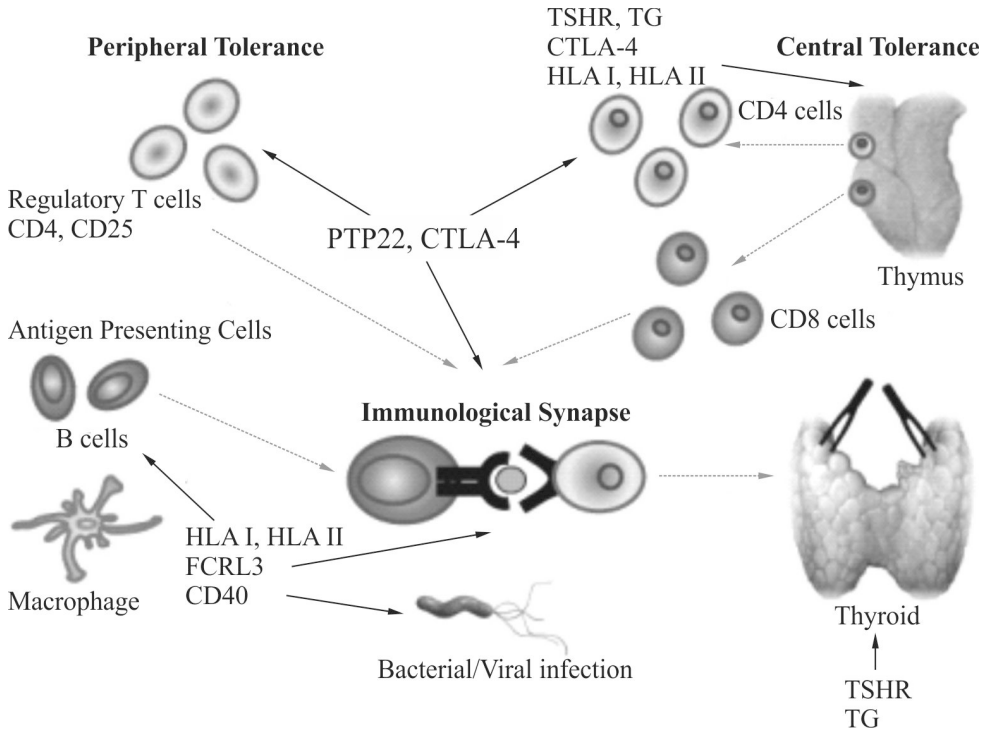

Fig. 2.5.2. *The cell types and tissues involved in Graves' disease pathogenesis. The proteins expressed by the different genes determine cell functions and activity. Thyroid antigens, TSHR and TG, are main autoantigenic targets in GD, may be presented by APCs to reactive T cells. HLA class I and II genes are expressed in many different cell types including B cells, dendritic cells and macrophages and bind antigens for presentation to T cells during central and peripheral tolerance. The PTPN22 and CTLA-4 are expressed on T cells whereas FCRL3 are known to be predominantly involved in B cell function. Adapted from [37].*

Production of TSHR autoantibodies by B lymphocytes leads to thyroid cell hypertrophy, hyperplasia and hyperfunction. The blood flow to the thyroid is increased due to increased vascularization, and vascular endothelial growth factor is thought to play a central role in the thyroid angiogenesis [199]. The pathological characteristics of thyroid tissue in GD are similar to that of orbital tissue in Graves' ophthalmopathy and can be characterized by diffuse infiltration by lymphocytes, the release of cytokines, and active inflammatory status that are involved in the pathogenesis [5].

2.6. Clinical features of Graves' disease

Typical symptoms of hyperthyroidism are caused by the excess of thyroid hormones and enhanced β-adrenergic activity. Patients usually have fatigue, weight loss, despite increased appetite and food intake [122], palpitations, heat intolerance, tremor, sleep disturbances, increased frequency of defecation, proximal muscle weakness, nervousness or anxiety [38, 270]. Women might have irregular menstrual cycle and decreased fertility, although amenorrhoea is rare [147]. Men might suffer from gynecomastia, reduced libido and erectile dysfunction (Table 2.6.1) [47]. Clinical findings include tachycardia or atrial fibrillation, tremor, warm, moist and smooth skin, hyperreflexia and the presence of goiter. Elderly patients have less obvious symptoms and signs than younger patients, including a lower frequency of goiter and a higher prevalence of cardiac manifestations such as atrial fibrillation or congestive heart failure [65].

Table 2.6.1. *Manifestations of Graves' disease. Adapted from [38, 40, 142, 178].*

System	Clinical finding or manifestation	Marker of direct or indirect thyroid hormone action
Cardiac	Increased heart rate and contractility	Increased expression of HCN2, voltage-gated potassium channel (Kv1.5, Kv4.2, Kv4.3), and SERCA; increased α-MHC and decreased β-MHCh expression; increased serum atrial natriuretic peptide
Hepatic	Increased peripheral T3 production; reduced total and LDL cholesterol, lipoprotein	Increased type 1 5′-deiodinase, LDL and VLDL receptor, lipase, SREBP-2, CYP7A, and CETP
Skeletal	Increased bone metabolism, osteopenia, osteoporosis, fractures	Increased osteocalcin, alkaline phosphatase, and urinary N-telopeptide
Muscle	Proximal muscle weakness, easy fatigue	Increased SERCA activity and serum creatine kinase
Reproductive Male Female	 Erectile dysfunction, reduced libido Irregular menstrual cycle	 Increased sex hormone globulin, reduced free testosterone Antagonism of estrogen action; impaired gonadotropin regulation
Metabolic	Increased thermogenesis and oxygen consumption Reduced fat mass	Increased fatty acid oxidation and sodium–potassium ATPase Increased adrenergic-mediated lipolysis

CETP – cholesterol ester transfer protein; CYP7A – cholesterol 7 α-hydroxylase; HCN2 – hyperpolarization-activated cyclic nucleotide-gated ion channel 2; LDL – low-density lipoprotein; MHCh – myosin heavy chain; SERCA – sarcoplasmic reticulum calcium-activated ATPase; SREBP 2 – sterol regulatory element-binding protein 2; VLDL – very-low-density lipoprotein.

Graves' ophthalmopathy, also called thyroid-associated ophthalmopathy, occurs in 30–50% of patients with Graves' disease; however, subclinical abnormalities can be demonstrated by computed tomography or magnetic resonance imaging in more than 80% of patients [137, 270]. Other extrathyroidal manifestations, pretibial myxedema (discoloured induration of the skin or non-pitting edema of the lower extremities) and thyroid acropachy (clubbing of the fingers and toes) are rare findings [85, 218]. An epidemiological study showed that 4% of the patients with ophthalmopathy

have dermopathy, and one in five patients with dermopathy has acropachy [28].

The diagnosis of GD is based on clinical symptoms and signs and laboratory findings, including suppressed serum TSH level, elevated serum free T4 and/or free T3 concentrations, and the presence of TRAb [38, 270]. The sensitivity and specificity of the TRAb assays have increased over the past 20 years; this improved the diagnosis of GD and probably contributed to the increase in reported incidence of GD [152]. The presence of GO or pretibial myxedema is sufficient to confirm the diagnosis of GD in a patient with hyperthyroidism and diffuse goiter. When in doubt, a thyroid radionuclide scan should be performed, which, in case of GD, demonstrates diffusely enhanced uptake in an enlarged thyroid [270].

2.7. The treatment options for Graves' disease

The optimal treatment of GD is still debatable. Current treatments for Graves' hyperthyroidism consist of antithyroid drugs, thyroid ablation with radioiodine, and surgery [91]. Initial treatment choice varies according to geographic location. In Europe and Asia, ATDs are the first line therapy, while RAI and surgery are used as a second line when ATDs fail or in the case of recurrence. In the United States RAI is the first line treatment prescribed to most adults with Graves' disease [267]. In only 1% of cases surgery is recommended as the first line treatment in all regions [227].

2.7.1. Antithyroid drug therapy

Propylthiouracil (PTU) and methimazole are the antithyroid drugs used in the United States. Methimazole is used in most Europe and Asia countries, and carbimazole, a methimazole analogue, is used in the United Kingdom [66]. Antithyroid drugs are effective in controlling hyperthyroidism, but the relapse rate after the withdrawal of therapy is very high. Relapse generally occurs within 6 months after discontinuation of ATD, but can occur later [109]. Approximately 30 to 60% of patients relapse within 1 year of ATD discontinuation [19, 86, 214] and only 30 to 40% of patients remain euthyroid 10 years after the treatment [270]. The primary effect of the antithyroid drugs is to inhibit the organification of iodine to tyrosine residues on the thyroglobulin molecule and the coupling of iodotyrosines, which are important steps in the synthesis of thyroxine and triiodothyronine. It is believed, that antithyroid drugs may have clinically important immunosuppressive effects because the treatment with ATD is associated with a significant fall in thyroid stimulating hormone receptor autoantibo-

dies levels. Propylthiouracil, but not methimazole or carbimazole, can block the conversion of thyroxine to triiodothyronine within the thyroid and in peripheral tissues [66]. PTU is preferred during pregnancy because it does not cross the placenta to the same extent as methimazole, which has been reported to have teratogenic effects [132]. The usual treatment strategy is a long-term ATD therapy of about 12–18 months. This requires careful monitoring of patients for side effects, such as rash, arthralgias, urticaria, and gastrointestinal symptoms [64]. Agranulocytosis, a potentially life-threatening toxicity, can occur in 0.37% of patients receiving propylthiouracil or methimazole; however, this rare side effect is reversible after discontinuation of medication [237]. Rare major toxicities include drug-induced hepatitis and antineutrophil cytoplasmic antibody-positive vasculitis, which are mainly observed in patients taking PTU [105, 207].

2.7.2. Radioactive iodine therapy

Radioactive iodine for the treatment of hyperthyroidism has been used since the 1940s. Initially radioiodine therapy included ^{130}I isotope, but in 1946 ^{131}I sodium iodide was introduced and became the preferred iodine isotope for treating hyperthyroidism [29, 130]. Radioactive iodine is a safe, effective therapy, but sometimes it is associated with a long latency period before its effect is seen. The principle of radioiodine treatment is based on the ability of the thyroid follicular cells to absorb and organify iodine. After intracellular uptake, radioiodine causes sterilization and destruction of a number of these cells, but the effect is to a large extent dependent on the absorbed dose of ^{131}I [73]. Although 70% of patients become euthyroid within 4 to 8 weeks, it may take up to 6 months to notice a full effect of RAI [11,270]. Most patients achieve euthyroid or hypothyroid state after a single dose of ^{131}I, and approximately 10–30% of patients require more than one dose [7]. Depending on the used dose of RAI, the risk of hyperthyroidism is 5% to 25%, while the risk of hypothyroidism is 20% during the first year, subsequently increasing to 3% to 5% per year [7, 268, 269]. RAI is sometimes followed by worsening of the thyrotoxicosis due to radiation- related thyroiditis; therefore, elderly patients and patients with cardiac disease should be pretreated with ATD before RAI. In patients with moderate to severe thyroid eye disease, especially the ones with severe hyperthyroidism and smokers, radioiodine worsens pre-existing ophthalmopathy, as compared to ATD or surgery. This might be related to the release of thyroid antigens into the circulation as a result of radiation-related thyroiditis. The worsening of ophthalmopathy may be prevented by prophylactic treatment with glucocorticoids [27]. Rare complications include thyrotoxic crisis and

hypoparathyroidism [203]. Radioiodine treatment also leads to the increased levels of TRAb, which can persist for years and in pregnant women cause fetal or neonatal hyperthyroidism [284]. Pregnancy and lactation are the two absolute contraindications for the use of RAI therapy and pregnancy testing must be performed before treatment. Although RAI therapy does not have any adverse effects on future fertility for women planning pregnancy, it is recommended to delay pregnancy at least 6 months after RAI treatment [130].

2.7.3. Surgery

In 1909 Kocher introduced surgical management of goiter, and he was the first to propose subtotal thyroidectomy as surgical treatment for GD. After the introduction of RAI therapy, surgery became less common as the primary treatment [217]. The indications for surgery are young age, poor response to ATD, pregnancy and lactation, the presence of a thyroid nodule or large goiter with compressive symptoms, and also patient's preference [233]. Total or subtotal thyroidectomy can be performed. In both cases, the patient should be pretreated with an ATD in order to avoid thyroid storm. Subtotal thyroidectomy seeks to cure the disease without inducing hypothyroidism but may be followed by a relapse, requiring removal of the thyroid remnants. The only argument in favor of subtotal thyroidectomy is the assumed lower complication rate. But comparison studies did not find any significant difference in the rate of complications after these two surgical procedures [26]. Total thyroidectomy and thyroxine substitution allows rapid return to the euthyroid state, without any possibility of recurrent hyperthyroidism. This is especially important in certain groups, such as women, who are planning pregnancy in the near future [26]. Specific complications of thyroid surgery include hypoparathyroidism, vocal cord paresis due to the damage to the recurrent laryngeal nerve, which occurs in 1–2% of patients, hemorrhage and infection [26,229]. Total thyroidectomy has been claimed to be preferable in patients with severe ophthalmopathy, as this procedure allows to remove any persistent thyroid antigens [233]. However, thyroid surgery is related to higher hospitalization costs than treatment with an antithyroid drug or radioactive iodine [163].

3. MATERIALS AND METHODS

The study protocol was approved by Kaunas Regional Biomedical Research Ethics Committee (reference number BE-2-21) with agreement for personal data processing by State Data Protection Inspectorate (reference number 2R-4146 (2.6.1)).

Scientific work consists of two studies:

I. Retrospective study of risk factors predicting ATD treatment failure in patients with Graves' disease.
II. The influence of genetic risk factors on Graves' disease phenotype and the outcome of antithyroid drug treatment.

3.1. Study population and methodology

3.1.1. Retrospective study of risk factors predicting antithyroid drug treatment failure in patients with Graves' disease

A retrospective study of all adult patients (≥18 yr.) with newly diagnosed Graves' disease who were referred to the Department of Endocrinology at the Hospital of Lithuanian University of Health Sciences in Kaunas between 2002 and 2007 was performed. Patients who fulfilled the following criteria were included in the study: clinical symptoms of hyperthyroidism, diffuse goiter detected by ultrasound examination, increased concentrations of free thyroxin (FT4), suppressed TSH concentrations, increased serum concentrations of TRAb, initial ATD treatment for a minimum of 12 months. The criteria for exclusion were:

- nodular goiter;
- normal serum concentrations of TRAb at the onset of the disease;
- the duration of the first course of ATD therapy less than 12 months.

All data was retrieved from medical documents and registered from the time of GD diagnosis until thyroid ablation or at least 12 months of follow - up after the discontinuation of ATD treatment. Complete remission was characterized by the disappearance of signs and symptoms of thyrotoxicosis and normalization of serum FT4 and TSH concentration. Remission of at least 12 months duration was regarded as long-term remission. Relapse was defined as the reappearance of signs and symptoms of thyrotoxicosis and increased serum concentration of FT4, suppressed concentration of TSH during the first year after the withdrawal of ATD therapy. The following parameters were recorded into the database at the onset of the disease for each patient: age, gender, goiter size by palpation, concentrations of FT4,

ATPO and TRAb; thyroid ultrasound examination (echogenicity and homogeneity). TRAb concentration at the end of ATD treatment and ultrasound examination of thyroid after ATD treatment were also recorded. The information about family history (FH) of thyroid disorders (including euthyroid, nodular goiter and unspecified thyroid disorders) and duration of ATD treatment was also obtained from medical documents.

According to the final outcome of the disease, patients were divided into remission (A) and treatment failure (B) groups.

A. **REMISSION group** (n=66) included patients who achieved a long-term (at least 12 months) complete remission after ATD withdrawal:

- **Group 1** (n=44) – patients who achieved long-term complete remission after initial ATD treatment with no relapses;
- **Group 2** (n=22) – patients who achieved long-term complete remission after two or three courses of ATD therapy. Patients from group 2 relapsed (one or two times) within 12 months after the withdrawal of initial ATD therapy and restarted ATD treatment until long-term (at least 12 months) complete remission was achieved.

B. **TREATMENT FAILURE group** (n=128) included patients who underwent thyroid ablation (surgery or radioiodine therapy):

- **Group 3** (n=44) – patients who underwent thyroid ablation due to relapses;
- **Group 4** (n=84) – patients who underwent thyroid ablation without ATD withdrawal.

One hundred and ninety four patients (38 males and 156 females) fulfilled the study criteria. Over three-quarters of patients were females, giving a ratio (male: female) of 1:4. The mean age of patients at the time of diagnosis was 42.4 ± 12.0 years (range 18–76). Remission group included sixty six patients, while treatment failure group – one hundred and twenty eight patients. Forty four patients achieved long-term complete remission after initial ATD treatment with no relapses (Group 1) and twenty two patients achieved long-term complete remission after two or three courses of ATD therapy (Group 2). Forty four patients underwent thyroid ablation due to relapses (Group 3); eighty four patients could not discontinue their ATD treatment and underwent thyroid ablation (Group 4) (Table 3.1.1.1). In the treatment failure group, 58 patients were treated with radioiodine, 65 patients underwent surgery, and 5 patients were treated with surgery after failed treatment with radioiodine.

