



# *CD28/CTLA-4/ICOS* haplotypes confers susceptibility to Graves' disease and modulates clinical phenotype of disease

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**Abstract** Graves' disease, an autoimmune disease with heterogeneous symptoms including Graves' orbitopathy, has a combined genetic/environmental background, where variations within *CD28/CTLA-4/ICOS* genes are considered as disease markers.

Association of *CD28c.17+3T>C*(rs3116496), *CTLA-4g.319C>T*(rs5742909), *CTLA-4c.49A>G*(rs231775), *CTLA-4g.\*642AT*(8\_33), *CT60*(rs3087243), *Jo31*(rs11571302), *ICOSc.1554+4GT*(8\_15) polymorphisms with susceptibility to Graves' disease and clinical outcome was investigated. The study group comprised of 561 Polish Caucasians, including 172 unrelated Graves' disease patients. *CTLA-4c.49A>G*, *CTLA-4g.319C>T*, and *CT60* were genotyped

by PCR–RFLP; *Jo31* and *CD28c.17+3C>T* by minisequencing; *CTLA-4g.\*642AT*(8\_33) and *ICOSc.1554+4GT*(8\_15)—PCR and fluorescence-based technique. *CD28c.17+3T>C*(rs3116496)*T/CTLA-4g.319C>T*(rs5742909)*C/CTLA-4c.49A>G*(rs231775)*G/CTLA-4g.\*642AT*(8\_33)(*AT*<sub>16–21</sub>)/*CT60*(rs3087243)*G/Jo31*(rs11571302)*G/ICOSc.1554+4GT*(8\_15)(m) and *TCA*(*AT*<sub><16</sub>)*GT*(m) haplotypes increased risk of Graves' disease, especially in males, as well as overall Graves' orbitopathy development with severe outcome. *TCG*(*AT*<sub>16–21</sub>)*GG*(l) haplotype increased risk of Graves' disease and reduced the chance of successful medical treatment. Although this haplotype was mainly observed in patients without signs of Graves' orbitopathy, if Graves' orbitopathy developed it favored a Graves' orbitopathy outcome. Haplotype *TCA*(*AT*<sub>>21</sub>)*GT*(m) increased Graves' disease risk in women and, in all patients, was linked to Graves' disease without Graves' orbitopathy. *TCG*(*AT*<sub><16</sub>)*GG*(m) haplotype was predominantly observed in patients without Graves' orbitopathy, whereas *TCA*(*AT*<sub>16–21</sub>)*GG*(m) was absent in those patients. *TCA*(*AT*<sub>16–21</sub>)*GG*(m) occurred in patients with a mild Graves' orbitopathy outcome. The marker *CTLA-4g.\*642AT*(8\_33) was the only independent Graves' disease risk factor, whereas *CT60* was an independent factor for disease progression. Sporadic Graves' disease was related to presence of *CTLA-4c.49A>G*[A] and the rare *CTLA-4g.319C>T*[T] allele variant. Familial background of the disease was exclusively associated with *CTLA-4g.\*642AT*(8\_33)[*AT*<sub>>21</sub>]/[*AT*<sub>>21</sub>] genotype. *CD28/CTLA-4/ICOS* loci may confer inherited susceptibility to Graves' disease or may be involved in susceptibility to Graves' disease and play a pathogenetic role.

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## Introduction

The pathogenesis of Graves' disease (GD) requires a specific sequence of events, involving both genetic factors and the ensuing environmental factors, for the final clinical phenotype to manifest [1–3]. The orbital inflammatory process in Graves' orbitopathy (GO) is believed to be driven by T cells in response to thyroid–orbit shared antigens accessing and infiltrating the orbital space [4]. As T cells play a key role in the pathogenesis of GO, and CD28/*CTLA-4*/*ICOS* co-stimulatory molecules are critical in T-cell activation, they may be considered to be candidate risk factors. Disruption to the precise balance of expression of co-stimulatory and inhibitory molecules is known to play a role in the pathogenesis of autoimmune diseases [5]. This is supported by studies demonstrating that adolescents and children newly diagnosed with GD had an abnormal percentage of lymphocytes expressing co-stimulatory molecules [6, 7]. Similarly, differences in the expression pattern of CD28 in T cells of the thyroid gland and peripheral blood T lymphocytes was observed [6]. Differential regulation of these molecules could easily affect T-cell function and hence the regulation of the immune response. Abnormal expression and/or dysfunction of these molecules may be caused by polymorphisms in the corresponding gene [8–26]. In previous work, we linked the presence of guanine (G) in CT60 and Jo31 single-nucleotide polymorphism (SNPs), located within the 3'UTR of *CTLA-4*, with an elevated level of the soluble *CTLA-4* isoform in GD, especially in cases of severe outcome [26]. Competitive binding of s*CTLA-4* with *CTLA-4* common ligands inhibited T-cell proliferation via increased activation of CD28 [27]. The prevalence of s*CTLA-4*-bearing genetics in GD may indicate abnormal expression of *CTLA-4* on T-regulatory cells (Treg). In turn, this may affect the precise balance of suppressive and effector activity of Treg, which is strongly linked to pathogenesis of autoimmune diseases [27].