Table 3.1.1.1. *The demographic characteristics of patients*

	Remission group (n=66)		**Treatment failure group (n=128)**		**Total (n=194)**
	Group 1 (n=44)	**Group 2 (n=22)**	**Group 3 (n=44)**	**Group 4 (n=84)**	
Male/Female, n (%)	13(19.7)/53(80.3)		25(19.5)/103(80.5)		38(19.6)/ 156(80.4)
	11(25.0)/ 33(75.0)	2(9.1)/ 20(90.9)	7(15.9)/ 37(84.1)	18(21.4)/ 66(78.6)	
Age (years), mean±SD	42.9±13.2		42.2±11.4		42.4±12.0
	44.2±12.4	40.2±14.6	41.4±13.5	42.6±10.2	

SD – standard deviation; Group 1 – patients achieved long-term complete remission after initial ATD treatment with no relapses; Group 2 –patients achieved long-term complete remission after two or three courses of ATD therapy; Group 3 – patients underwent thyroid ablation due to relapses; Group 4 – patients underwent thyroid ablation without ATD therapy withdrawal.

From the start of ATD treatment all patients received thiamazole (methimazole) 30 mg daily. Some patients were treated with thiamazole alone (57.7%) by dose titration regimen; others (42.3%) were supplemented by levothyroxine, continuing low dose antithyroid drugs after initial high dose ATD therapy (low dose block-replace treatment). The duration of the first course of ATDs was 21.7±14.9 months and the total duration of ATD therapy was 28.0±17.6 months. No statistically significant difference between groups 1, 2 and 3 was observed in initial duration of ATD treatment ($p=0.408$). The total ATD therapy duration was similar between group 2 and group 3 ($p=0.27$); between other groups difference in total duration was statistically significant ($p<0.05$). The initial and total duration of ATD therapy was significantly shorter in the remission group than treatment failure group ($p=0.02$) (Table 3.1.1.2.).

Table 3.1.1.2. *Details of the ATD therapy duration and relapse rates between patient groups*

Characteristics	Remission group		Treatment failure group	
	Group 1	**Group 2**	**Group 3**	**Group 4**
The duration of the first course of ATD therapy (months; mean ± SD)	18.0±10.0*		23.6±16.6*	
	19.2±11.6	15.6±5.1	19.1±10.4	26.3±18.9
Total duration of ATD therapy (months; mean ± SD)	23.4±13.1°		30.3±19.2°	
	19.2±11.6	31.9±11.9	38.1±17.5	26.3±18.9
Number of relapses (mean, range)	–	1.27 (1-2)	1.45 (1-3)	–

*,° p=0.02; SD, standard deviation; Group 1 – patients achieved long-term complete remission after initial ATD treatment with no relapses; Group 2 – patients achieved long-term complete remission after two or three courses of ATD therapy; Group 3 – patients underwent thyroid ablation due to relapses; Group 4 – patients underwent thyroid ablation without ATD therapy withdrawal.

Patients who underwent thyroid ablation due to relapses (group 3) more frequently than those who achieved a long-term remission (group 2) had early relapses (within 6 months) after ATD withdrawal (p=0.041). Remission group and treatment failure group did not significantly differ in different drug regimens (low dose block-replace and dose titration regimen) (p=0.52).

3.1.2. The influence of genetic risk factors on Graves' disease phenotype and the outcome of antithyroid drug treatment

The case-control study was performed in the Institute of Endocrinology of Lithuanian University of Health Sciences. All patients from the retrospective study population were invited by telephone to participate in the genetic study. Patients who agreed to participate were asked to attend a visit at which blood samples were collected for genetic investigation. The study included one hundred and five patients with GD. Forty eight patients (from remission group) achieved and sustained complete remission for a minimum 1 year after ATD withdrawal and fifty seven patients (from treatment failure group) underwent thyroid ablation (radioiodine therapy or surgery) due to poor response to ATD therapy. Mean age and gender of patients participating in the study did not differ significantly from the patients who did not participate in the genetic study. Control group was selected from adult subjects (n=90) with normal ultrasound of the thyroid, normal TSH concentration, normal concentration of thyroid peroxidase antibodies, and

negative family history of thyroid diseases. Written informed consents were obtained from all participants.

3.2. Methods of the study

3.2.1. Goiter size assessment

The goiter size was assessed by clinical examination according to the criteria recommended by The World Health Organization (1960): grade 0 – no goiter; grade IA – palpable but not visible goiter; grade IB – goiter palpable and visible only when the neck is fully extended; grade II – goiter visible with the neck in normal position; grade III – very large goiter visible at a distance [75]. Goiter grade I (IA and IB) we called small goiter, grade II – medium goiter and grade III – large goiter.

3.2.2. Ultrasound of the thyroid

Thyroid ultrasound examination was performed with high frequency real-time linear transducer (12 MHz). Echogenicity of the thyroid was described as normal (normoechogenic), decreased (hypoechogenic) or increased (hyperechogenic) compared with the echogenicity of prethyroid muscles and submandibular gland. Typically, a normal thyroid gland presents a higher echogenicity as compared with prethyroid muscles, and a similar or slightly higher echogenicity as compared with submandibular glands [280]. Homogeneity of the thyroid was described as homogeneous or inhomogeneous thyroid tissue. An ultrasound examination of the thyroid was performed on all patients at the time of diagnosis and after ATD therapy (in Group 4 ultrasound examination was performed at the time of diagnosis and before thyroid ablation).

3.2.3. Hormonal analysis

The hormonal analysis was performed in the Hormonology Laboratory of the Institute of Endocrinology of the Lithuanian University of Health Sciences. The serum TSH concentrations (normal range 0.17–4.05 mIU/l) were measured by immunoradiometric assay (IRMA), FT4 concentrations (normal range 11.5–23.0 pmol/l) and ATPO concentrations (normal range <20 IU/ml; borderline 20–100 IU/ml; positive >100 IU/ml) were determined by radioimmunoassay (RIA) using commercially available kits by IMMUNOTECH (Beckman Coulter, Prague, Czech Republic) with sensitivity of 0.025 mIU/l for TSH, 0.4 pmol/l for FT4, and 4 IU/ml for ATPO. TSH receptor antibodies (thyroid binding inhibiting immunoglobulins)

(normal range <9 U/l; borderline 9–14 U/l; positive >14 U/l) were measured by radioreceptor assay (RIAZEN TSH-R-Ab, ZenTech S.A., Belgium) with sensitivity of 0.8 U/l. The intra-assay and inter-assay coefficients of variation were 3.7% and 5.7% for TSH, 6.7% and 6.5% for FT4, 9.7% and 10% for ATPO, 9.7% and 9.2% for TRAb.

3.2.4. Genotyping

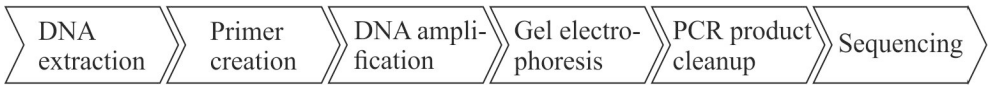

Fig. 3.2.4.1. *Genotyping workflow.*

DNA samples and extraction

From each subject 9 ml of blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes. The tubes were centrifuged and preserved at (-70 ºC) until further laboratory procedures. Genomic DNA was extracted from 100 µl of peripheral blood buffy coat (leukocytes) using DNeasy® Blood & Tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Reagents:

- DNeasy® Blood and tissue kit (Qiagen GmbH, Hilden, Germany);
- PBS, pH 7.2 (50 mM potassium phosphate, 150 mM NaCl).

Equipment:

- 10/100/1000 µl single-channel pipettes (Eppendorf AG, Germany);
- 1.5 ml tubes (Eppendorf AG, Germany)
- Centrifuge 5415 (Eppendorf AG, Germany);
- Thermomixer comfort (Eppendorf AG, Germany);
- Minishaker (IKA, Wilmington, USA);
- Spectrophotometer Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, USA);

Protocol:

1. Pipet 20 µl of proteinase K into a 1.5 ml microcentrifuge tube. Add 100 µl of buffy coat. Adjust the volume to 220 µl with PBS.
2. Add 200 of µl Buffer AL (without added ethanol). Mix thoroughly by vortexing and incubate at 56°C for 10 min.
3. Add 200 µl of ethanol (96–100%) to the sample and mix thoroughly by vortexing.
4. Pipet the mixture from step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at 6000 × g (8000 rpm) for 1 min. Discard flow-through and collection tube.
5. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl of Buffer AW1 and centrifuge for 1 min at 6000 × g (8000 rpm). Discard flow-through and collection tube.
6. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl of Buffer AW2, and centrifuge for 3 min at 20,000 × g (14,000 rpm) in order to dry the DNeasy membrane. Discard flow-through and collection tube.
7. Place the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube, and pipet 200 µl of Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min and then centrifuge for 1 min at 6000 × g (8000 rpm) to elute.
8. The DNA can be stored at -20 ºC.

2 µl of extracted DNA was analyzed on a spectrophotometer (Nanodrop 2000) and A260/280 ratio was ≥ 1.8.

DNA amplification

Specific primers for PCR were designed with the assistance of the *Primer3* web based software (Fig. 3.2.4.2) [205].

A. Thyroglobulin (TG) gene on chromosome 8 (GenBank accession number NG_015832.1). Analyzed fragment length 207 bp (underlined marker). Used primer location highlighted in gray. SNP location rs2076740 (bold) in exon 33 (104835..104914).

```
104581 gcatgatgaa tgaatgggtg aataaatgat taatggcagg acagtattcc tgagaggagt
104641 agaattttgt tttcagaaag aacctcaaag ctaagaatcc actcatgcat attgaccaaa
104701 gcacccccag tttaagtagg gggtaaaaaa ataagctagt tccccaaagc aagaatgact
104761 accatcagcc cacccagtag gtcctgggtc accagtgaaa accttcaggc ctgctctttc
104821 tcttcctatg ccaggttctt tgaatgtgaa cgaCggtgcg atgcggaccc atgctgcact
104881 ggctttggat ttctaaatgt ttcccagtta aaaggtaata atggtaacaa cttcctctcc
104941 cctgcgcaca gtactctgca gtttagaaaa cacattcaca tctatgcttt tacaaatcct
105001 cagcatctcc tatcctcgtt cacagataag gaaatggggg cttggagagg gcaactggct
105061 aataaatgtg gtagctgaga catggctcct agtccagtga tcatcacttt acatgtgcag
105121 ttctacctgg ccaaacttcc ttccttctac tcttggcttg gatatctttt gttcatcttt
```

B. Cytotoxic T-lymphocyte-associated protein - 4 (CTLA-4) gene on chromosome 2 (GenBank accession number NG_011502.1). Analyzed fragment length 215 bp (underlined marker). Used primer location highlighted in gray. SNP location rs231775 (bold) in exon 1 (1..264).

```
1   cttctgtgtg tgcacatgtg taatacatat ctgggatcaa agctatctat ataaagtcct
61  tgattctgtg tgggttcaaa cacatttcaa agcttcagga tcctgaaagg ttttgctcta
121 cttcctgaag acctgaacac cgctcccata aagccatggc ttgccttgga tttcagcggc
181 acaaggctca gctgaacctg gctAccagga cctggccctg cactctcctg ttttttcttc
241 tcttcatccc tgtcttctgc aaaggtgagt gagacttttg gagcatgaag atggaggagg
301 tgtttctcct acctgggttt catttgttc agcagtcaaa ggcagtgatt tatagcaaag
361 ccagaagtta aaggtaaaac tccaatctgg cttggctggc tctgtattcc agggccagca
421 gggagcagtt gggcggcagc aaataaggca aagagatagc tcagaacaga gcgccaggta
481 tttagtaggg gcttcatgaa tgcatgtgag ttggtttagt agagagacac aggcaatttc
```

C. Cytotoxic T-Lymphocyte-associated protein - 4 (CTLA-4) gene on chromosome 2 (GenBank accession number NG_011502.1. Abalyzed fragment length 185 bp (underlined marker). Used primer location highlighted in gray. SNP location rs3087243 (bold) in 3'UTR (untranslated region).

```
11221 tttttgattg attcattcag tatctggtgg agtctccaat attagaaata ctggaaacaa
11281 actgaaaaac cacaaaagga caaataatgc ttcatgagtc agctttgcac cagccattac
11341 ctgcaagtca ttcttggaag gtatccatcc tctttccttt tgatttcttc accactattt
11401 gggatataac Ggtgggttaac acagacatag cagtccttta taaatcaatt ggcatgctgt
11461 ttaacacagg ttcttcacct ccccttctt accgcctgct ttctcagctc aactatcaca
11521 ggcattacag ttgtcatggc aaccccaatg ttggcaacca cgtcccttgc agccattttg
11581 atctgccttc ctgaaatata gagcttttcc ctgtggcttc caaatgaact attttgcaaa
11641 tgtggggaaa acacacacct gtggtcctat gttgctatca gctggcacac ctaggcctgg
11701 cacactaagc cctctgtgat tcttgcttaa ccaatgtata gtctcagcac atttggtttc
```

Fig. 3.2.4.2. *Wild type sequences of studied regions with positioned PCR primers*

PCR was performed in a 0.2 ml PCR tubes. The ready mix was vortexed and centrifuged briefly, then distributed to PCR strips, followed by the addition of 100 ng genomic DNA. Final volume in each PCR tube was ca. 50 µl. DNA amplification reaction conditions and PCR primers are shown in Tables 3.2.4.1 and 3.2.4.2.

Equipment:

- Thermocycler – Mastercycler Epgradient (Eppendorf AG, Germany);
- 10/100 single-channel pipettes (Eppendorf AG, Germany);
- Centrifuge 5415 (Eppendorf AG, Germany);
- Minishaker (IKA, Wilmington, USA);
- PCR tubes, 0.2 ml. (Axygen).

PCR master mix for one sample:*

PCR Master Mix (2x) (Fermentas, Lithuania)	25 µl
Primer (forward) 10 pmol	1 µl
Primer (reverse) 10 pmol	1 µl
Water	12 µl
DNA (100 ng)	2 µl
Total volume	50 µl

*Use approximately 10% more reagents than needed due to pipetting errors.

Table 3.2.4.1. *Primers used for the amplification of genomic DNA*

SNP localization	Primer	Sequence	Annealing T (°C)
CTLA-4 49A/G (rs231775)	forward	CTGAACACCGCTCCCATAAA	58
	reverse	CACTGCCTTTGACTGCTGAA	
CTLA-4 CT60 (rs3087243)	forward	CTTTGCACCAGCCATTACCT	60
	reverse	CTGAGAAAGCAGGCGGTAAG	
TG E33 C/T (rs2076740)	forward	GTTCCCCAAAGCAAGAATGA	60
	reverse	GCAGGGGAGAGGAAGTTGTT	

Table 3.2.4.2. *Genomic DNA amplification reaction conditions*

Step	Temperature, °C	Duration	The number of cycles
Initial DNA denaturation	95	1 min	–
DNA denaturation	95	30 s	35
Annealing	X	30 s	
Extension	72	1 min	
Final Extension	72	10 min	–
END (pause)	4	–	–

X – primer specific annealing temperature presented in the Table 3.2.4.1.

Product can stay in the thermocycler overnight at temperatures 4°C to 10°C without loss of performance.

The amplified products were analyzed on 2% agarose gel with ethidium bromide, and after electrophoresis gels were visualized under UV.

Equipment:

- Electrophoresis Power Supply and horizontal submarine unit (BIO-RAD Laboratories, USA);
- Molecular imager Gel Doc 2000 system (BIO-RAD Laboratories, USA);
- Microwave 700 W (Samsung, South Korea);
- 10/100 single-channel pipettes (Eppendorf AG, Germany); 10/100 single-channel pipettes (Eppendorf AG, Germany).

Reagents:

- Buffer 10×TBE UltraPure™ (Invitrogen, USA);
- Ethidium bromide (Merck, Darmstadt, Germany);
- 6X DNA Loading Dye (Thermo Scientific, Lithuania);
- GeneRuler^{TM} 100 bp DNA Ladder (Thermo Scientific, Lithuania);
- Agarose (PeQlab Biotechnologie, Erlagen, Germany);
- Distilled water.

Procedure

Making the gel:

1. place the casting platform with well-formed sideways in the gel stand where you wish to pour the gel
2. add 2g of agarose per 100 ml for making 2% gel;
3. add 100 ml of 1×TBE.
4. mix and microwave for about 1–3 minutes until boiling;
5. add 15 mg of ethidium bromide/ml of gel solution from stock solution (10 mg/ml);
6. pour hot gel into the gel cast on a flat surface avoiding bubble formation.

Loading the gel:

1. load the loading dye onto the parafilm;
2. add the appropriate volume of sample and mix with one pipette;
3. very carefully load the sample with the loading pipette into the appropriate well carefully.

2 µl of loading dye was added to each 5 µl of PCR product and this mixture was pipetted into wells of a 2% agarose gel. A DNA ladder was loaded at the same time to confirm that products' sizes were as expected.

Gels were run for 25 minutes at 150 volts. Each DNA sample was visualized with UV light using a molecular imager (Fig. 3.2.4.3).