A previously published, candidate gene strategy indicated and validated several immune-related and thyroid-specific susceptibility genes for GD. In regard to immune-related genes, comprehensive fine mapping of the *CD28/CTLA-4/ICOS* region showed an association signal within a 6.1-Kb 3' region of *CTLA-4* [18]. To date, most research has focused on univariate association studies of single polymorphisms—especially the highly polymorphic *CTLA-4* [8, 9, 16, 18, 21, 25, 28, 29, 30, 31, 32, 33, 34, 35, 36], with several functional polymorphisms having been observed within this gene [8–26]. Similar findings concerning GO and *CTLA-4* polymorphisms have been reported [37]. In the case of the co-stimulatory receptors CD28 and *ICOS*, definitive determination of their role in GD and GO pathogenesis is still impossible due to the

paucity of research published to date. In our opinion, treating the 2q33 chromosomal region where *CD28*, *CTLA-4*, and *ICOS* are located as a *locus* is more appropriate and takes into account the possible effects of interactions between polymorphisms located within those genes on their function and/or expression and, consequently, on the pathogenesis and clinical outcome of GD. Such a relationship was previously observed between genetic variants in *ICOS*IVS1+173T/C and *CTLA-4* mRNA expression levels [38].

Based on the concept that the associations of GD and GO with this region may represent the effects of any of these three genes alone, or in combination, due to linkage disequilibrium (LD), we attempted to verify the possible associations between *CD28*c.17+3T>C(rs3116496)/*CTLA-4*g.319C>T(rs5742909)/*CTLA-4*c.49A>G(rs231775)/*CTLA-4*g.\*642AT(8\_33)(AT<sub>16-21</sub>)/CT60(rs3087243)/Jo31(rs11571302)/*ICOS*c.1554+4GT(8\_15) gene polymorphisms and susceptibility to GD, as well as the severity and activity of GO defined as one susceptible *locus*, expressed as the haplotype.

## Materials and methods

### Study population

#### Patients

One hundred and seventy-two unrelated Polish Caucasian GD patients consecutively admitted to the *tertiary care academic medical center* were enrolled into the study. The initial diagnosis of GD was confirmed by routine clinical and laboratory tests. Detailed patient characteristics are presented in Table 1. Ninety-six patients responded to first-line medical treatment and required no further therapy for relapse within next 18 months.

#### Assessment of ocular changes

Ophthalmological examination was performed at the time of blood collection. The severity and activity of orbitopathy was assessed using criteria proposed by Bartalena [39] (detailed in Table 1).

#### Control group

The control group consisted of 389 apparently healthy volunteers from the same geographical area (225 (57.8 %) females and 164 (42.2 %) males).

**Table 1** GD patients ( $n = 172$ ) clinical characteristics

	Parameter		$n$ (%)	
Without GO ( $n = 28$ )	CAS + orbitopathy index	0	28 (16.28)	
With GO ( $n = 144$ )	CAS	Non-active ( $n = 73$ )	0	
			1	
			2	
			3	
			4	
			5	
			6	
	Orbitopathy index	Non-severe ( $n = 64$ )	Mild	29 (16.86)
			Moderate	35 (20.35)
			Severe	80 (46.50)
Gender		Female/male	134 (77.90)/38 (22.10)	
Familial autoimmune thyroid history		Yes/no	138 (80.23)/34 (19.77)	
Smoking status	Non-smokers ( $n = 65$ )	Never	65 (37.79)	
		Smokers ( $n = 107$ )	In past	
		$\leq 10$	40 (23.26)	
		10–20	25 (14.53)	
		$\geq 20$	4 (2.33)	
Response to anti-thyroid treatment		Yes	96 (55.82)	
	No ( $n = 76$ )	Thyroidectomy	16 (9.30)	
		$^{131}\text{I}$ therapy	60 (34.88)	
Age	Mean $\pm$ SD		49 $\pm$ 12.73	
	Median		48	
	Range		23–82	

### Ethical approvals

The local ethics committee approved the study protocol.

### Genotyping of polymorphisms

The *CTLA-4g.319C>T* (rs5742909), *CTLA-4c.49A>G* (rs231775), *CT60 (CTLA-4g.\*6230G>A*, rs3087243), and *Jo31 (CTLA-4g.\*10223G>T*, rs11571302) polymorphisms were examined as described by Daroszewski et al. [26].

The *CD28c.17+3T>C* SNP (rs3116496, IVS3+17C/T), *CTLA-4g.\*642AT(8\_33)* and *ICOSc.1554+4GT(8\_15)* short tandem repeat polymorphisms were genotyped according to Suwalska et al. [40].

### Statistical analyses

The Hardy–Weinberg equilibrium (HWE) was tested for the studied group. Categorical data between GD patients and controls, and within the GD group according to clinical outcome, were compared using the  $\chi^2$  test with appropriate correction. In case of multiple comparisons, Bonferroni

multiple adjustments were employed. Haplotype analysis and LD coefficients were estimated using the SHEsis software (<http://202.120.7.14/analysis/myAnalysis.php>).

The combined effects of polymorphic markers and environmental factors (gender and smoking habits) on the development of GD and GD outcome were analyzed by unconditional multivariate logistic regression analysis with a logit regression model using a quasi-Newton estimation method (software EPIINFO Ver. 7.1.1.14).

Differences were considered statistically significant if the  $p$ -value  $< 0.05$ .

### Results

Genotype frequencies in both groups were in HWE (Table 2).

For *CTLA-4g.\*642AT(8\_33)* polymorphism, 20 alleles and 90 genotypes were identified (data not shown). Alleles were grouped into three groups for further analysis: [AT<sub><16</sub>] with less than 16, [AT<sub>16–21</sub>] with 16–21, and [AT<sub>>21</sub>] with more than 21 AT repeats.

**Table 2** *CTLA-4*g.\*642AT(8\_32), CT60 (rs3087243, *CTLA-4*g.\*6230G>A), and *ICOSc.1554+4GT(8\_15)* genotype and allele frequencies in GD patients and control group

	GD patients n (%)	Control group n (%)	$p_{\text{global}}^{\#}$	$\chi^2$	$p$	Odds ratio	95 % confidence interval
<i>CTLA-4</i> g.*642AT(8_33)							
<i>Genotype<sup>a</sup></i>							
[AT <sub>&lt;16</sub> ]/[AT <sub>&lt;16</sub> ]	31 (18.0)	99 (25.4)	0.08 <sup>^</sup>	<i>Referent</i>			
[AT <sub>&lt;16</sub> ]/[AT <sub>16-21</sub> ]	52 (30.2)	104 (26.7)		3.10	0.08	1.60	0.95–2.69
[AT <sub>&lt;16</sub> ]/[AT <sub>&gt;21</sub> ]	22 (12.8)	64 (16.5)		0.08	0.77	1.10	0.58–2.06
[AT <sub>16-21</sub> ]/[AT <sub>16-21</sub> ]	35 (20.3)	50 (12.9)		<b>7.26</b>	<b>0.007</b>	<b>2.24</b>	<b>1.24–4.04</b>
[AT <sub>16-21</sub> ]/[AT <sub>&gt;21</sub> ]	30 (17.4)	54 (13.9)		3.53	0.06	1.77	0.97–3.24
[AT <sub>&gt;21</sub> ]/[AT <sub>&gt;21</sub> ]	2 (1.2)	18 (4.6)		1.21*	0.27*	0.35	0.08–1.62
<i>Allele<sup>b</sup></i>							
[AT <sub>&lt;16</sub> ]	136 (39.5)	366 (47.0)	<b>0.006<sup>^</sup></b>	<i>Referent</i>			
[AT <sub>16-21</sub> ]	152 (44.2)	258 (33.2)		<b>10.41</b>	<b>0.001</b>	<b>1.59</b>	<b>1.20–2.10</b>
[AT <sub>&gt;21</sub> ]	56 (16.3)	154 (19.8)		0.01	1.00	0.98	0.68–1.41
<i>Dominant model</i>							
[AT <sub>&lt;16</sub> ]/[AT <sub>16-21</sub> ] + [AT <sub>16-21</sub> ]/[AT <sub>16-21</sub> ] + [AT <sub>16-21</sub> ]/[AT <sub>&gt;21</sub> ] vs. others	117 (68.0)	208 (53.5)		<b>10.36</b>	<b>0.001</b>	<b>1.85</b>	<b>1.27–2.70</b>
	55 (32.0)	181 (46.5)				<b>0.54</b>	<b>0.37–0.79</b>
CT60 ( <i>CTLA-4</i> g.*6230G>A, rs3087243)							
<i>Genotype<sup>c</sup></i>							
[GG]	74 (43.0)	133 (34.3)	0.08 <sup>^</sup>	<i>Referent</i>			
[GA]	80 (46.5)	187 (48.2)		1.78	0.18	0.77	0.52–1.13
[AA]	18 (10.5)	68 (17.5)		<b>6.19</b>	<b>0.01</b>	<b>0.48</b>	<b>0.26–0.86</b>
<i>Allele</i>							
[G]	228 (66.3)	453 (58.4)		<b>6.09</b>	<b>0.01</b>	<b>1.41</b>	<b>1.08–1.84</b>
[A]	116 (33.7)	323 (41.6)				<b>0.72</b>	<b>0.55–0.93</b>
<i>Dominant model</i>							
[GG] + [GA] vs. [AA]	154 (89.7)	320 (82.5)		<b>4.57</b>	<b>0.03</b>	<b>1.82</b>	<b>1.05–3.16</b>
	18 (10.3)	68 (17.5)				<b>0.55</b>	<b>0.32–0.96</b>
<i>Recessive model</i>							
[AA] + [GA] vs. [GG]	98 (57.0)	255 (65.7)		<b>3.91</b>	<b>0.05</b>	<b>0.69</b>	<b>0.48–1.00</b>
	74 (43.0)	134 (34.4)				<b>1.45</b>	<b>1.00–2.09</b>
<i>ICOSc.1554+4GT(8_15)</i>							
<i>Genotype<sup>d</sup></i>							
(s)/(s)	1 (0.6)	4 (1.0)	ns <sup>^</sup>	0.00*	1.00*	0.58	0.06–5.29
(s)/(m)	5 (2.9)	35 (9.0)		<b>4.46*</b>	<b>0.03*</b>	<b>0.33</b>	<b>0.13–0.88</b>
(s)/(l)	7 (4.1)	17 (4.4)		0.009	1.00	0.96	0.38–2.41
(m)/(m)	71 (41.3)	166 (42.9)		<i>Referent</i>			
(m)/(l)	77 (44.8)	137 (35.4)		1.77	0.18	1.31	0.88–1.94
(l)/(l)	11 (6.4)	28 (7.2)		0.06	0.81	1.10	0.52–2.32
<i>Allele<sup>e</sup></i>							
(s)	14 (4.1)	60 (7.8)	ns <sup>^</sup>	<b>4.58</b>	<b>0.03</b>	<b>0.52</b>	<b>0.29–0.96</b>
(m)	224 (65.1)	504 (65.1)		<i>Referent</i>			
(l)	106 (30.8)	210 (27.1)		0.74	0.39	0.88	0.67–1.17
<i>Dominant model</i>							
(m)/(m) + (m)/(l) vs. others	153 (89.0)	338 (87.3)		0.29	0.59	0.86	0.49–1.50
	19 (11.0)	49 (12.7)				1.17	0.67–2.05