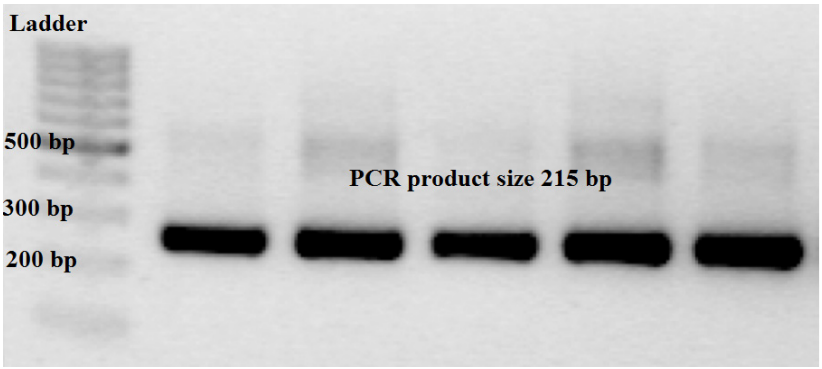

Fig. 3.2.4.3. *DNA amplification products (CTLA-4 SNP rs3087243) on a 2 % agarose gel*

PCR product cleanup

PCR product cleanup from leftover primers and unreacted nucleotides before sequencing was performed with a DNA Clean & Concentrator™-25 kit (Zymo Research corp., Orange, USA) according to the manufacturer's recommendations.

Equipment:

- 100/1000 µl single-channel pipettes (Eppendorf AG, Germany);
- 1.5 ml tubes (Eppendorf AG, Germany);
- Centrifuge 5415 (Eppendorf AG, Germany);

Procedure:

1. In a 1.5 ml microcentrifuge tube add 5 volumes of DNA Binding Buffer to each volume of DNA sample. Mix briefly by vortexing.
2. Transfer mixture to a Zymo-SpinTM Column in a Collection Tube.
3. Centrifuge for 30 seconds at 16,000×g. Discard the flow-through.
4. Add 200 µl of the DNA wash buffer to the column. Centrifuge at max.speed for 30 seconds. Repeat the wash step.
5. Add 25 µl of the DNA elution buffer directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge for 30 seconds at 16,000×g to elute the DNA.

PCR product after cleanup was stored at (-20°C) until further analysis by sequencing.

Sequencing

DNA fragments preparation for scanning was performed by MACROGEN Company (Seoul, Korea, #60–24, Gasan-dong Geumchen-gu). DNA sequencing was performed with the HiSeq® 2500 Sequencing System (Illumina Inc., USA). Results of sequencing were analyzed with the assistance of the Finch TV 1.4.0 software (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Representative chromatograms are presented in Fig. 3.2.4.4–3.2.4.6.

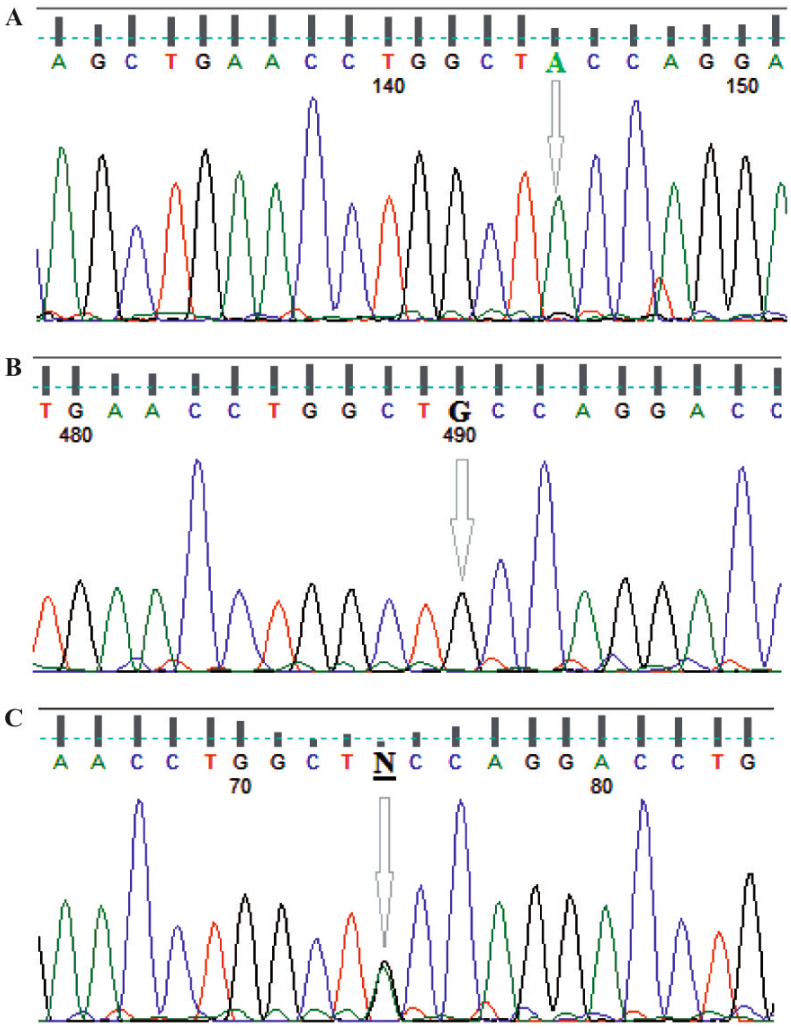

Fig. 3.2.4.4. *Representative chromatograms of CTLA-4 49A/G SNP analysis: (A) – AA homozygote, (B) – GG homozygote, (C) – polymorphism AG represented as N.*

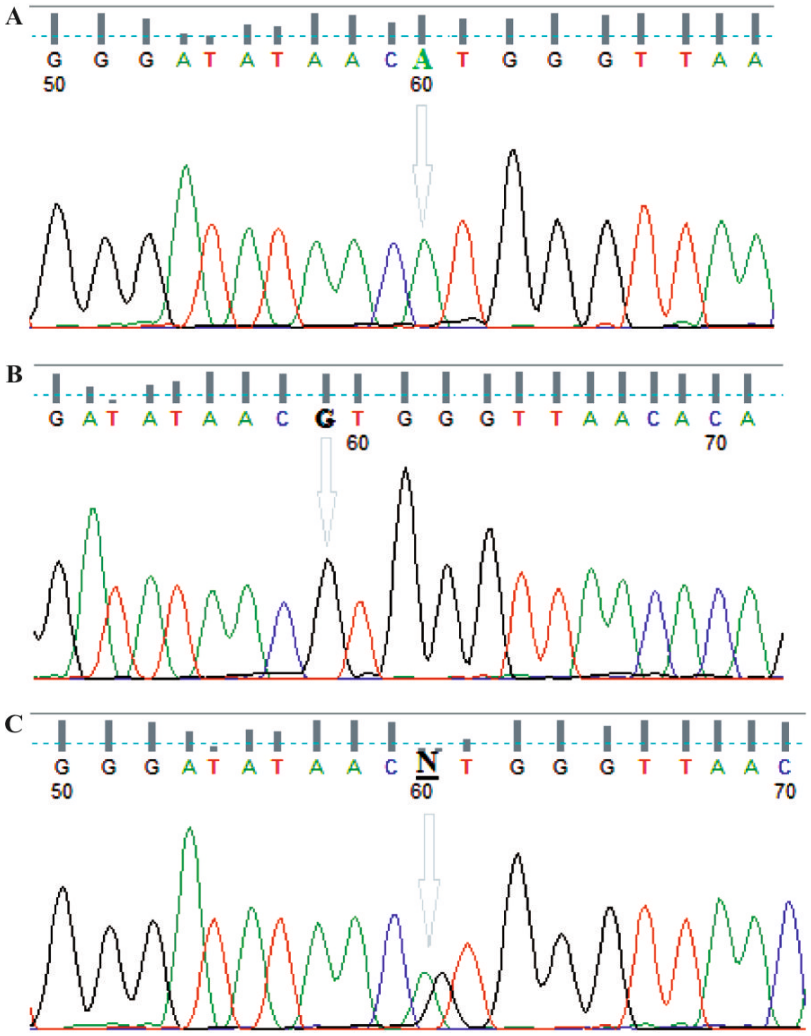

Fig. 3.2.4.5. *Representative chromatograms of CTLA-4 CT60 SNP analysis: (A) – AA homozygote, (B) – GG homozygote, (C) – polymorphism AG represented as N.*

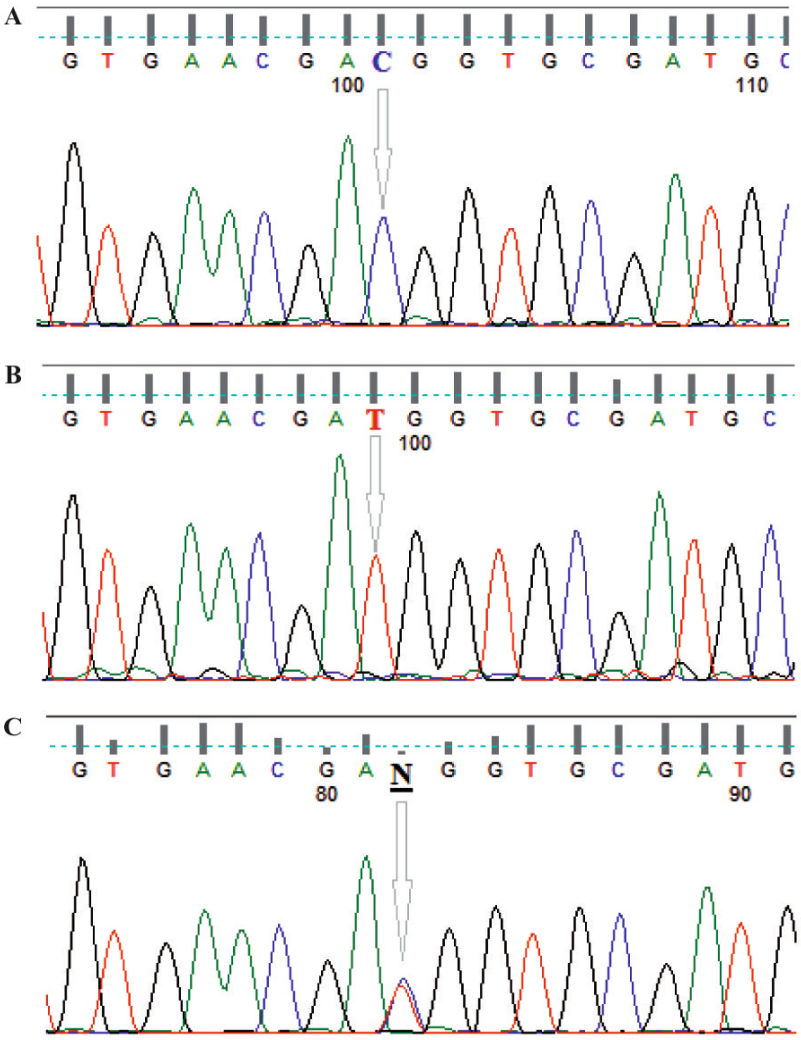

Fig. 3.2.4.6. *Representative chromatograms of TG E33 C/T SNP analysis: (A) – CC homozygote, (B) – TT homozygote, (C) – polymorphism CT represented as N.*

3.3. Sample size determination

The retrospective study of 120 patients was performed to calculate a minimum sample size. Mean concentration of TRAb at the onset of the disease was 28.7 (U/l) in remission group and 75.4 (U/l) in treatment failure group, SD±71.4. To determine a minimum sample size we used Snedecor and Cochran formula [225]:

$$n = 1 + 2C \cdot \left(\frac{SD}{d}\right)^2$$

Here, SD is the standard deviation, d is the difference between means, and C is a constant dependent on the selected α and β values. C for α =0.05 and 1–β =0.9, is 10.51; d= (m_1–m_2) =46.7, then:

$$n = 1 + 21 \cdot \left(\frac{71.4}{46.7}\right)^2 = 50$$

A minimum sample size for each group with statistical power 0.9 is 50 patients and for the whole study – 100 patients.

The sample size for genetic study and the power of the current sample size were calculated using web based (http://sampsize.sourceforge.net/iface/s3.html#cc) software considering the allele frequencies of all studied SNPs in European populations. According to the SNP database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/SNP), the frequency of G allele of CTLA-4 49A/G SNP is 38.9%, G allele of CTLA-4 CT60 SNP – 54.0% and T allele of TG E33 C/T SNP – 38.5%. A minimum sample size for each group with an anticipated odds ratio of 2.5 and 80% statistical power at a significance level of p=0.05 is 77 patients for CTLA-4 49A/G SNP (for the whole study – 154 patients), 84 patients for CTLA-4 CT60 SNP (for the whole study – 168 patients) and 77 patients for TG E33 C/T SNP (for the whole study – 154 patients).

Considering allele frequencies in the study population, our study with its current sample size has ≥80% power to detect significant ($p<0.05$) effects with OR of ≥2.2 (or ≤0.43) for the G allele of CTLA-4 49A/G SNP, with OR of ≥2.3 (or ≤0.45) for the G allele of CTLA-4 CT60 SNP and with OR of ≥2.2 (or ≤0.42) for the T allele of TG E33 C/T SNP.

3.4. Statistical analysis

Descriptive analyses were performed for demographic, clinical and laboratory variables. One sample Kolmogorov-Smirnov test was used to test

for normality of the variable. The distribution of continuous variables is presented as means ± standard deviation (SD). Chi-square (χ^2) test or Fisher's exact test were used for analysis of data when variables were categorical. For continuous data we used the Mann-Whitney test. The Kruskal-Wallis test was used for the comparison of continuous variables between more than 2 independent groups. For bivariate correlations, Pearson (r) correlation test (for parametric data) or Spearman (r) correlation test (for non-parametric data) were used. Wilcoxon signed rank test was used for within-group changes. Odds ratios (OR) with 95% confidence interval (CI) were calculated by logistic regression to estimate associations of different variables with outcome of GD. Multiple logistic regression analysis was performed to assess the independent predictors of GD outcome. A receiver-operating characteristic (ROC) curve analysis was conducted to assess the prognostic value of TRAb in predicting the outcome of GD. Cut-off values were determined with MedCalc software (MedCalc, Belgium). Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows (version 17.0.0).

We tested Hardy-Weinberg equilibrium for each genetic marker among controls and GD patients using the χ^2 test. CubeX analysis software (http://www.oege.org/software/cubex) was used to estimate the haplotype frequencies, the normalised linkage disequilibrium (LD) parameter (D'), and the LD correlation coefficient between two loci (r^2) [97]. Genetic association analyses were performed using SNPStats software (http://bioinfo.iconcologia.net/snpstats/) [226]. Based on the logistic regression method the case control association of genotypes in five inheritance models for each SNP were tested: codominant (major allele homozygotes vs. heterozygotes and major allele homozygotes vs. minor allele homozygotes), dominant (major allele homozygotes vs. heterozygotes + minor allele homozygotes), recessive (major allele homozygotes + heterozygotes vs. minor allele homozygotes), overdominant (major allele homozygotes + minor allele homozygotes vs. heterozygotes) and log-additive (major allele homozygotes vs. heterozygotes vs. minor allele homozygotes). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated. Akaike's information criterion (AIC) was used to select the genetic model that best fits the data (the model with the lowest AIC score was the best fitting). Bonferroni correction for number of studied CTLA-4 SNPs was applied. Haplotype analysis was performed using SNPStats [226]. P value less than 0.05 was considered as statistically significant.

4. RESULTS

4.1. Retrospective study of risk factors predicting antithyroid drug treatment failure in patients with Graves' disease

4.1.1. Gender and age related clinical and laboratory differences in patients with Graves' disease

The peak incidence of Graves' disease was observed in the fifth decade of life for both genders (Fig. 4.1.1.1).

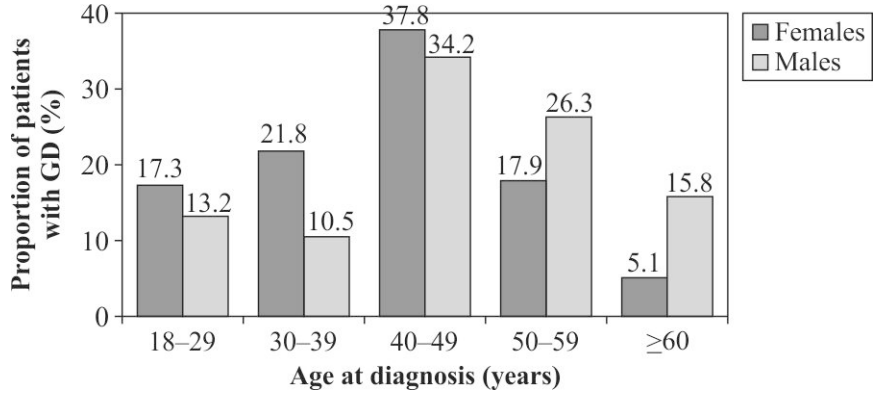

Fig. 4.1.1.1. *Age and gender distribution of patients at the onset of Graves' disease.*

The mean age of males was greater than females (46.5±12.5 years vs. 41.4±11.7 years, p=0.022, respectively). The biochemical severity of thyroid dysfunction (serum FT4) and antibodies concentrations (ATPO and TRAb) at diagnosis were similar in females and males (p=0.87, p=0.15 and p=0.63, respectively). The thyroid eye disease was documented in 46% of patients with similar proportions of females and males (p=0.351) (Table 4.1.1.1).

Table 4.1.1.1. *The relationship of gender with demographic and clinical characteristics at presentation of Graves' disease.*

Characteristics	Males	Females	p value
Age at diagnosis (years; mean ± SD)	46.5 ± 12.5	41.4 ±11.7	**0.022**
Family history of thyroid disorders Yes (%) No (%)	 15.8 84.2	 19.2 80.8	 0.625
Diffuse goiter n (%) Small (n=31) Medium (n=126) Large (n=37)	 8 (21.1) 27 (71.1) 3 (7.9)	 23 (14.7) 99 (63.5) 34 (21.8)	 0.13
Ophthalmopathy n (%)	20 (52.6)	69 (44.2)	0.351
FT4 (pmol/l; mean±SD)	58.1 ± 22.8	59.1 ± 23.3	0.87
ATPO (IU/ml; mean ± SD)	317.1 ± 309.1	327.5 ± 568.2	0.15
TRAb (U/l; mean ± SD)	57.0 ± 65.5	64.7 ± 76.4	0.63

SD – standard deviation; FT4 – free thyroxine; ATPO – antithyroid peroxidase antibodies; TRAb – TSH receptor antibodies.