Bold indicates statistical significant association

*ICOSc.1554+4GT(8\_15)*: (s)-short alleles with 8 and 9 repeats of GT pair; (m)-medium alleles with 10 and 11 repeats of GT pair; (l)-long alleles with 12 or more repeats of GT pair

OR odds ratio, 95 % CI 95 % confidence intervals, HWE Hardy–Weinberg equilibrium

<sup>^</sup>*p*-value after Bonferroni correction; \**p*-value after Yate’s correction

<sup>a</sup>  $\chi^2 = 13.85$ ,  $df = 5$ ,  $p \sim = 0.02$ ,  $n_{\text{GDpatients}} = 172$ ,  $n_{\text{controls}} = 389$

<sup>b</sup>  $\chi^2 = 12.51$ ,  $df = 3$ ,  $p \sim = 0.002$

<sup>c</sup>  $\chi^2 = 6.40$ ,  $df = 2$ ,  $p \sim = 0.04$ ,  $n_{\text{GDpatients}} = 172$ ,  $\text{HWE}_{\text{GDpatients}}: \chi^2 = 0.28$ ,  $p = 0.60$ ,  $n_{\text{controls}} = 388$ ,  $\text{HWE}_{\text{controls}}: \chi^2 = 0.03$ ,  $p = 0.87$

<sup>d</sup>  $\chi^2 = 9.46$ ,  $df = 5$ ,  $p \sim = 0.09$ ,  $n_{\text{GDpatients}} = 172$ ,  $n_{\text{controls}} = 387$

<sup>e</sup>  $\chi^2 = 6.02$ ,  $df = 3$ ,  $p \sim = 0.05$

For *ICOS*.1554+4GT(8\_15) polymorphism, 8 alleles and 19 genotypes were observed (data not shown); alleles were grouped into three cohorts: short alleles (s) with 8–9, medium (m) with 10–11, and long alleles (l) with 12 or more GT repeats.

Smokers were statistically more prone to severe eye symptoms ( $p=0.04$ ), and patients with non-severe GO were the best responders to anti-thyroid treatment ( $p=0.001$ ) (Supplementary Table 1).

### CD28/CTLA-4/ICOS gene polymorphisms and GD risk

In univariate analysis a significant association with GD was observed only with two of the studied polymorphisms: g.\*642AT(8\_33) and CT60 (rs3087243) (Table 2).

The *CTLA-4*g.\*642AT(8\_33)[AT<sub>16–21</sub>] variant increased the risk of disease 2.24-fold ( $p=0.007$ , 95 % CI: 1.24–4.04), and the carriers of this allele variant were 1.85 times more prone to GD ( $p=0.001$ , 95 % CI: 1.27–2.70).

CT60 (rs3087243) [AA] homozygosity significantly protected against GD ( $p=0.01$ , OR = 0.48, 95 % CI: 0.26–0.86). Weaker association was seen for overall presence of this allele ( $p=0.03$ , OR = 0.69, 95 % CI: 0.48–1.00) (Table 2).

Other studied polymorphisms were not associated with GD risk (Supplementary Table 2).

In the controls robust LD was observed between the pair of *CTLA-4* polymorphisms, and weaker association was seen between *CTLA-4* polymorphisms and *CD28c.17+3T>C* (rs3116496), while in patients such relations were observed only between *CTLA-4* gene polymorphisms (Supplementary Table 3).

Significantly different haplotype distribution among GD patients and controls was observed ( $\chi^2 = 25.028397$ ,  $p_{\text{corrected}} = 0.00069$ ). Haplotypes TCA[AT<sub><16</sub>]AT(l), TCG[AT<sub>>21</sub>]GG(m) [in order: *CD28c.17+3T>C/g.319C>T/c.49A>G/g.\*642AT(8\_33)/CT60/Jo31/ICOS.1554+4GT(8\_15)*] decreased the risk of GD ( $p=0.029116$ , OR = 0.499, 95 % CI: 0.265–0.941;  $p=0.000456$ , OR = 0.195, 95 % CI: 0.071–0.535), whereas TCG[AT<sub>16–21</sub>]GG(l) and TTA[AT<sub>>21</sub>]GG(m) amplified such risk ( $p=0.053837$ , OR = 1.455, 95 % CI: 0.993–2.133;  $p=0.050925$ , OR = 2.030, 95 % CI: 0.985–4.185) (Supplementary Table 4).

### CD28/CTLA-4/ICOS gene polymorphisms and gender

The *CTLA-4*g.\*642AT(8\_33)[AT<sub>16–21</sub>]/[AT<sub>16–21</sub>] genotype increased risk of disease in women ( $p=0.01$ , OR = 2.48), whereas [AT<sub>16–21</sub>] allele increased the risk of disease in both gender ( $p=0.007$ ;  $p=0.05$ ) (Supplementary Table 5).

Moreover, women possessing the CT60 (rs3087243) [AA] genotype have lower risk of developing GD ( $p=0.02$ ,

OR = 0.43, 95 % CI: 0.22–0.86), similarly was for the allele and dominant model ( $p=0.02$ ;  $p=0.02$ ) (Supplementary Table 5).

There also the *ICOS*.1554+4GT(8\_15)(l) allele increased risk of GD in women ( $p=0.003$ , OR = 1.72, 95 % CI: 1.21–2.46) (Supplementary Table 5).

TCG[AT<sub>>21</sub>]GG(m) haplotype protected against disease after stratification according to gender ( $p=0.007$ ,  $p=0.008$ ). Moreover, TCA[AT<sub><16</sub>]GT(m) and CTA[AT<sub>16–21</sub>]GG(l) haplotypes were observed only in male patients, and TCA[AT<sub><16</sub>]AG(m) and TCG[AT<sub>16–21</sub>]GG(m) haplotypes increased disease risk in male ( $p=0.03$ , OR = 5.93, 95 % CI: 0.94–37.51;  $p=0.05$ , OR = 1.93, 95 % CI: 1.00–3.74). Contrary, haplotypes TCA[AT<sub><16</sub>]AT(l), TCG[AT<sub>>21</sub>]GG(m), and CTA[AT<sub>>21</sub>]GG(m) were absent in male patients (Supplementary Table 6).

Additionally, stratification by gender showed differed frequency of TCA[AT<sub><16</sub>]AG(m) and CTA[AT<sub>16–21</sub>]GG(l) within GD ( $p=0.04$ ,  $p=0.001$ ), whereas within controls this was observed for CTA[AT<sub>>21</sub>]GG(m) haplotype ( $p=0.02$ ) (Supplementary Table 6).

### GD risk factors—multivariate associations

The unconditional stepwise multivariate logistic regression analysis of all studied polymorphisms, including three prone genetic parameters: presence of T allele at *CTLA-4*g.319C>T (rs5742909) (genotype TT and CT), presence of *CTLA-4*g.\*642AT(8\_33)[AT<sub>16–21</sub>] allele, presence of G allele at CT60 (rs3087243) [genotype (GG) and (GA)], and environmental factor—female gender, showed that only the *CTLA-4*g.\*642AT(8\_33) was an independent GD risk factor ( $\chi^2 = 33.3$ ,  $p=0.0008$ ). In detail, presence of *CTLA-4*g.\*642AT(8\_33)[AT<sub>16–21</sub>] allele 1.94-fold increased risk for GD ( $p=0.00081$ , 95 % CI: 1.32–2.86).

### CD28/CTLA-4/ICOS gene polymorphisms and clinical data in GD

#### Familial autoimmune thyroid history

The *CTLA-4*g.319C>T (rs5742909) genotype and allele were differentially distributed ( $p=0.0002$ ;  $p=4.07 \times 10^{-5}$ ), and lack of the rare [T] allele increased GD in patients with familial autoimmune thyroid incidence by 4.85-fold ( $p=0.00005$ , 95 % CI: 2.18–10.76). *CTLA-4*g.\*642AT(8\_33)[AT<sub>>21</sub>]/[AT<sub>>21</sub>] genotype was also present only in patients with familial autoimmune thyroid background ( $p_{\text{corrected}} = 0.04$ ), whereas presence of the *CTLA-4*c.49A>G (rs231775) [A] allele variant was significantly associated with sporadic GD ( $p_{\text{corrected}} = 0.05$ ) (Table 3 and Supplementary Table 7).

A significant difference in haplotype frequency in GD patients with respect to familial thyroid autoimmune history

**Table 3** *CTLA-4*g.319C>T, *CTLA-4*c.49A>G, *CTLA-4*g.\*642AT(8\_32), *ICOS*c.1554+4GT(8\_15) genotype and allele frequencies in GD patients according to occurrence of familial autoimmune thyroid history in first-line relatives