The presence of the large goiter was associated with lower mean age at diagnosis in both gender groups (females with large goiter 37.6±10.7 years vs. females with small goiter 44.7±9.2 years, p=0.006; males with large goiter 27.3±3.5 years vs. males with medium goiter 46.9±11.8 years and vs. males with small goiter 52.5±10.4 years, p=0.019 and p=0.014, respectively) (Fig. 4.1.1.2.).

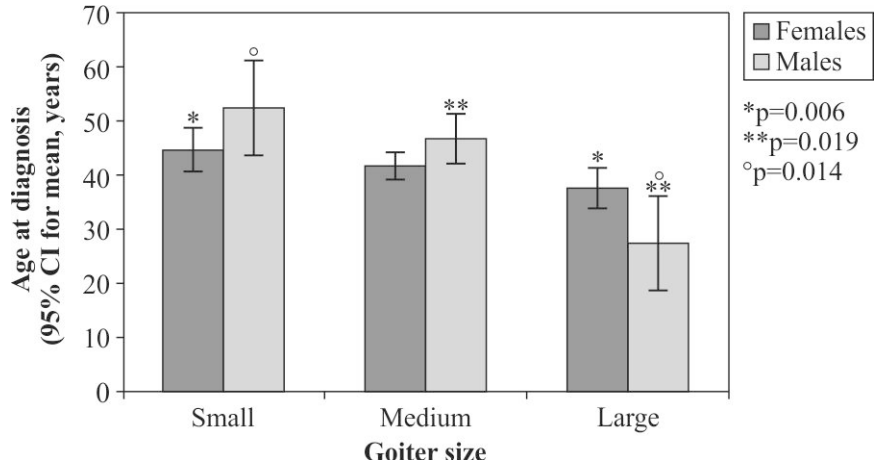

Fig 4.1.1.2. *Age at diagnosis (years) in males and females with Graves'disease according to the goiter size. Charts represent mean values; error bars represent 95% confidence intervals (CI).*

Males and females had the same outcome after ATD treatment with a remission rate of 34%. In males, the presence of larger goiter size was associated with greater biochemical disease severity (males with medium goiter FT4 64.4±20.9 pmol/l vs. males with small goiter FT4 39.4±19.4 pmol/l, p=0.008) (Fig. 4.1.1.3).

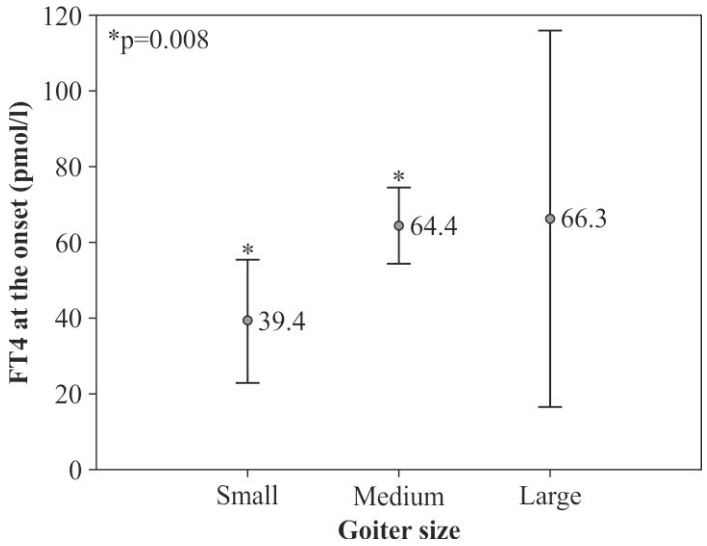

Fig. 4.1.1.3. *Serum FT4 concentrations (pmol/l) at the onset of the disease in male subjects with Graves' disease according to the goiter size. Charts represent mean values and 95% confidence intervals (CI).*

Age at the onset of the disease did not correlate with ATPO and TRAb concentrations before treatment (p=0.268 and p=0.248, respectively), but showed a weak negative correlation with initial FT4 concentrations (r=–0.249, p=0.046), although this relationship was lost after adjustment for gender (p=0.101).

For analysis, patients were divided into two age groups: younger than 40 years at presentation and 40 years and older. Age at the onset of the disease did not predict the ATD treatment outcome. Younger patients had the same remission rate as older (p=0.79). Age less than 40 years was a significant predictor of the presence of large goiter at diagnosis. Patients younger than 40 years of age were more likely to have large goiter than medium or small goiter compared with patients in an older age group (OR 2.45, 95% CI, 1.16–5.16 and OR 5.47, 95% CI, 1.82–16.48, respectively). It should be noted that when age was analyzed as a continuous variable, the result were identical to those above (p<0.05). Patients younger than 40 years had insignificantly higher mean FT4 concentrations (63.9±24.2 vs.

55.7±21.9, p=0.057) and TRAb concentrations (69.7± 81.5 vs. 59.4±69.9, p=0.347) before treatment than the older age group patients.

4.1.2. Influence of family history of thyroid disorders on Graves' disease outcome

18.6% of patients with GD (30 females and 6 males) reported FH of thyroid disorders (including euthyroid, nodular goiter and unspecified thyroid disorders). The overall prevalence of positive FH was similar in females and males (19.2% and 15.8%, respectively, p=0.625). A positive FH of thyroid disorders was associated with poor outcome of GD. Patients, who failed ATD treatment, reported FH of thyroid disorders more frequently than patients who achieved long-term remission (22.7% vs. 10.6%, p=0.041). Patients with positive FH were 2.5 times more likely to fail to respond to medical treatment (OR 2.5; 95% CI, 1.02–5.99).

4.1.3. Goiter size predicts the outcome of antithyroid drug treatment

All patient groups did not differ significantly in mean age, gender, and mean FT4 and ATPO concentrations before the ATD treatment (Table 3.1.1.1 and Table 4.1.3.1).

Table 4.1.3.1. *Comparison of mean FT4 and ATPO concentrations between patient groups*

Characteristics	**Remission group (n=66)**		**Treatment failure group (n=128)**	
	Group 1 (n=44)	**Group 2 (n=22)**	**Group 3 (n=44)**	**Group 4 (n=84)**
FT4 (pmol/l) (mean±SD)	57.3±22.2		59.9±23.8	
	58.6±22.9	55.0±21.3	59.3±20.4	60.3±25.5
ATPO(IU/ml) (mean±SD)	284.9±479.7		346.8±555.3	
	296.3±562.0	265.3±302.9	632.2±844.6	219.5±287.4

SD – standard deviation; FT4 – free thyroxine; ATPO – antithyroid peroxidase antibodies; Group 1 – patients achieved long-term complete remission after initial ATD treatment with no relapses; Group 2 – patients achieved long-term complete remission after two or three courses of ATD therapy; Group 3 – patients underwent thyroid ablation due to relapses; Group 4 – patients underwent thyroid ablation without ATD therapy withdrawal.

We detected significant differences in the goiter size at the onset of the disease in all patient groups. Small goiter was more frequent in group 1 than in group 2 ($p=0.012$) or group 3 ($p=0.025$). The prevalence of medium goiter was higher in group 2 and group 3 than in group 4 ($p<0.001$ and $p=0.012$, respectively), but also higher in group 4 than in group 1 ($p=0.002$). Large goiter was more frequent in group 4 than in group 1, group 2, and group 3 ($p<0.001$, $p<0.001$ and $p=0.009$, respectively), and more frequent in group 3 than in group 2 ($p=0.011$) (Fig.4.1.3.1.).

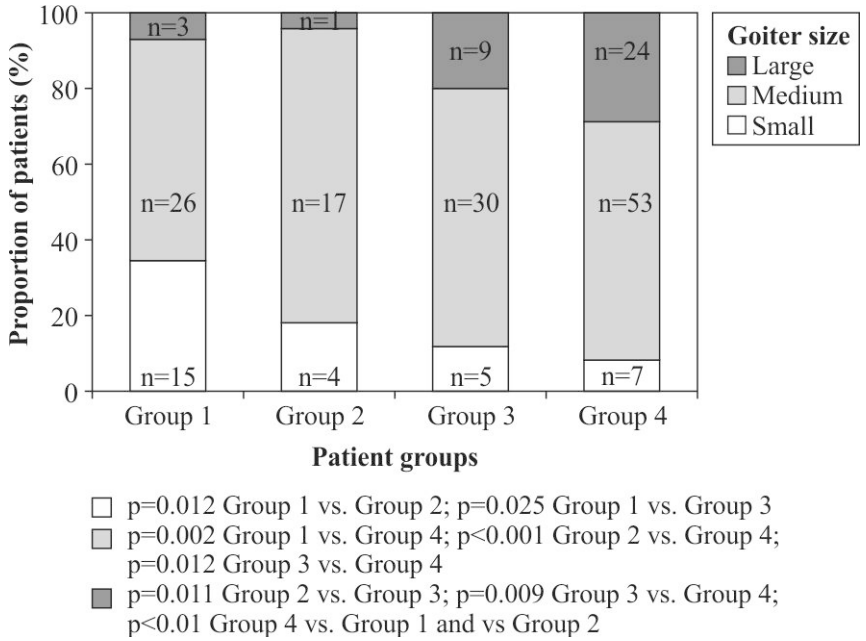

Fig. 4.1.3.1. *The prevalence of different size of goiters between patient groups at the onset of the disease.*

Group 1 – patients achieved long-term complete remission after initial ATD treatment with no relapses; Group 2 – patients achieved long-term complete remission after two or three courses of ATD therapy; Group 3 – patients underwent thyroid ablation due to relapses; Group 4 – patients underwent thyroid ablation without ATD therapy withdrawal.

Treatment failure group had higher frequency of larger goiter (medium or large) than the remission group ($p<0.001$) (Fig. 4.1.3.2).

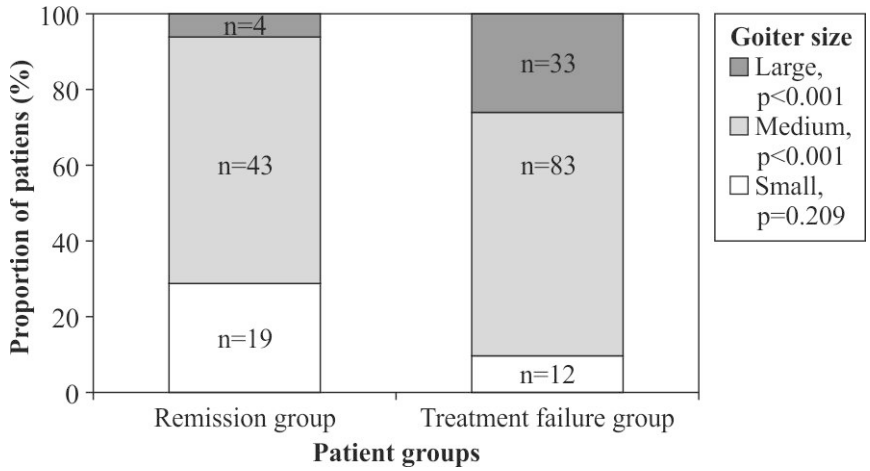

Fig. 4.1.3.2. *The prevalence of diferent size of goiters at the onset of the disease between remission and treatment failure groups*

The goiter size was not associated with serum FT4 and ATPO concentrations at the time of diagnosis (p=0.325 and p=0.272, respectively). Significant relationship was observed between serum TRAb concentrations at the time of diagnosis and goiter size: patients with large goiter had higher mean concentrations of initial TRAb than patients with small goiter (81.2±89.6 vs. 53.0±83.2 U/l, p=0.005, respectively). The presence of larger goiter was significantly associated with failure of ATD treatment. Presence of medium goiter (OR 3.1, 95% CI, 1.4–6.9) and especially large goiter (OR 13.1, 95% CI, 3.7–46.3) compared with small goiter increased the risk of failure.

4.1.4. Influence of thyroid echogenicity on the outcome of Graves' disease

At baseline, 172 (88.7%) patients had a hypoechogenic thyroid and 22 (11.3%) patients had normoechogenic thyroid. After ATD therapy hypoechogenic thyroid was observed in 146 (75.3%) patients, and normoechogenic thyroid in 48 (24.7%) patients. In 154 (79.4%) patients before treatment thyroid echogenicity was decreased in combination with an inhomogeneous thyroid echotexture, while after treatment hypoechogenic thyroid with inhomogeneous echotexture was observed in 138 (71.1%) patients. The changes in thyroid echogenicity after ATD therapy were statistically significant in all patient groups (p<0.05) (Fig.4.1.4.1–4.1.4.2).

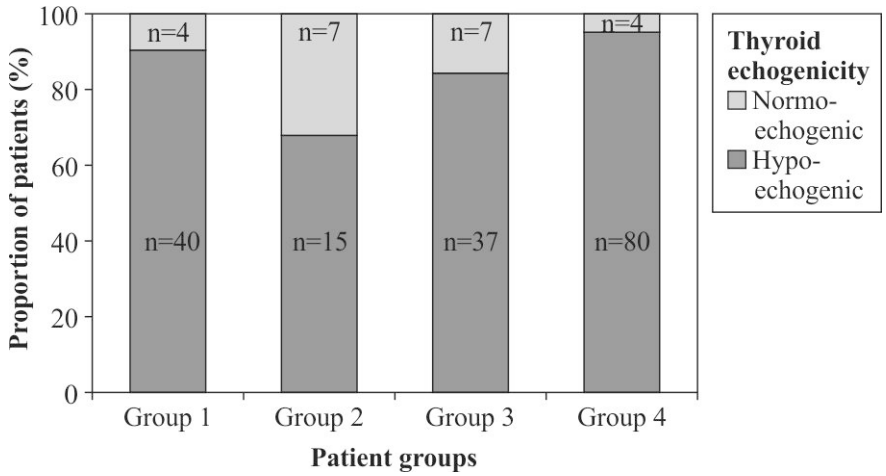

Fig. 4.1.4.1. *Distribution of the thyroid echogenicity at the onset of the disease between patient groups.*

Group 1 – patients achieved long-term complete remission after initial ATD treatment with no relapses; Group 2 – patients achieved long-term complete remission after two or three courses of ATD therapy; Group 3 –patients underwent thyroid ablation due to relapses; Group 4 – patients underwent thyroid ablation without ATD therapy withdrawal.

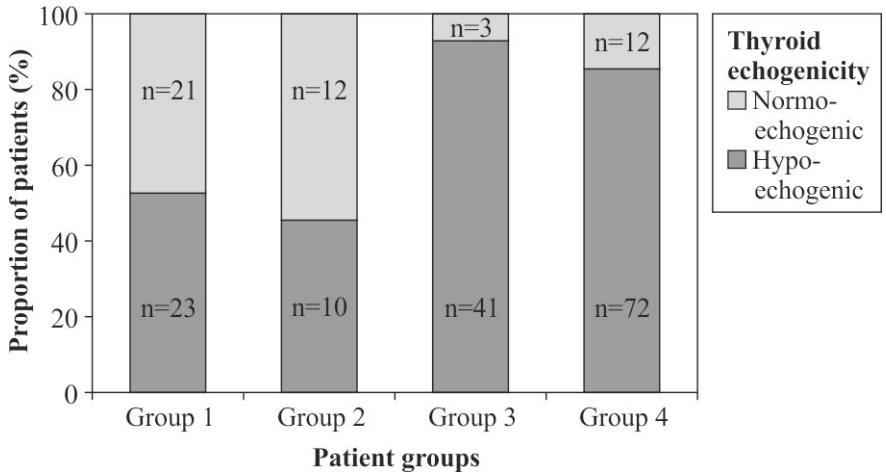

Fig. 4.1.4.2. *Distribution of the thyroid echogenicity after ATD treatment between patient groups*

Group 1 – patients achieved long-term complete remission after initial ATD treatment with no relapses; Group 2 – patients achieved long-term complete remission after two or three courses of ATD therapy; Group 3 – patients underwent thyroid ablation due to relapses; Group 4 – patients underwent thyroid ablation without ATD therapy withdrawal.

Our results revealed, that the echogenicity of the thyroid before ATD therapy did not predict the outcome of GD. Patients with hypoechogenic and normoechogenic thyroid before treatment had the same frequency of failure (p=0.093). Patients with hypoechogenic thyroid after ATD therapy more frequently failed to respond to treatment than patients with normoechogenic thyroid (p<0.001) (Table. 4.1.4.1).

Table 4.1.4.1. *The relationship between thyroid echogenicity and the outcome of GD*

Echogenicity of the thyroid		**Outcome**			
Thyroid echogenicity before ATD therapy	**Thyroid echogenicity after ATD therapy**	**Remission group, n (%)**		**Treatment failure group, n (%)**	
Hypoechogenic	Hypoechogenic	27 (20.0)’	33 (22.6)°	108 (80.0)’	113 (77.4)°
Normoechogenic		6 (54.5)		5 (45.5)	
Hypoechogenic	Normoechogenic	28 (76.0)	33 (68.8)*	9 (24.0)	15 (31.2)*
Normoechogenic		5 (45.5)		6 (54.5)	

’p=0.008; *p=0.009; °p<0.001

The presence of hypoechogenic thyroid after ATD therapy increased the risk of failure more than 7.5 times (OR 7.53, 95% CI 3.7–15.5; p<0.001) with sensitivity of 88% and specificity of 50% (positive predictive value of 77% and negative predictive value of 73%). The changes in thyroid echogenicity after ATD therapy revealed that persistent hypoechogenicity increased the risk of failure 7.8 times (OR 7.8, 95% CI 3.93–15.46).

We investigated the possible combinations of both factors before treatment, the thyroid echogenicity and its size, in affecting the outcome of GD. Although only goiter size before treatment showed the statistical significance in predicting the poor outcome, the overall test of interaction of these two factors was significant. The impact of thyroid size was significant only in the case of hypoechogenic thyroid, while in case of normoechogenic thyroid, the goiter size did not have the significant influence on the outcome of GD. The presence of hypoechogenic medium and especially hypoechogenic large goiter comparing with hypoechogenic small goiter was associated with an increased likelihood of failure (p=0.019 and p<0.001, respectively) (Table 4.1.4.2).

Table 4.1.4.2. *The association of thyroid echogenicity and goiter size with antithyroid drug treatment failure*

Characteristics	Failure, n (%)	Failure OR (95% CI)	p value
Hypoechogenic thyroid			
Small goiter	26 (15.1)	1.0 (reference)	
Medium goiter	111 (64.5)	2.84 (1.2–6.8)	**0.019**
Large goiter	35 (20.3)	10.6 (2.9–38.8)	**<0.001**
Normoechogenic thyroid			
Small goiter	5 (22.7)	1.0 (reference)	
Medium goiter	15 (68.2)	4.57 (0.4–51.1)	0.217
Large goiter	2 (9.1)	3.0 (0.6–14.9)	0.053

OR – odds ratio; CI – confidence interval.

4.1.5. Prognostic value of TRAb in predicting antithyroid drug treatment failure

Mean concentrations of TRAb before and at the end of ATD treatment were significantly higher in group 3 and group 4 than in group 1 and group 2 and also were higher in group 4 than in group 3 ($p<0.05$), but the difference between group 1 and group 2 was insignificant (Fig. 4.1.5.1, 4.1.5.2).

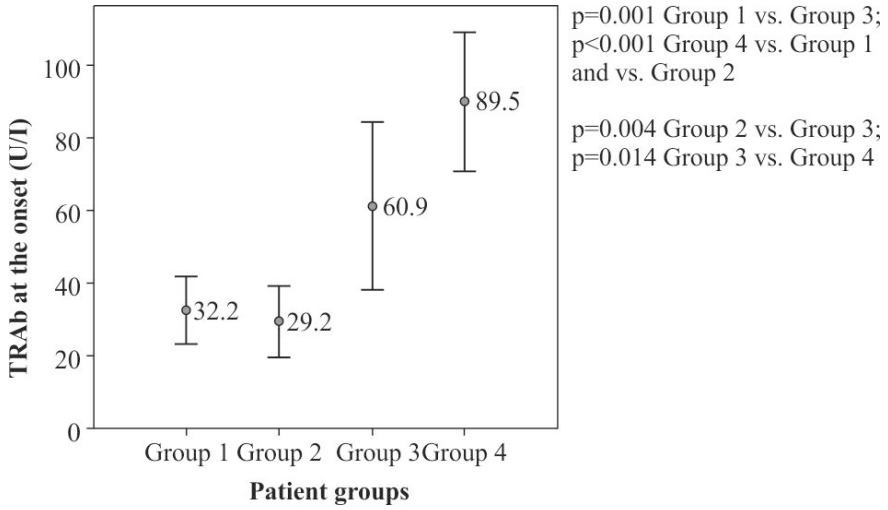

Fig. 4.1.5.1. *Serum TRAb concentrations (U/l) at the onset of the disease between patient groups. Charts represent mean values and 95% confidence intervals (CI).*

Group 1 – patients achieved long-term complete remission after initial ATD treatment with no relapses; Group 2 – patients achieved long-term complete remission after two or three courses of ATD therapy; Group 3 – patients underwent thyroid ablation due to relapses; Group 4 – patients underwent thyroid ablation without ATD therapy withdrawal.

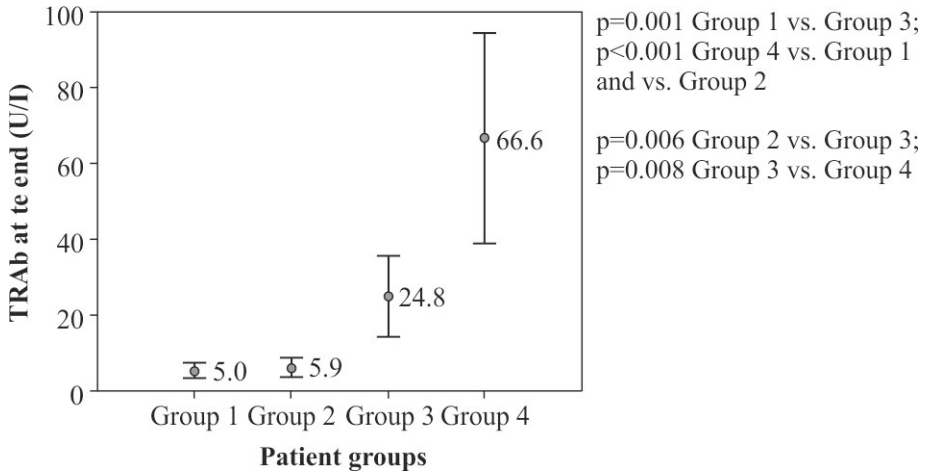

Fig 4.1.5.2. *Serum TRAb concentrations (U/l) at the end of ATD treatment between patient groups. Charts represent mean values and 95% confidence intervals (CI).*

Group 1 – patients achieved long-term complete remission after initial ATD treatment with no relapses; Group 2 – patients achieved long-term complete remission after two or three courses of ATD therapy; Group 3 – patients underwent thyroid ablation due to relapses; Group 4 – patients underwent thyroid ablation without ATD therapy withdrawal.

Mean concentrations of initial TRAb and TRAb at the end of ATD therapy were significantly higher in treatment failure group than in the remission group (p<0.001) (Fig. 4.1.5.3, 4.1.5.4).

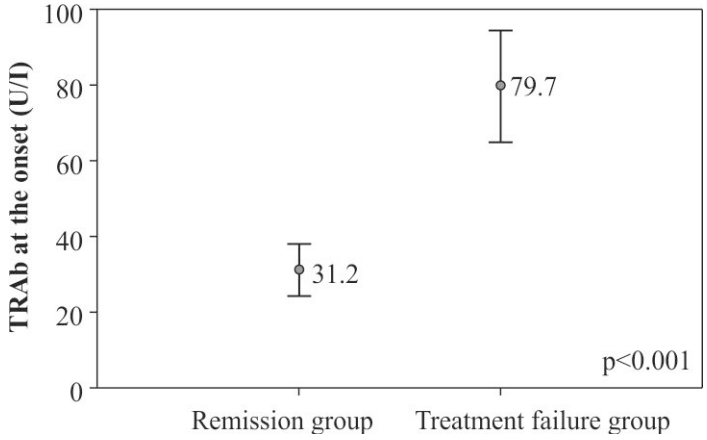

Fig 4.1.5.3. *Serum TRAb concentrations (U/l) at the onset of the disease between remission and treatment failure groups. Charts represent mean values and 95% Confidence intervals (CI).*

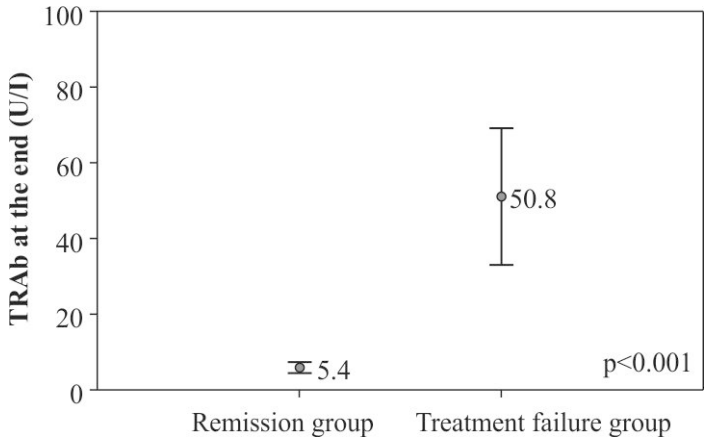

Fig 4.1.5.4. *Serum TRAb concentrations (U/l) at the end of ATD treatment between remission and treatment failure groups. Charts represent mean values and 95% Confidence intervals (CI).*

During the treatment period, serum TRAb concentrations at the end of ATD therapy decreased significantly in all patient groups ($p<0.05$). A weak positive correlation was found between TRAb and FT4 concentrations at disease onset after adjustment for gender and age ($r=0.2$, $p=0.022$) (Fig. 4.1.5.5).

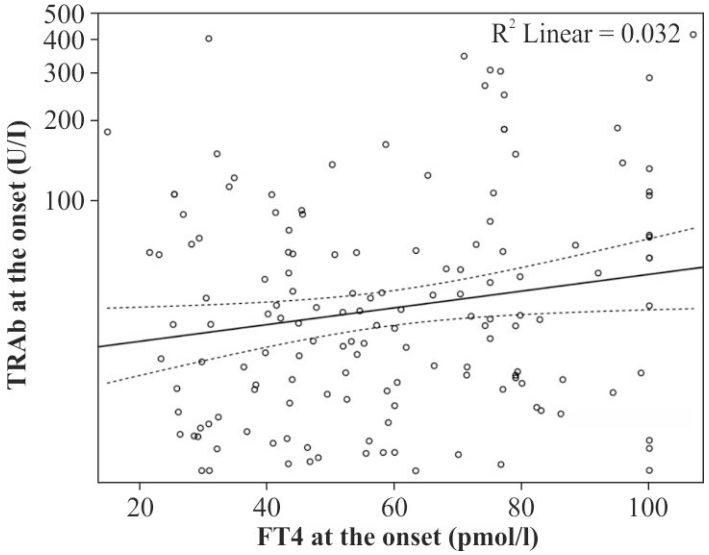

Fig 4.1.5.5. *Correlation between serum concentrations of TRAb (U/l) and FT4 (pmol/l) at the onset of the disease (in logarithmic scale) (r=0.2, p=0.022)*

ROC curve analysis was used to determine prognostic value of TRAb in predicting the outcome of GD (Fig.4.1.5.6).

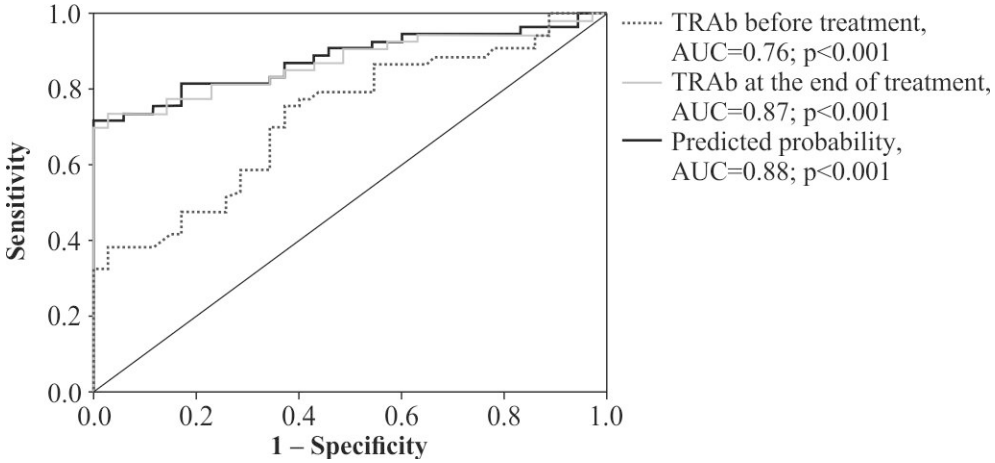

Fig. 4.1.5.6. *Receiver-operating characteristic (ROC) curves comparing serum TRAb levels (U/l) at different time and the combined predicted probability of ATD treatment failure. AUC – area under the curve*

The area under the ROC curve (AUC) of TRAb levels before treatment was 0.76 (95% CI, 0.7–0.82, p<0.001); and TRAb concentration of 30.2 U/l was found to be the best cut off value. Initial TRAb concentrations above 30.2 U/l identified patients who had never achieved long-term remission with sensitivity of 74% and specificity of 68% (positive predictive value of 82% and negative predictive value of 58%). The AUC value of TRAb levels at the end of ATD therapy was 0.87 (95% CI, 0.78–0.93, p<0.001). The difference in AUC values between initial TRAb levels and TRAb levels at the end of ATD therapy was significant (p=0.011). The TRAb concentrations at the end of ATD therapy above 12.97 U/l (as the best cut off value) identified patients who had never achieved long-term remission with sensitivity of 74% and specificity of 97% (positive predictive value of 98% and negative predictive value of 71%). The calculated predicted probability of treatment failure with logistic regression analysis, based on a combination of initial TRAb levels and TRAb levels at the end of ATD therapy, had AUC=0.88 (95% CI, 0.79–0.94, p<0.001), which was significantly higher than initial TRAb levels alone (p=0.0017), but difference from TRAb levels at the end of ATD therapy was not statistically significant (p=0.679).

4.1.6. Independent baseline prognostic factors predicting the failure of medical treatment in Graves' disease

Multiple logistic regression analysis was performed to select the independent baseline prognostic parameters. Variables that were chosen for inclusion in the analysis were those with significant univariate associations with ATD treatment failure in this study, as well as those found in other studies to be significant predictors of failure (gender, age at diagnosis (less than 40 years), positive family history of thyroid disorders, goiter size before treatment, initial TRAb and FT4 levels, pretreatment thyroid echogenicity). Analysis results revealed that only TRAb levels and the presence of large goiter were independent predictors of ATD treatment failure (Table 4.1.6.1).

Table 4.1.6.1. *Multiple logistic regression analysis of baseline prognostic factors associated with ATD treatment failure*

Risk factor	OR (95% CI)	p value
Gender (male)	1.68 (0.63–4.49)	0.303
Age (<40 yr)	0.58 (0.24–1.39)	0.222
Positive FH of thyroid disorders	2.29 (0.75–7.02)	0.147
Goiter size Medium Large	 2.42 (0.87–6.71) 14.7 (2.91–73.7)	 0.09 **0.001**
TRAb level (U/l)	1.02 (1.01–1.03)	**<0.001**
FT4 level (pmol/l)	0.998 (0.98–1.01)	0.855
Hypoechogenic thyroid	1.37 (0.42–4.41)	0.603

OR – odds ratio; CI – confidence interval; FH – family history; TRAb– TSH receptor antibodies; FT4– free thyroxine.

In summary, the study results revealed that gender and age at the onset of the disease did not predict the ATD treatment outcome. Age less than 40 years was a significant predictor of the presence of large sized goiter at the onset of the disease. Positive FH of thyroid disorders was associated with an increased probability of medical treatment failure. The echogenicity of the thyroid before ATD therapy did not predict the outcome of GD**.** Hypoechogenic thyroid after ATD therapy and persistent hypoechogenicity increased the risk of failure. Large sized goiter at the onset of the disease and levels of TRAb were independent baseline predictors of medical treatment failure. Initial TRAb concentration of 30.2 U/l and concentration at the

end of ATD therapy of 12.97 U/l were cutoff values that defined high risk patients for ATD therapy failure.

4.2. The influence of genetic risk factors on Graves' disease phenotype and the outcome of antithyroid drug treatment

4.2.1. Characteristics of patients with Graves' disease and control group

The control group included 9 males (10.0%) and 81 females (90.0%), while GD patients group included 18 males (17.1%) and 87 females (82.9%) (p=0.15). The mean age of GD patients at disease onset and control subjects were 42.4±12.0 and 41.4±10.1 years, respectively (p=0.688). Positive family history of thyroid disorders (including euthyroid or nodular goiter and unspecified thyroid disorders) was reported by 15.2% of patients. Forty eight patients (45.7%) achieved and sustained remission for a minimum 1 year after ATD withdrawal and fifty seven (54.3%) patients submitted to thyroid ablation (radioiodine therapy or surgery) due to poor response to ATD therapy.

4.2.2. Association of CTLA-4 gene 49A/G and CT60 polymorphisms with Graves' disease

The genotype distribution in GD patients and control group was consistent with Hardy-Weinberg equilibrium for 49A/G SNP (χ^2=1.44; p=0.23 in patients and χ^2=1.55; p=0.21 in controls). Statistically significant deviation from Hardy-Weinberg equilibrium was found only in GD patients (χ^2=4.2; p=0.04) but not in controls (χ^2=0.51; p=0.48) for CT60 SNP.

Significant associations with GD risk were observed for both CTLA-4 SNPs (49A/G and CT60) in genotype analysis using different inheritance models (Table 4.2.2.1).