	Familial autoimmune thyroid history		$\chi^2$	<i>p</i> -value	Odds ratio	95 % confidence interval
	Without ( <i>n</i> = 34) <i>n</i> (%)	With ( <i>n</i> = 138) <i>n</i> (%)				
<i>CTLA-4</i> g.319C>T (rs5742909)						
<i>Genotype<sup>a</sup></i>						
[CC]	16 (47.1)	112 (81.2)			<i>Referent</i>	
[CT]	16 (47.1)	24 (17.4)	14.95	<b>0.0001</b>	<b>0.21</b>	<b>0.09–0.49</b>
[TT]	2 (5.9)	2 (1.4)	2.00*	0.16*	0.14	0.02–1.09
<i>Allele</i>						
[C]	48 (70.6)	248 (89.9)	<b>16.87</b>	<b>4.07 × 10<sup>-5</sup></b>	<b>3.69</b>	<b>1.92–7.08</b>
[T]	20 (29.4)	28 (10.1)			<b>0.27</b>	<b>0.14–0.52</b>
<i>Dominant model</i>						
[CC] + [CT] vs. [TT]	32 (94.1)	136 (98.6)	0.81*	0.37*	4.25	0.58–31.32
	2 (5.9)	2 (1.4)			0.24	0.03–1.73
<i>Recessive model</i>						
[TT] + [CT] vs. [CC]	18 (52.9)	26 (18.8)	<b>16.66</b>	<b>4.5 × 10<sup>-5</sup></b>	<b>0.21</b>	<b>0.09–0.46</b>
	16 (47.1)	112 (81.2)			<b>4.85</b>	<b>2.18–10.76</b>
<i>CTLA-4</i> c.49A>G (rs231775)						
<i>Genotype<sup>b</sup></i>						
[AA]	12 (35.3)	39 (28.3)			<i>Referent</i>	
[AG]	19 (55.9)	70 (50.7)	0.09	0.76	0.88	0.39–2.01
[GG]	3 (8.8)	29 (21.0)	1.79*	0.18*	2.97	0.77–11.51
<i>Allele</i>						
[A]	43 (63.2)	148 (53.6)	2.04	0.15	0.67	0.39–1.16
[G]	25 (36.8)	128 (46.4)			1.49	0.86–2.57
<i>Dominant model</i>						
[AA] + [AG] vs. [GG]	31 (91.2)	109 (79.0)	<b>3.85*</b>	<b>0.05*</b>	<b>0.27</b>	<b>0.08–0.94</b>
	3 (8.8)	29 (21.0)			<b>3.70</b>	<b>1.07–12.78</b>
<i>Recessive model</i>						
[GG] + [AG] vs. [AA]	22 (64.7)	99 (71.7)	0.65	0.42	1.38	0.63–3.07
	12 (35.3)	39 (28.3)			0.72	0.33–1.60
<i>CTLA-4</i> g.*642AT(8_33)						
<i>Genotype<sup>c</sup></i>						
[AT <sub>&lt;16</sub> ]/[AT <sub>&lt;16</sub> ]	6 (17.6)	25 (18.1)			<i>Referent</i>	
[AT <sub>&lt;16</sub> ]/[AT <sub>16–21</sub> ]	10 (29.4)	42 (30.4)	0.00	1.00	1.01	0.33–3.11
[AT <sub>&lt;16</sub> ]/[AT <sub>&gt;21</sub> ]	6 (17.6)	16 (11.6)	0.46	0.50	0.64	0.18–2.33
[AT <sub>16–21</sub> ]/[AT <sub>16–21</sub> ]	6 (17.6)	29 (21.0)	0.05	0.82	1.16	0.33–4.06
[AT <sub>16–21</sub> ]/[AT <sub>&gt;21</sub> ]	6 (17.6)	24 (17.4)	0.004	1.00	0.96	0.27–3.39
[AT <sub>&gt;21</sub> ]/[AT <sub>&gt;21</sub> ]	0 (0.0)	2 (1.4)	<b>4.27*</b>	<b>0.04*</b>	–	–
<i>Allele<sup>d</sup></i>						
[AT <sub>&lt;16</sub> ]	28 (41.2)	108 (39.1)			<i>Referent</i>	
[AT <sub>16–21</sub> ]	28 (41.2)	124 (44.9)	0.22	0.64	1.15	0.64–2.06
[AT <sub>&gt;21</sub> ]	12 (17.6)	44 (15.9)	0.02	1.00	0.95	0.44–2.04
<i>Dominant model</i>						
[AT <sub>&lt;16</sub> ]/[AT <sub>16–21</sub> ] + [AT <sub>16–21</sub> ]/[AT <sub>16–21</sub> ] + [AT <sub>&lt;16</sub> ]/[AT <sub>&gt;21</sub> ] vs. others	34 (100.0)	136 (98.6)	0.04*	0.85*	–	–
	0 (0.0)	2 (1.4)			–	–
<i>ICOS</i> c.1554_4GT(8_15)						
<i>Genotype<sup>e</sup></i>						
(s)/(s)	0 (0.0)	1 (0.7)	0.39*	0.53*	–	–
(s)/(m)	0 (0.0)	5 (3.6)	0.47*	0.49*	–	–
(s)/(l)	3 (8.8)	4 (2.9)	0.41*	0.52*	0.42	0.09–2.07
(m)/(m)	17 (50.0)	54 (39.1)			<i>Referent</i>	
(m)/(l)	10 (29.4)	67 (48.6)	2.97	0.08	2.11	0.89–4.98
(l)/(l)	4 (11.8)	7 (5.1)	0.26*	0.61*	0.55	0.14–2.11
<i>Allele<sup>f</sup></i>						
(s)	3 (4.4)	11 (4.0)	0.03*	0.85*	0.90	0.24–3.35
(m)	44 (64.7)	180 (65.2)			<i>Referent</i>	
(l)	21 (30.9)	85 (30.8)	0.001	1.00	0.99	0.55–1.77