Table 4.2.2.1. *Association analysis of the CTLA-4 gene 49A/G and CT60 SNPs with Graves' disease*

SNP	Type	Control, n (%)	GD, n (%)	Inheri-tance model	OR (95% CI)	p	p*
49A/G rs231775							
Geno-types	AA AG GG	26 (28.9) 50 (55.6) 14 (15.6)	18 (17.1) 58 (55.2) 29 (27.6)	Codo-minant	1.0 (reference) 1.68 (0.82–3.4) 2.99 (1.25–7.19)	**0.044**	0.086
	AA AG+GG	26 (28.9) 64 (71.1)	18 (17.1) 87 (82.9)	Dominant	1.0 (reference) 1.96 (0.99–3.88)	0.05	
	AA+AG GG	76 (84.4) 14 (15.6)	76 (72.4) 29 (27.6)	Recessive	1.0 (reference) 2.07 (1.02–4.22)	**0.041**	0.08
	AA+GG AG	40 (44.4) 50 (55.6)	47 (44.8) 58 (55.2)	Overdo-minant	1.0 (reference) 0.99 (0.56–1.74)	0.96	
				Log-additive'	1.73 (1.12–2.68)	**0.012**	**0.024**
Alleles	A allele G allele	102 (56.7) 78 (43.3)	94 (44.8) 116 (55.2)		1.61 (1.08–2.41)	**0.019**	**0.038**
CT60 rs3087243							
Geno-types	GG AG AA	25 (27.8) 48 (53.3) 17 (18.9)	42 (40.0) 56 (53.3) 7 (6.7)	Codo-minant	1.0 (reference) 0.69 (0.37–1.3) 0.25 (0.09–0.67)	**0.017**	**0.034**
	GG AG+AA	17 (18.9) 73 (81.1)	7 (6.7) 98 (93.3)	Dominant	1.0 (reference) 0.58 (0.32–1.06)	0.072	
	GG+AG AA	65 (72.2) 25 (27.8)	63 (60.0) 42 (40.0)	Recessive	1.0 (reference) 0.31 (0.12–0.78)	**0.009**	**0.018**
	GG+AA AG	42 (46.7) 48 (53.3)	49 (46.7) 56 (53.3)	Overdo-minant	1.0 (reference) 1.0 (0.57–1.76)	1.0	
				Log-additive'	0.55 (0.35–0.86)	**0.008**	**0.016**
Alleles	A allele G allele	82 (45.6) 98 (54.4)	70 (33.3) 140 (66.7)		1.67 (1.11–2.52)	**0.013**	**0.026**

SNP – single nucleotide polymorphism; GD – Graves' disease; Codominant – major allele homozygotes vs. heterozygotes and major allele homozygotes vs. minor allele homozygotes; Dominant – major allele homozygotes vs. heterozygotes+minor allele homozygotes; Recessive – major allele homozygotes+heterozygotes vs. minor allele homozygotes; Overdominant – major allele homozygotes+ minor allele homozygotes vs. heterozygotes; Log-additive major allele homozygotes vs. heterozygotes vs. minor allele homozygote; OR – odds ratio; CI – confidence interval; p* – p value after Bonferroni correction; ' – The model with the smallest AIC value was defined as the best model for each SNP.

Patients with GG genotype had about three-fold increase in risk of GD (OR 2.99, 95% CI 1.25–7.19) compared to patients with AA genotype, when 49A/G SNP exhibited a codominant effect. Patients with GG genotype had two-fold increase in GD risk (OR 2.07, 95% CI 1.02–4.22) compared to patients with AA and AG genotypes when 49A/G exhibited a recessive effect. But these statistically significant differences did not remain after Bonferroni correction (p=0.086 in codominant model, p=0.08 in recessive model). In the case of 49A/G SNP, the log-additive model had an association of 1.73 with GD risk (OR 1.73, 95% CI 1.12–2.68) and this statistically significant difference remained after Bonferroni correction (p=0.024). For CT60 SNP, AA genotype showed reduced odds of GD assuming a codominant (OR 0.25, 95% CI 0.09–0.67) and recessive (OR 0.31, 95% CI 0.12-0.78) models. In the case of CT60 SNP, the log-additive model also showed reduced odds of GD (OR 0.55, 95% CI 0.35–0.86). Following Bonferroni correction, these associations remained significant (p=0.034 in codominant model, p=0.018 in recessive model and p=0.016 in log-additive model).

The association of 49A/G and CT60 SPNs with GD was also observed in the allele analysis (Table 4.2.2.1.). The frequency of the G allele was higher in GD patients than in controls for 49A/G SNP (55% vs. 43%) and for CT60 SNP (67% vs. 54%). The G allele of both SNPs was significantly associated with higher GD risk (OR 1.61, 95% CI 1.08–2.41 for 49A/G SNP and OR 1.67, 95% CI 1.11–2.52 for CT60 SNP).

4.2.3. Haplotype association analysis

Results of linkage disequilibrium for the association of the studied 49A/G and CT60 polymorphisms in the CTLA-4 locus indicated D'=0,858 and r^2=0,465 and we subsequently conducted haplotype analysis to understand the role of these two SNPs better. Haplotype analysis revealed three predominant haplotypes (AA, AG, GG) and the less common haplotype GA, accounting for less than 3% of the total study population gene pool (Table 4.2.3.1).

Table 4.2.3.1. *Haplotype frequencies of CTLA-4 gene in controls and in GD patients*

Haplotype (49A/G:CT60)	Total (%)	Control (%)	GD (%)	OR (95% CI)	p
GG	47.0	38.6	54.1	1.0 (reference)	
AA	36.2	40.7	32.2	0.5 (0.3–0.83)	**0.008**
AG	14.0	15.9	12.6	0.54 (0.28–1.02)	0.059
GA	2.8	4.8	1.1	0.15 (0.03–0.78)	**0.025**

GD – Graves' disease; OR – odds ratio; CI – confidence interval.

The frequency of haplotype AA was higher in the healthy controls than in GD patients (41% vs. 32%), while haplotype GG was more frequent in GD patients (54% vs. 39%). Test of haplotype association was significantly positive (global p=0.0055). The haplotypes AA and GA significantly decreased the susceptibility to GD (OR 0.5, 95% CI 0.3–0.83 and OR 0.15, 95% CI 0.03–0.78, respectively).

4.2.4. Association of thyroglobulin gene E33 C/T polymorphism with Graves' disease

The genotypes distribution of the TG E33 C/T SNP was consistent with Hardy-Weinberg equilibrium for both GD patients and controls (χ^2=1.17; p=0.68 in GD patients and χ^2=1.34; p=0.25 in controls). Logistic regression analysis using different inheritance models did not show TG E33 C/T SNP association with GD risk (Table 4.2.4.1).

Table 4.2.4.1. *Association analysis of the* TG E33 C/T SNP *with Graves' disease*

SNP	Type	Control, n (%)	GD, n (%)	Inheritance model	OR (95% CI)	p
E33 SNP rs2076740						
Genotypes	CC CT TT	31 (34.4) 48 (53.3) 11 (12.2)	32 (30.5) 50 (47.6) 23 (21.9)	Codominant	1.0 (reference) 1.01 (0.54–1.9) 2.03 (0.85–4.84)	0.2
	CC CT+TT	31 (34.4) 59 (65.6)	32 (30.5) 73 (69.5)	Dominant	1.0 (reference) 1.2 (0.66–2.19)	0.55
	CC+CT TT	79 (87.8) 11 (12.2)	82 (78.1) 23 (21.9)	Recessive'	1.0 (reference) 2.01 (0.92–4.4)	0.072
	CC+TT CT	42 (46.7) 48 (53.3)	55 (52.4) 50 (47.6)	Overdominant	1.0 (reference) 0.8 (0.45–1.4)	0.43
				Log-additive	1.34 (0.88–2.02)	0.17
Alleles	C T	110 (61.1) 70 (38.9)	114 (54.3) 96 (45.7)		1.32 (0.88–1.98)	0.175

SNP – single nucleotide polymorphism; GD – Graves' disease; Codominant – major allele homozygotes vs. heterozygotes and major allele homozygotes vs. minor allele homozygotes; Dominant – major allele homozygotes vs. heterozygotes+minor allele homozygotes; Recessive – major allele homozygotes+heterozygotes vs. minor allele homozygotes; Overdominant – major allele homozygotes+ minor allele homozygotes vs. heterozygotes; Log-additive major allele homozygotes vs. heterozygotes vs. minor allele homozygote; OR – odds ratio; CI – confidence interval; ' – The model with the smallest AIC value was defined as the best model.

4.2.5. Relationship of CTLA-4 gene 49A/G and CT60 polymorphisms with clinical features of Graves' disease

The differences in clinical and laboratory features between GD patients with different allele compositions were compared. No significant differences were observed between different genotypes of 49A/G and CT60 in mean age, gender, mean concentrations of FT4 and ATPO before the treatment and mean concentrations of TRAb at the end of ATD therapy. Mean concentrations of TRAb before treatment were significantly higher in GG genotype carriers than AG genotype carriers for 49A/G SNP (83.5±79.9 vs. 50.9±60.6, p=0.02, respectively). At the end of treatment, serum TRAb concentrations decreased significantly in all genotypes of 49A/G SNP. The initial mean TRAb concentrations in AA genotype of 49A/G SNP dropped by 43% (p=0.006), in AG genotype by 58% (p<0.001), and in GG genotype by 46% (p=0.015) (Table 4.2.5.1).

Table 4.2.5.1. *Comparison of clinical and laboratory features between Graves' disease patients with different genotypes of 49 A/G SNP*

Characteristics	Genotypes of 49 A/G SNP		
	AA	**AG**	**GG**
F/M, n (%)	12(66.7)/ 6(33.3)	51(87.9)/ 7(12.1)	24(82.8)/ 5(17.2)
Age at diagnosis (years; mean ± SD)	43.2±8.5	42.6±12.9	43.1±11.5
FT4 (pmol/l, mean±SD)	55.5±18.4	60.2±23.4	66.1±22.7
ATPO (IU/ml; mean±SD)	214.0±330.1	306.4±533.6	185.3±260.7
TRAb at disease onset (U/l; mean±SD)	54.8±77.0	50.9±60.6	**83.5±79.9***
TRAb at the end of ATD treatment (U/l; mean±SD)	31.3±56.7	21.6±33.9	45.2±47.7
Diffuse goiter n (%) Small (n=22) Medium (n=70) Large (n=13)	 6 (33.3) 9 (50.0) 3 (16.7)	 10 (17.2) **43 (74.1)** [01] 5 (8.6)	 6 (20.7) 18 (62.1) 5 (17.2)

*p=0.02 GG vs. AG; [0]p<0.001 AG vs. AA; [1]p=0.001 AG vs. GG; SD, standard deviation; SNP, single nucleotide polymorphism; FT4, free thyroxine; ATD, antithyroid drugs; TRAb, TSH receptor antibodies, ATPO, antithyroid peroxidase antibodies.

GG genotype carriers of CT60 SNP also had higher mean concentrations of initial TRAb than AA or AG genotype carriers (77.6±71.1 vs. 27.1±18.6 and vs. 52.1±71.0, p=0.013 and p=0.004, respectively). However, for CT60 SNP, initial TRAb concentrations at the end of ATD therapy decreased significantly only in patients with AG and GG genotypes. In AG genotype of this SNP TRAb concentrations decreased by 49% (p<0.001), while in GG genotype by 57% (p<0.001). In AA genotype TRAb concentrations dropped insignificantly (p=0.273) (Table 4.2.5.2).

Table 4.2.5.2. *Comparison of clinical and laboratory features between GD patients with different genotypes of CT60 SNP*

Characteristics	Genotypes of CT60 SNP		
	AA	**AG**	**GG**
F/M n (%)	5(71.4)/ 2(28.6)	46(82.1)/ 10(17.9)	36(85.7)/ 6(14.3)
Age at diagnosis (years; mean ± SD)	37.9±7.3	43.9±12.4	42.2±11.5
FT4 (pmol/l, mean±SD)	64.7±17.8	59.6±22.6	61.3±23.4
ATPO (IU/ml; mean±SD)	229.5±387.8	246.4±355.9	269.2±532.5
TRAb at disease onset (U/l; mean±SD)	27.1±18.6	52.1±71.0	**77.6±71.1*°**
TRAb at the end of ATD treatment (U/l; mean±SD)	20.8±28.9	26.5±45.8	33.6±41.5
Diffuse goiter n (%)			
Small (n=22)	**2 (28.6)[12]**	11 (19.6)	9 (21.0)
Medium (n=70)	**5 (71.4)[3]**	37 (66.1)	28 (66.7)
Large (n=13)	0 (0)	8 (14.3)	5 (12.4)

*p=0.013 GG vs. AA; °p=0.004 GG vs. AG; [1]p=0.013 AA vs. AG; [2]p=0.035 AA vs. GG; [3]p<0.001 AA vs. AG and vs. GG; SD – standard deviation; SNP – single nucleotide polymorphism; FT4 – free thyroxine; ATD – antithyroid drugs; TRAb – TSH receptor antibodies, ATPO – antithyroid peroxidase antibodies.

Results for goiter size between different genotypes of 49A/G SNP revealed that patients with AG genotype had medium goiter more frequently than patients with AA or GG genotype (74% vs. 50% and vs. 62%, p<0.001 and p=0.001, respectively) (Table 4.2.5.1). For CT60 SNP, AA genotype carriers more frequently than AG and GG genotype carriers had small goiter (29% vs. 20% and vs. 21%, p=0.013 and p=0.035, respectively) and medium goiter (71% vs. 66% and vs. 67%, p<0.001, respectively). It should be noticed that patients with AA genotype of CT60 SNP did not have large goiter (Table 4.2.5.2).

4.2.6. Relationship of thyroglobulin E33 C/T polymorphism with clinical features of Graves' disease

GD patients with different genotypes of TG E33 C/T SNP did not significantly differ in mean age, gender, mean concentrations of FT4 and ATPO before ATD treatment and mean concentrations of TRAb before and at the end of ATD therapy (Table 4.2.6.1).

Table 4.2.6.1. *Comparison of clinical and laboratory features between GD patients with different genotypes of TG E33 C/T SNP*

Characteristics	Genotypes of TG E33 C/T SNP		
	CC	**CT**	**TT**
F/M, n (%)	26(81.2)/ 6(18.8)	44(88.0)/ 6(12.0)	17(73.9)/ 6(26.1)
Age at diagnosis (years; mean ± SD)	45.2±12.7	42.8±12.0	39.5±9.4
FT4 (pmol/l, mean±SD)	59.4±21.2	57.4±21.56	69.2±24.3
ATPO (IU/ml; mean±SD)	191.9±377.1	318.6±502.2	174.1±299.6
TRAb at disease onset (U/l; mean±SD)	46.4±34.9	63.8±83.9	73.6±73.1
TRAb at the end of ATD treatment (U/l; mean±SD)	19.3±23.1	33.5±49.2	32.5±50.4
Diffuse goiter n (%)			
Small (n=22)	8 (36.4)	**12 (54.5)***	2 (9.1)
Medium (n=70)	21 (30.0)	31 (44.3)	18 (25.7)
Large (n=13)	3 (23.1)	7 (53.8)	3 (23.1)

*p=0.008 CT vs. TT; SD – standard deviation; SNP – single nucleotide polymorphism; GD – Graves' disease; FT4 – free thyroxine; ATD – antithyroid drugs; TRAb – TSH receptor antibodies, ATPO – antithyroid peroxidase antibodies; OR – odds ratio; CI – confidence interval.

At the end of ATD treatment serum TRAb concentrations decreased significantly in patients with CC and CT genotypes. The initial mean TRAb concentrations in CC genotype decreased by 58% ($p<0.001$), in CT genotype by 47% ($p<0.001$). Results of goiter size between different genotypes of TG E33 C/T SNP revealed that patients with CT genotype more frequently than patients with TT genotype had small-sized goiter ($p=0.008$).

4.2.7. Association of the CTLA-4 gene polymorphisms with clinical outcome of Graves' disease

The patients in treatment failure group had significantly higher frequency of GG genotype and a lower frequency of AA genotype than patients in the remission group for both SNPs (49A/G and CT60) (Table 4.2.7.1).

Table 4.2.7.1. *Analysis of the CTLA-4 gene 49A/G and CT60 SNPs with failure of medical treatment in Graves' disease*

SNP	Type	Remission group, n (%)	Treatment failure group, n (%)	OR (95% CI)	p	p*
49A/G rs231775						
Genotypes	AA AG GG	11 (22.9) 30 (62.5) 7 (14.6)	7 (12.3) 28 (49.1) 22 (38.6)	1.0 (reference) 1.47 (0.5–4.31) 4.94 (1.38–17.65)	 0.49 **0.014**	 **0.028**
	AA AG+GG	11 (22.9) 37 (77.1)	7 (12.3) 50 (87.7)	1.0 (reference) 0.47 (0.17–1.33)	 0.155	
Alleles	A G	52 (54.2) 44 (45.8)	42 (36.8) 72 (63.2)	 2.03 (1.17–3.52)	 **0.012**	 **0.024**
CT60 rs3087243						
Genotypes	AA AG GG	6 (12.5) 31 (64.6) 11 (22.9)	1 (1.8) 25 (43.9) 31 (54.4)	1.0 (reference) 4.84 (0.55–42.87) 16.9 (1.83–156.6)	 0.157 **0.013**	 **0.026**
	AA AG+GG	6 (12.5) 42 (87.5)	1 (1.8) 56 (98.2)	1.0 (reference) 8.0 (0.93–68.99)	 0.059	
Alleles	A G	43 (44.8) 53 (55.2)	27 (23.7) 87 (76.3)	 2.61 (1.45–4.71)	 **0.0014**	 **0.003**

SNP – single nucleotide polymorphism; GD – Graves' disease; OR – odds ratio; CI – confidence interval; p* – p value after Bonferroni correction.

Patients with GG homozygous genotype had more than four-fold (OR 4.94, 95% CI 1.38–17.65) and more than sixteen-fold (OR 16.9, 95% CI 1.83–156.6) increase in the risk of ATD treatment failure compared to patients with AA genotype for 49A/G SNP and for CT60 SNP, respectively. The frequency of the G allele was higher in treatment failure group than remission group for 49 A/G SNP (63% vs. 46%) and for CT60 SNP (76% vs. 55%). The frequency of the G allele at both polymorphic sites was significantly associated with higher probability of poor outcome (OR 2.03, 95% CI 1.17–3.52 for 49A/G SNP and OR 2.61, 95% CI 1.45–4.71 for CT60 SNP). G-containing genotype (AG+GG) frequency for both SNPs did not significantly differ between GD patient groups.

4.2.8. Association of thyroglobulin E33 C/T polymorphism with clinical outcome of Graves' disease

There were no significant differences in the genotypes distribution of the TG E33 C/T SNP between remission and treatment failure groups (p=0.056). The frequency of T-containing genotype (CT+TT) was similar between GD patient groups (p=0.155). Only allele analysis revealed significant information in predicting the outcome of GD. The frequency of the T allele was significantly associated with failure of ATD treatment (OR 2.4, 95% CI 1.37–4.19) (Table 4.2.8.1).

Table 4.2.8.1. *Analysis of TG gene E33 C/T SNP with failure of medical treatment in Graves' disease*

SNP	Type	Remission group, n (%)	Treatment failure group, n (%)	OR (95% CI)	p value
Genotypes of TG E33 SNP (rs2076740)	CC	14 (29.2)	18 (31.6)	1.0 (reference)	
	CT	28 (58.3)	22 (38.6)	0.61 (0.25–1.49)	0.28
	TT	6 (12.5)	17 (29.8)	2.2 (0.69–7.06)	0.183
	CC	11 (22.9)	7 (12.3)	1.0 (reference)	
	CT+TT	37 (77.1)	50 (87.7)	0.47 (0.17–1.33)	0.155
Alleles	C	56 (58.3)	42 (36.8)		
	T	40 (41.7)	72 (63.2)	2.4 (1.37–4.19)	**0.002**

SNP – single nucleotide polymorphism; OR – odds ratio; CI – confidence interval.

4.2.9. Prediction of antithyroid drug treatment failure using different risk factors

We performed a multiple logistic regression analysis simultaneously analyzing the influence of clinical factors (age (less than 40 yr. and older), gender, family history of thyroid diseases, goiter size), laboratory variables at disease onset (serum FT4 and TRAb concentrations) and genetic markers (CTLA-4 49A/G, CTLA-4 CT60 and TG E33 C/T genotypes) in predicting the clinical outcome of GD. When association of CTLA-4 49A/G and CT60 SNPs genotypes and TG E33 C/T SNP alleles with the outcome of GD was analyzed separately it provided significant prognostic information, but after multiple logistic regression analysis these genetic markers were not independent of other factors. Only TRAb levels before treatment were independently associated with elevated odds of failure (OR 1.05, 95% CI 1.02–1.08) (Table 4.2.9.1).

Table 4.2.9.1. *Multiple logistic regression analysis of prognostic factors associated with ATD treatment failure*

Risk factor	OR (95% CI)	p value
Age	0.4 (0.78–2.03)	0.267
Gender (male)	1.77 (0.31–10.26)	0.525
Positive FH of thyroid disorders	4.78 (0.7–32.64)	0.111
Goiter size	3.13 (0.85–11.51)	0.086
TRAb level (U/l)	1.05 (1.02–1.08)	**0.001**
FT4 level (pmol/l)	0.999 (0.97–1.03)	0.943
CTLA-4 49A/G SNP	1.31 (0.35–4.94)	0.688
CTLA-4 CT60 SNP	2.66 (0.61–11.64)	0.195
TG E33 C/T SNP	1.03 (0.42–2.55)	0.949

ATD – antithyroid drugs; OR – odds ratio; CI – confidence interval; FH – family history; TRAb – TSH receptor antibodies; FT4 – free thyroxine, CTLA-4 – cytotoxic T-lymphocyte-associated protein 4 gene; TG – thyroglobulin, SNP – single nucleotide polymorphism.

In summary, the study results revealed that CTLA-4 gene 49A/G and CT60 polymorphisms confer genetic susceptibility to Graves' disease. GG genotype and G allele of CTLA-4 gene 49A/G and CT60 polymorphisms were associated with the development of Graves' disease. The haplotypes AA and GA significantly decreased the susceptibility to GD. Thyroglobulin gene E33 C/T polymorphism was not associated with susceptibility to Graves' disease. GG genotype and G allele of CTLA-4 gene 49A/G and CT60 polymorphisms and T allele of thyroglobulin E33 C/T polymorphism were significantly associated with the higher probability of medical treatment failure.

5. DISCUSSION

5.1. Retrospective study of risk factors predicting antithyroid drug treatment failure in patients with Graves' disease

ATD still remains the first line treatment option for Graves' hyperthyroidism in many countries, but the response to treatment is unpredictable. In many cases long-term results of ATD treatment are unsatisfactory. Clinical decisions concerning the long-term treatment of patients with GD are difficult. Thus the identification of factors that predict the outcome before starting the treatment would help to choose the most appropriate form of therapy in individual patients. In this study, we examined the relationship between clinical, laboratory and radiological features as well as the outcome of GD and identified several independent baseline predictive factors of medical treatment failure for patients with GD. In this study we have observed the remission rate of 34% after ATD treatment. This finding is in accordance with earlier European studies, where remission rates after ATD treatment ranged between 30 and 60% [13, 100].

Graves' disease occurs with greater frequency in females, what may be related to the influence of estrogens on the immune system, particularly the B cell repertoire [70]. In our study over three-quarters of patients were females, giving a ratio (male: female) of 1:4. It has been suggested previously that males suffer worse biochemical hyperthyroidism with less severe symptoms [8, 12]. Allahabadia et al. and Cinemre et al. reported that males with GD are less likely to enter remission after ATD therapy, perhaps because of poorer compliance and biochemical control [8, 59]. Our analysis of gender demonstrated that males with larger goiter had more biochemically severe disease, although gender did not have significant effect on the treatment outcome in agreement with other authors [46, 180].

Age related differences in clinical presentation of Graves' hyperthyroidism have been reported. Severity of hyperthyroidism and prevalence of antibodies tend to decrease with advancing age [4]. Our analysis of the influence of age confirmed previously reported data, that younger patients have more severe biochemical hyperthyroidism and a higher prevalence of TRAb, although these differences did not reach statistical significance. Conflicting data exists concerning the influence of age on the outcome of ATD therapy. Some studies have reported that patients aged less than 40 years at presentation are more likely to fail to respond to ATD treatment [8, 260], although other authors did not confirm any age related associations [36, 102]. In our study, age of onset did not have significant effect on the

treatment outcome, although age less than 40 years was a significant predictor of the presence of large-sized goiter at the onset of the disease. In our study women had a significantly lower age at diagnosis than men. This might be influenced by delayed disease presentation in men, as recorded in other diseases. These observations suggest that relatively higher levels of testosterone in young men after puberty may provide temporary protection from the autoimmune disease in those men who were genetically predisposed to develop the disease [261]. But neither age at diagnosis nor gender had a significant influence on the outcome in our study.

Previous studies have demonstrated an inverse correlation between the patient's age and serum FT4 levels at the onset of hyperthyroidism [4,165], suggesting that the autoimmune process in thyroid was more likely to subside in patients older than 40 years. We also found a negative correlation between patient's age and serum FT4 levels at the onset of hyperthyroidism, although this relationship was lost after adjustment for gender. Severity of biochemical hyperthyroidism has been suggested to indicate a poor prognosis after medical treatment [46, 59]. Our data could not confirm this hypothesis.

Early evidence exists that GD has a hereditary component stems from familial studies. In our study a positive family history of thyroid disorders was observed in 18.6% of patients, in contrast with reports of other authors, where frequency of around 50% has been observed [165]. Although the association between positive family history of thyroid disorders and poor outcome of GD was significant, multiple logistic regression analysis showed that this was not an independent factor. Family data have some limitations because the design of this study was retrospective and patients reported a family history of all types of thyroid diseases, including euthyroid, nodular goiter and unspecified thyroid disorders.

Goiter prevalence has been reported to be inversely related to age at diagnosis of hyperthyroidism [165]. Our analysis of the influence of age confirmed previously reported associations. Our data has shown that the presence of larger goiter was associated with more biochemically severe disease, although this association was observed only in males. These findings correspond to the findings in Maniji et al. [165] study, where the presence of large goiter was associated with greater disease severity in both genders. This association may be related to the stimulatory effect of TSH and/or TRAb. Relationship between large goiter and higher TRAb concentrations may be explained by the stronger growth stimulation potential of higher TRAb levels on thyroid gland. An effect of goiter size on remission rate has been reported in many studies [180, 260], but some

researchers have been unable to confirm this association [46, 59]. Differences in goiter size assessment may account for such discrepancies. Despite the limitations of assessment of goiter size by clinical examination, our results for goiter size showed a significant association between the presence of larger goiter and failure of medical treatment. The present finding, showing that large goiter independently predicts the failure of ATD treatment, is plausible, suggesting that goiter size may be a significant marker of the severity of autoimmune process.

Ultrasonography is the most widely used imaging method for the diagnosis and follow-up of thyroid disorders. It can be used to measure thyroid gland volume, to detect focal lesions such as nodules or cysts or to characterize the tissue echogenicity, which provides information about the cellularity and vascularization of the organ. This method includes a major disadvantage – judgment of alterations depends on the visual impression of an observer. Because of the retrospective design of this study, ultrasonography had been performed by different physicians. This means that the results of the test were partly subjective and depended on the experience of the different investigators. An additional limitation of the study is that thyroid size was assessed by palpation, rather than ultrasound, which is more precise in diagnosing goiter. Hypoechogenicity of the tissue is caused by the reduced colloid content, lymphocytic infiltration and an increase in the intrathyroidal flow [166]. These features are characteristic to Graves' disease. Zingrillo et al [286] reported that thyroid hypoechogenicity before treatment could not be used as a predictor of relapse of GD. They announced that antithyroid drugs may alter the follicular structure and influence changes in thyroid hypoechogenicity. The absence of thyroid hypoechogenicity after ATD treatment is a favorable prognostic sign of remission, because it directly reflects inflammatory status of the thyroid gland [286]. In our study, the thyroid echogenicity before treatment did not predict the outcome of treatment for Graves' hyperthyroidism, in accordance with Zingrillo et al. However, hypoechogenic thyroid after ATD therapy and persistent hypoechogenicity were associated with greater risk of medical treatment failure, in accordance with other investigators.

Rubello et al. [206] reported that elevated TRAb levels were correlated with hypoechogenicity of the thyroid, but we did not find significant correlation between TRAb values and hypoechogenic tissue. Some authors suggest, that thyroid echogenicity may predict the outcome of radioiodine therapy – normoechogenic gland is much more radioresistant, when compared with the relatively radiosensitive hypoechogenic gland [166].

TSH receptor antibodies are considered the hallmark of Graves' disease and their presence in the serum of patients treated with ATD may reflect ongoing disease activity. When sensitive bioassays are used, 95-96% of patients with hyperthyroidism caused by GD have thyroid stimulating antibodies in their serum [212]. Assays used in clinical routine differ considerably in their ability to detect such antibodies, but recent generations of assays show high sensitivity and specificity. In the present study, TRAb concentrations were measured using a radioreceptor assay, which detects autoantibodies that interfere with the binding of thyroid stimulating hormone to TSH receptor, but does not differentiate between thyroid stimulating antibodies and thyroid blocking antibodies in serum samples. All studies using in vitro assays for detecting TRAb in patients with Graves' disease have found a subgroup of patients being TRAb-negative. In general, TRAb-negative patients tend to have a milder disease [134]. As the aim of our study was to determine predictive value of TRAb, we excluded patients that were TRAb-negative before therapy. The role of TRAb as a predictor of outcome after ATD treatment is still controversial [168]. There are many factors, such as design of the studies (prospective vs. retrospective), laboratory methods adopted to evaluate TRAb (thyroid stimulating antibodies vs. thyroid blocking antibodies) [239], time of TRAb measurement (before treatment vs. after treatment) that complicate the overall interpretation of this issue. Early attempts of using TRAb to predict remission of GD were followed by a meta-analysis, which suggested that the absence of TRAb after antithyroid drug therapy may predict disease remission [285]. In the study published in 2004, the predictive cutoff value of 1.5 U/L for TRAb was low and their clinical utility was limited [215]. Subsequent studies attempted to use TRAb thresholds that were higher, and measured them at various points during the course of the disease to improve the predictive value. Cappelli et al. studied 216 patients with GD for 120 months. They measured TRAb at diagnosis and every 6 months thereafter. Initial TRAb level >46.5U/L identified patients who had never achieved long-term remission with sensitivity of 52% and specificity of 78% [46]. In the present study, TRAb levels at diagnosis and at the end of ATD treatment were strongly associated with final outcome. A lower cutoff value above 30.2 U/l at the onset of the disease defined high risk patients and had a higher sensitivity than has been reported by Capelli. The effect of TRAb levels on the response to ATD treatment was independent of other baseline factors, although TRAb levels at the end of ATD therapy provide higher accuracy in predicting medical treatment failure in ROC curve analysis. Because of the retrospective design of this study some laboratory variables were measured

in less than 50% of patients. Although some of these variables, including TRAb levels at the end of ATD therapy, were significant predictors of medical treatment failure in the univariate analysis, they could not be included in the multivariate analysis without excessively restricting the sample size.

Laurberg et al. followed the variations in TRAb after therapy, comparing the three common treatment options of patients with Graves' disease [159]. They reported that during medical treatment or after surgery the majority of patients with Graves' disease gradually entered remission of TSH receptor autoimmunity, with no difference between the two types of therapy. Radioiodine therapy led to a year-long worsening of autoimmunity against the TSH receptor. The number of patients who entered remission of TSH-receptor autoimmunity without any traces of TRAb in the serum during the following years was considerably lower than with the other types of therapy.

Conflicting data exists regarding the effect of the duration of ATD therapy on the relapse of GD. In earlier studies the effect of 12 or 18 months of ATDs treatment was compared to 6-months therapy, but the results were inconsistent. In Allannic et al. study, 18 months therapy using the titration regimen was administered and relapse rate was significantly lower, when compared to the 6 month therapy [10]. The six month block-replace regimen was found to be as effective as the 12 month treatment in one subsequent quasi-randomised study [273]. Studies comparing longer durations of therapy, 12 versus 24 months [96] or 18 versus 42 months [167], did not find any difference in the relapse rates after therapy discontinuation. Ishtiaq O. et al. recommended alternative treatment options to patients who could not achieve remission after 12–18 months of ATD treatment [120]. Although a recent retrospective study reported that duration of ATDs therapy of more than 24 months independently predicts a long-term remission of GD [13]. If the remission of Graves' disease during ATD therapy was caused by an immunosuppressive effect of the drugs, it would be expected that the use of higher doses of drugs would lead to a more rapid and probably more sustained remission of the disease. This hypothesis was proposed by Romaldini et al. [204]. Subsequently, a number of prospective randomized studies have been performed to elucidate the dose response effect of ATD on the remission of Graves' hyperthyroidism. Studies comparing the effect of high dose block-replace regimen versus titration regimen did not find any difference in the relapse rates, but adverse effects of ATD were significantly higher in the block-replace group [32, 180, 202]. Also the continuation of thyroxine treatment after the initial antithyroid therapy did not provide any benefits in terms of recurrence of hyperthyroidism [191, 198]. A recent meta-analysis

[2] suggested that the duration of antithyroid drug therapy for the titration regimen should be between 12 to 18 months. The titration regimen is as effective as the block-replace regimen with a significantly lower incidence of adverse effects, and should be considered as the first-line regimen in most situations. In our study all patients were treated with ATD for a minimum period of 12 months. The initial duration of ATD therapy was significantly shorter in remission group than treatment failure group, suggesting that longer initial treatment duration did not have influence on treatment outcome. Total duration of ATD treatment was longer in patients who underwent thyroid ablation comparing to the patients in remission group; obviously this result is due to the fact that subjects with more relapses had to restart the therapy more times. Combined administration of thyroxine and antithyroid drug after initial high dose antithyroid therapy did not have positive influence on treatment outcome.

Finally, an additional limitation of the study is that patients were followed for only 1 year after discontinuation of antithyroid medications. It is possible that some patients experienced a relapse of the disease after the 1-year follow-up period; however, data from previous studies indicated that the frequency of such late relapses is lower [109]. We also were unable to follow ophthalmopathy progression in patients with GD, because of the absence of standardized clinical assessments of eye disease severity in medical documents (e.g. NOSPECS classification).

In summary, we have demonstrated that goiter of medium or large size, higher TRAb levels, and a positive family history of thyroid disorders are significant baseline predictors of medical treatment failure. After multiple logistic regression analysis, large-sized goiters and higher TRAb levels remained predictors for failure. These factors should be taken into account when determining the treatment plan, especially for patients who are less likely to achieve long-term remission; consideration of alternative therapeutic options may be advisable. Although a positive family history of thyroid disorders failed to reach statistical significance as a predictor in multiple logistic regression analysis, it showed a significant univariate association with poor outcome of ATD treatment. These findings provide some insight into the complex interactions between genetic factors, which account for a major part of disease susceptibility.