**Table 3** continued

	Familial autoimmune thyroid history		$\chi^2$	<i>p</i> -value	Odds ratio	95 % confidence interval
	Without ( <i>n</i> = 34) <i>n</i> (%)	With ( <i>n</i> = 138) <i>n</i> (%)				
<i>Dominant model</i>						
(s)/(m) + (m)/(m) + (m)/(l) vs. others	27 (79.4)	126 (91.3)	<b>3.92</b>	<b>0.05</b>	<b>2.72</b>	<b>0.98–7.55</b>
	7 (20.6)	12 (8.7)			<b>0.37</b>	<b>0.13–1.02</b>
<i>CTLA-4g.319C&gt;T</i> (rs5742909)	Whole genotype	a	$\chi^2 = 16.89$ , <i>df</i> = 2, <i>p</i> = <b>0.0002</b>			
<i>CTLA-4c.49A&gt;G</i> (rs231775)	Whole genotype	b	$\chi^2 = 2.78$ , <i>df</i> = 2, <i>p</i> = 0.25			
<i>CTLA-4g.*642AT</i> (8_33)	Whole genotype	c	$\chi^2 = 1.44$ , <i>df</i> = 5, <i>p</i> = 0.92			
	Whole allele	d	$\chi^2 = 0.33$ , <i>df</i> = 2, <i>p</i> = 0.85			
<i>ICOSc.1554+4GT</i> (8_15)	Whole genotype	e	$\chi^2 = 8.75$ , <i>df</i> = 5, <i>p</i> = 0.12			
	Whole allele	f	$\chi^2 = 0.026678$ , <i>df</i> = 2, <i>p</i> = 0.986749			

Bold indicates statistical significant association

*ICOSc.1554+4GT*(8\_15): (s)-short alleles with 8 and 9 repeats of GT pair; (m)-medium alleles with 10 and 11 repeats of GT pair; (l)-long alleles with 12 or more repeats of GT pair

<sup>^</sup>*p*-value after Bonferroni correction; \**p*-value after Yate's correction; <sup>~</sup>global *p*-value for genotype

was observed ( $\chi^2_{\text{Global}} = 18.40$ ,  $p_{\text{corrected}} = 0.03$ ). In particular, haplotypes CCA[AT<sub><16</sub>]AT(m) and TTA[AT<sub>>21</sub>]GG(m) were significantly associated with sporadic GD ( $p = 0.055078$ ;  $p = 0.003176$ ), whereas TCG[AT<sub>16–21</sub>]GG (m) was over-represented in patients with familial thyroid autoimmune history ( $p = 0.025898$ ) (Supplementary Table 8).

#### Response to medical treatment

Univariate analysis revealed significant associations of two *CTLA-4* 3'UTR SNPs: CT60 (rs3087243) and Jo31 (rs11571302)-genotypes at both polymorphic sites were differentially distributed with regard to sustained response to medical treatment ( $p = 0.007$ ;  $p = 0.03$ ). Both, the CT60 (rs3087243) [AA] and Jo31 (rs11571302) [TT] homozygosity significantly increased the response rate ( $p_{\text{corrected}} = 0.007$ , OR = 0.16, 95 % CI: 0.04–0.60;  $p_{\text{corrected}} = 0.01$ , OR = 0.30, 95 % CI: 0.12–0.79). This was also confirmed at the allele level ( $p = 0.002$ ;  $p = 0.008$ ), and lack of those alleles statistically significantly reduces the success of medical treatment ( $p = 0.01$ , OR = 2.24, 95 % CI: 1.21–4.15;  $p = 0.02$ , OR = 2.02, 95 % CI: 1.10–3.89) (Table 4 and Supplementary Table 9).

Haplotype TTA[AT<sub>16–21</sub>]GT(l) significantly reduced, whereas TCA[AT<sub><16</sub>]AT(m) increased the success of anti-thyroid treatment ( $p = 0.008184$ , OR = 9.048, 95 % CI: 1.287–63.593;  $p = 0.007347$ , OR = 0.438, 95 % CI: 0.238–0.806, Supplementary Table 10).

#### Response to medical treatment—multivariate associations

Finally, unconditional stepwise multivariate logistic regression analysis of all studied polymorphisms, including

three prone genetic parameters: presence of G allele at CT60 (rs3087243) (genotype [GG] and [GA]), Jo31 (rs11571302) [GG] genotype, and presence of *CTLA-4g.\*642AT*(8\_33)[AT<sub>16–21</sub>] allele, indicated only the CT60 (rs3087243) marker as an independent disease relapse factor ( $\chi^2 = 114.6$ ,  $p = 0.009$ ). CT60 (rs3087243) [G] allele 4.51-fold decreased success of anti-thyroid treatment ( $p = 0.02$ , 95 % CI: 1.24–16.3).

#### CD28/CTLA-4/ICOS gene polymorphisms and GO

##### The course of GO—univariate associations

The *CTLA-4g.\*642AT*(8\_33)[AT<sub>16–21</sub>] homozygosity increased the risk of GO 2.42-fold ( $p = 0.004$ , 95 % CI: 1.31–4.46). Moreover, the same genotype was associated with GO severity ( $p = 0.0001$ , OR = 4.55, 95 % CI: 2.01–10.31;  $p = 0.01$ , OR = 3.91, 95 % CI: 1.33–11.49), but favored the inactive outcome ( $p = 0.004$ , OR = 3.37, 95 % CI: 1.44–7.89). The same observation was true for allele (Table 5, Supplementary Tables 11 and 12).