5.2. The influence of genetic risk factors on Graves' disease phenotype and the outcome of antithyroid drug treatment

Single nucleotide polymorphisms can be used as a tool for investigating genetic variations and disease susceptibility. CTLA-4 is an important negative regulator of T cell activation and several CTLA-4 gene polymorphisms have been found to be associated with thyroid autoimmunity. CTLA-4 provides a negative signal to the T cell, thereby restricting immune response to self-antigens [103]. A/G SNP at position 49 in the first exon [161] and CT60 SNP in 3' untranslated region [253] of the CTLA-4 gene have been shown to have biological relevance. Both of these SNPs have A and G alleles, and G is the risk allele for either of the two loci [264]. 49A/G SNP results in the substitution of amino acid threonine to alanine in the peptide leader sequence of the CTLA-4 protein. This leads to the expression of defective receptor with impaired inhibitory effect of CTLA-4 on lymphocyte T cell activation [144]. CT60 SNP is associated with an alteration in the ratio of splice forms of the CTLA4 gene and this ratio may affect disease susceptibility [253]. The association of 49A/G and CT60 polymorphisms of the CTLA-4 gene with GD was studied by several groups in different populations, but studies, investigating roles of these SNPs (especially CT60 SNP) on remission of Graves' hyperthyroidism, particularly in European populations, are lacking.

More recently, the 8q24 locus, which contains the thyroglobulin gene, has been shown to be strongly associated with autoimmune thyroid diseases [56]. All 48 exons of the TG gene have been sequenced and 14 SNPs have been identified. It has been shown that among these SNPs the exon 10–12 and exon 33 SNPs are significantly linked with autoimmune thyroid diseases, including GD [56]. However, studies of different populations gave conflicting results [62, 116].

In this study we analyzed associations of CTLA-4 gene 49A/G SNP in exon 1 (rs231775), CT60 A/G SNP in 3'UTR (rs3087243) and TG gene C/T SNP in exon 33 (rs2076740) with susceptibility to GD in Lithuanian population and roles of these SNPs on remission of Graves' hyperthyroidism after ATD withdrawal. We tested Hardy-Weinberg equilibrium for each studied SNP in patients and controls. The observed control genotype frequencies were consistent with Hardy-Weinberg equilibrium for all markers. This confirms that the controls were suitable. No significant deviation from Hardy-Weinberg equilibrium was observed for CTLA-4 49A/G SNP and TG E33 C/T SNP in patients with Graves' disease. A significant deviation from Hardy-Weinberg equilibrium was observed for CTLA-4 CT60 SNP in

GD patients. If deviation from Hardy-Weinberg equilibrium was observed only in the genotype distribution of the patient group, but not controls, this could mean that there is additional support for an association of the marker locus with the disease [83].

In our study results of CTLA-4 A/G SNP at position 49 revealed the significant association with GD, in accordance with other studies in different populations, including Japanese [141], Koreans [186], Chinese [283], Taiwanese [274] and in South Indian population [256], although some studies have produced conflicting results [58, 76, 92, 139, 179, 274]. A comprehensive meta-analysis using data from 42 studies comprising of 8.288 GD cases and 9.372 controls (three studies were eliminated from the total 42 studies due to a p-value of <0.05 for Hardy-Weinberg equilibrium in control group) found association between 49A/G SNP and GD in log-additive, recessive and dominant genetic models [219]. In our study a significant association between 49A/G SNP and risk for GD was observed in codominant, recessive and log-additive models, but after Bonferroni correction the initially observed association was statistically significant only in log-additive model.

Our finding that the G allele and GG genotype of the CTLA-4 CT60 SNP are significantly more frequent in GD patients than controls is consistent with previous studies performed in other populations including Italian [190], Slovenian [35], British [253], Taiwanese [274] and Japanese [24], whereas study in Korean population did not indicate correlation of the CT60 SNP with GD [58].

Some studies have reported an association between E33 SNP of TG gene and increased susceptibility to autoimmune thyroid diseases [23, 115, 241]. Ban et al. suggested that the substitution of a hydrophobic amino acid (tryptophane) to a hydrophilic amino acid (arginine) due to TG C/T SNP changes the structure of TG at that region and this may cause increased susceptibility to autoimmune thyroid diseases, including GD [23]. The SNP in exon 33 of TG gene has been shown to have a statistical interaction with the Arg74 polymorphism of HLA-DR, resulting in increased susceptibility to GD [112]. However, some studies failed to detect any contribution of TG gene to the genetic component of GD pathogenesis [31, 62]. Our study results of genotype and allele frequencies showed no significant differences of E33 SNP in TG gene between GD patients and controls.

The frequencies of alleles and genotypes of CTLA-4 gene 49A/G and CT60 SNPs and TG gene E33 SNP are different in various ethnic groups. According to the SNP database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/SNP), the risk for G allele of CTLA-4

49A/G SNP is higher in Asians (63.5%) than in Europeans (38.9%) or Sub-Saharan Africans (36.3%). Similarly, the G allele of CTLA-4 CT60 is more common in Asians (79.1%) and Sub-Saharan Africans (81.9%) than Europeans (54.0%). T allele of TG E33 SNP is more frequent in Sub-Saharan Africans (59.7%) or Europeans (38.5%) than in Asians (16.3%). In our healthy controls the frequency of G allele of CTLA-4 49A/G SNP (43.3%) was similar to Polish (40.3%) control subjects [92], was higher when compared to British (35.5%) [254], Italian (31.0%) [190], Slovenian (32.5%) [35] and Turkish (34.4%) [241] control subjects and was lower compared to Russian (52.7%) population [57]. The G allele frequency of CTLA-4 CT60 SNP in our controls (54.4%) was similar to Slovenian population (51.7%) [35], was higher compared to Italian (48.0%) [190] and Polish (35.0%) [187] control subjects; and was lower compared to northern Sweden population (61.3%) [169]. T allele frequency of TG E33 SNP in our controls was similar to Turkish (33.3%) control subjects [241] and was lower compared to British (46.1%) [62] or Taiwanese (60.3%) populations [116].

Some previous studies indicated that 49A/G and CT60 polymorphisms of CTLA-4 gene may influence the course of the disease. Studies in Japanese population reported that Graves' patients with GG genotype [140] and the G allele [140, 141] in exon 1 of the CTLA-4 gene frequently relapse after ATD therapy withdrawal, are required to continue ATD treatment for longer periods [141] and in many cases are suitable for radioiodine therapy [140]. Subsequent studies in Taiwanese population demonstrated that GG genotype and G allele of both SNPs (49A/G and CT60) are associated with early relapses [264], whereas study in Korean population did not find significant association between 49A/G SNP of the CTLA-4 gene and clinical outcome of GD [138]. In our study, although G allele and GG genotype of both SNPs (49A/G and CT60) of CTLA-4 gene significantly increased the risk of ATD treatment failure, these genetic markers were not independent prognostic factors. The importance of these genetic markers may be reduced by relatively small sample size of our study population. Recent study with larger sample size in Polish population showed, that CT60 polymorphic marker was independently associated with disease progression [71].

Hsiao et al. found a higher frequency of TT genotype of TG E33 C/T SNP in GD patients compared to control group. However, CC genotype of the TG gene was strongly associated with higher relapse rate and higher levels of persisting TRAb at the end of treatment [116]. In our study the association of TG E33 C/T SNP with GD was not observed in the allele frequency, but the T allele of TG E33 C/T SNP in GD group seemed to be higher than that in control, suggesting that this SNP might be weakly

associated with GD. The higher frequency of T allele showed significant association with poor outcome of ATD treatment.

Tomer et al. reported that CTLA-4 leads to the production of thyroid autoantibodies but it does not contribute specifically to Graves' or Hashimoto's thyroiditis [247]. Kinjo et al. showed the association of CTLA-4 49A/G SNP with the changes of levels of antibodies to TSH receptor during antithyroid treatment in Japanese patients. Whereas remission of GD can be predicted by a smooth decrease in the levels of autoantibodies after treatment [240], their results suggested that 49A/G SNP may affect the remission of Graves' hyperthyroidism [141]. It was reported that the frequencies of GG genotype and G allele were significantly higher in patients with persistently positive TRAb after 5 years of ATD treatment [141]. Results of our study showed that TRAb levels before treatment were significantly higher in GG genotype carriers of both CTLA-4 SNPs compared to other genotype carriers. Interestingly, patients with GG genotype of 49A/G and CT60 SNPs compared to other genotypes also had higher TRAb levels at the end of ATD treatment. Unfortunately, this difference did not reach statistical significance.

Some authors proposed that ATD may have immunosuppressive effect, because the treatment with ATD is associated with a significant fall in TRAb levels [66]. In our study decreased TRAb concentrations at the end of treatment were observed among different genotypes of all studied SNPs. The percentage of reductions in TRAb concentrations was significantly greater in AG genotype of CTLA-4 49A/G SNP, GG genotype of CTLA-4 CT60 SNP and CC genotype of TG E33 C/T SNP carriers compared with other genotype carriers. We hypothesize that the different effect of ATD on the changes of TRAb levels during treatment may be associated with genotype selective activity.

In a comprehensive meta-analysis by Kavvoura et al. [133] haplotypes of CTLA-4 gene 49A/G and CT60 SNPs and associations with GD have been studied. It was reported that compared with the AA haplotype, the risk conferred by the GG haplotype was 1.49 for GD in Asian and Caucasian descent subjects. Our study results also revealed differences in the frequency of the CTLA-4 haplotypes between patients and controls. Haplotype AA was more common in controls than in GD patients, while frequency of GG haplotype was higher in GD patients. Similar results were obtained in a study of Slovenian population [35]. The haplotype association analysis in our study revealed a protective effect of the haplotypes AA and GA, although the latter haplotype was very rare.

In summary, our data suggest that CTLA-4 49A/G and CT60 SNPs are associated with the development of GD and may provide important information when identifying patients with high risk of failure to ATD therapy. The TG E33 C/T polymorphism is not related to susceptibility to GD, but higher frequency of T allele is associated with poor outcome of ATD treatment. Further studies to identify other disease susceptibility loci are needed. Combinations of alleles at a variety of candidate loci may provide greater accuracy than individual markers in predicting clinical outcome in patients with Graves' disease.

CONCLUSIONS

1. Gender and age did not predict the outcome of medical treatment in patients with Graves' disease. Positive family history of thyroid disorders was associated with the higher probability of medical treatment failure.
2. Large sized goiter at the onset of the disease was an independent baseline predictor of medical treatment failure. Thyroid echogenicity at the onset of Graves' disease did not predict the outcome of antithyroid drug treatment. Thyroid hypoechogenicity after medical treatment and persistent hypoechogenicity were significantly associated with the higher probability of medical treatment failure.
3. Thyroid stimulating hormone receptor antibodies are useful tool for predicting the outcome of GD. Higher levels of TRAb at the onset of the disease and at the end of medical treatment were associated with failure of ATD treatment. Levels of thyroid stimulating hormone receptor antibodies before treatment were independently associated with the higher probability of medical treatment failure.
4. CTLA-4 gene 49A/G and CT60 polymorphisms confer genetic susceptibility to Graves' disease. GG genotype and G allele of CTLA-4 gene 49A/G and CT60 polymorphisms are associated with the development of Graves' disease. Thyroglobulin gene E33 C/T polymorphism is not associated with susceptibility to Graves' disease.
5. CTLA-4 gene 49A/G and CT60 polymorphisms and thyroglobulin E33 C/T polymorphism provide significant prognostic information in predicting the medical treatment outcome in Graves' disease. GG genotype and G allele of CTLA-4 gene 49A/G and CT60 polymorphisms and T allele of thyroglobulin E33 C/T polymorphism are significantly associated with the higher probability of medical treatment failure.

PRACTICAL RECOMMENDATIONS

In our study we identified risk factors that may predict the response to antithyroid drug treatment in patients with Graves' disease. Our findings should help to improve the treatment planning process in the clinical practice. Patients with positive family history of thyroid disorders, who have large-sized goiter at disease presentation, higher levels of thyroid stimulating hormone receptor antibodies at the onset of the disease and at the end of medical treatment, hypoechogenic thyroid after antithyroid drug treatment, GG genotype or G allele of CTLA-4 gene 49A/G and CT60 polymorphisms and T allele of TG E33 C/T polymorphism, are unlikely to achieve long-term remission. These factors should be taken into account when determining the treatment plan. For patients who are less likely to achieve remission, consideration of alternative therapeutic options (surgery or radioiodine therapy) may be advisable. In other cases, patients should be encouraged to continue antithyroid drugs, given the good prognosis for remission.

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2. Daukšienė, Dalia; Daukša, Albertas; Mickuvienė, Narseta. Greivso ligos medikamentinio gydymo nesėkmės prognozės žymenys / D. Daukšienė, A. Daukša, N. Mickuvienė // Lietuvos endokrinologija. Kaunas: UAB „Medicininės informacijos centras“. (Mokslinis straipsnis). ISSN 1392-7213. 2011, t. 19, Nr. 4, p. 69-74. [Index Copernicus].

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1. Jurkštienė, Vilma; Pavilonis, Alvydas; Garšvienė, Daiva; Juozulynas, Algirdas; Samsonienė, Laimutė; Daukšienė, Dalia; Jankauskienė, Konstancija; Šimonienė-Kazlauskienė, Genovaitė; Stankevičius, Edgaras. Investigation of the antimicrobial activity of Rhaponticum (Rhaponticum Carthamoides D.C. Iljin) and Shrubby Cinquefoil (Potentilla Fruticosa L.) / Vilma Jurkštienė, Alvydas Pavilonis, Daiva Garšvienė, Algirdas Juozulynas, Laimutė Samsonienė, Dalia Daukšienė, Konstancija Jankauskienė, Genovaitė Šimonienė-Kazlauskienė, Edgaras Stankevičius // Medicina. Kaunas : Lietuvos sveikatos mokslų universitetas. (Eksperimentiniai tyrimai). ISSN 1010-660X. 2011, t. 47, Nr. 3, p. 174-179. Prieiga per internetą: <http://medicina.kmu.lt/1103/1103-06e.pdf>. [Science Citation Index Expanded (Web of Science); MEDLINE; Index Copernicus; DOAJ]. [Citav. rod.: 0,423].
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APPENDIX

KAUNO REGIONINIS BIOMEDICININIŲ TYRIMŲ ETIKOS KOMITETAS
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LEIDIMAS ATLIKTI BIOMEDICININĮ TYRIMĄ

2011-05-10 Nr. BE-2-21

Biomedicininio tyrimo pavadinimas: **„Greivso ligos patogenezėje dalyvaujančių genų polimorfizmo įtaka ligos fenotipinių požymių išraiškai".**	
Protokolo Nr.:	**1**
Data:	**2011-04-20**
Versija:	**1**
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Išvada:
Kauno regioninio biomedicininių tyrimų etikos komiteto posėdžio, įvykusio **2011 m. gegužės 3 d.** (protokolo Nr. 57/2011) sprendimu pritarta biomedicininio tyrimo vykdymui.

Mokslinio eksperimento vykdytojai įsipareigoja: (1) nedelsiant informuoti Kauno Regioninį biomedicininių Tyrimų Etikos komitetą apie visus nenumatytus atvejus, susijusius su studijos vykdymu, (2) iki sausio 15 dienos – pateikti metinį studijos vykdymo apibendrinimą bei, (3) per mėnesį po studijos užbaigimo, pateikti galutinį pranešimą apie eksperimentą.

Kauno regioninio biomedicininių tyrimų etikos komiteto nariai			
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4.	Prof. Irayda Jakušovaitė	filosofija	taip
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Pirmininkė

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2012 m. lapkričio 14 d. Nr. 2R- 1416 (2.6.1)
Vilnius

Valstybinė duomenų apsaugos inspekcija, išnagrinėjusi Lietuvos sveikatos mokslų universiteto 2012-09-27 Pranešimą dėl išankstinės patikros ir Duomenų apsaugos priemonių aprašą bei kitus dokumentus (Inspekcijoje gauta 2012-10-01, reg. Nr. 1R-3792) (toliau – Pranešimas), bei 2012 m. lapkričio 7 d. raštu (Inspekcijoje gauta 2012-11-08, reg. Nr. 1R-4410) pateiktus paaiškinimus ir Pranešimo patikslinimus,

n u s t a t ė,

kad Pranešime ir jo patikslinime nurodyti asmens duomenų tvarkymo veiksmai atitinka Lietuvos Respublikos asmens duomenų teisinės apsaugos įstatyme (Žin., 1996, Nr. 63-1497; 2008, Nr. 22-804; 2011, Nr. 65-3046) nustatytus asmens duomenų tvarkymo ir duomenų subjektų teisių įgyvendinimo reikalavimus, bei numatytos tinkamos organizacinės ir techninės duomenų saugumo priemonės.

Valstybinė duomenų apsaugos inspekcija, vadovaudamasi Lietuvos Respublikos asmens duomenų teisinės apsaugos įstatymo 33 straipsniu, Išankstinės patikros atlikimo taisyklių, patvirtintų Valstybinės duomenų apsaugos inspekcijos direktoriaus 2006 m. vasario 2 d. įsakymu Nr.1T-6 (Žin., 2006, Nr. 18-653; 2009, Nr. 11-447) 11 ir 18.1 punktais,

n u s p r e n d ž i a

Lietuvos sveikatos mokslų universitetui išduoti leidimą atlikti Pranešime ir jo patikslinime nurodytų asmens duomenų tvarkymo mokslinio medicininio tyrimo „***Greivso ligos patogenezėje dalyvaujančių genų polimorfizmo įtaka ligos fenotipinių požymių išraiškai***“ tikslu, veiksmus.

Direktorius dr. Algirdas Kunčinas

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Juridinių asmenų registre
Kodas 188607912