Concerning CT60 (rs3087243), the [AA] genotype significantly reduced the risk of GO and heavy GO outcome: severe eye symptoms as well as active GO course ( $p = 0.02$ , OR = 0.48, 95 % CI: 0.26–0.90;  $p = 0.01$ , OR = 0.35, 95 % CI: 0.15–0.83;  $p = 0.02$ , OR = 0.35, 95 % CI: 0.14–0.86), which was also true for presence of [A] allele ( $p = 0.02$ , OR = 0.64, 95 % CI: 0.43–0.94;  $p = 0.02$ , OR = 0.55, 95 % CI: 0.34–0.90;  $p = 0.05$ , OR = 0.60, 95 % CI: 0.36–1.00) (Table 5, Supplementary Tables 11 and 12).

Although the association of *CTLA-4g.319C>T* (rs5742909) with GO development was observed only at the allele level ( $p = 0.04$ ) (Table 5), this SNP was associated with GO outcome (Supplementary Table 11). The rare [T]

**Table 4** *CTLA-4*g.\*642AT(8\_32), CT60 (rs3087243, *CTLA-4*g.\*6230G>A), Jo31 (*CTLA-4*g.\*10223G>T) genotype and allele frequencies in GD patients with respect to response to anti-thyroid treatment

	Anti-thyroid treatment		$\chi^2$	<i>p</i> -value	Odds ratio	95 % confidence interval
	Response <i>n</i> (%)	No response <i>n</i> (%)				
<i>CTLA-4</i> g.*642AT(8_33)						
<i>Genotype<sup>c</sup></i>						
[AT<16]/[AT<16]	22 (22.9)	9 (11.8)			<i>Referent</i>	
[AT<16]/[AT16-21]	28 (29.2)	24 (31.6)	2.38	0.12	2.10	0.81–5.41
[AT<16]/[AT>21]	13 (13.5)	9 (11.8)	0.81	0.37	1.69	0.54–5.35
[AT16-21]/[AT16-21]	20 (20.8)	15 (19.7)	1.36	0.24	1.83	0.66–5.11
[AT16-21]/[AT>21]	13 (13.5)	17 (22.4)	<b>4.76</b>	<b>0.03</b>	<b>3.20</b>	<b>1.11–9.22</b>
[AT>21]/[AT>21]	0 (0.0)	2 (2.6)	1.66*	0.20*	–	–
<i>Allele<sup>d</sup></i>						
[AT<16]	85 (44.3)	51 (33.6)			<i>Referent</i>	
[AT16-21]	81 (42.2)	71 (46.7)	2.49	0.11	1.46	0.91–2.34
[AT>21]	26 (13.5)	30 (19.7)	<b>4.20</b>	<b>0.04</b>	<b>1.92</b>	<b>1.03–3.61</b>
<i>Dominant model</i>						
[AT<16]/[AT16-21] + [AT16-21]/[AT16-21] + [AT<16]/[AT>21] vs. others	61 (63.5)	56 (73.7)	2.01	0.16	1.61	0.83–3.10
	35 (36.5)	20 (26.3)			0.62	0.32–1.20
CT60( <i>CTLA-4</i> )(g.*6230G > A, rs3087243)						
<i>Genotype<sup>e</sup></i>						
[GG]	33 (34.4)	41 (53.9)			<i>Referent</i>	
[GA]	48 (50.0)	32 (42.1)	3.66	0.06	0.54	0.28–1.02
[AA]	15 (15.6)	3 (3.9)	<b>7.22*</b>	<b>0.007*</b>	<b>0.16</b>	<b>0.04–0.60</b>
<i>Allele</i>						
[G]	114 (59.4)	114 (75.0)	<b>9.27</b>	<b>0.002</b>	<b>2.05</b>	<b>1.29–3.27</b>
[A]	78 (40.6)	38 (25.0)			<b>0.49</b>	<b>0.31–0.78</b>
<i>Dominant model</i>						
[GG] + [GA] vs. [AA]	81 (84.4)	73 (96.1)	<b>4.99*</b>	<b>0.03*</b>	<b>4.51</b>	<b>1.25–16.20</b>
	15 (15.6)	3 (3.9)			<b>0.22</b>	<b>0.06–0.80</b>
<i>Recessive model</i>						
[AA] + [GA] vs. [GG]	63 (63.6)	35 (46.1)	<b>6.63</b>	<b>0.01</b>	<b>0.45</b>	<b>0.24–0.83</b>
	33 (34.4)	41 (53.9)			<b>2.24</b>	<b>1.21–4.15</b>
Jo31 ( <i>CTLA-4</i> )(g.*10223G>T, rs11571302)						
<i>Genotype<sup>f</sup></i>						
[GG]	28 (29.2)	35 (46.1)			<i>Referent</i>	
[GT]	47 (49.0)	33 (43.4)	2.89	0.09	0.56	0.29–1.10
[TT]	21 (21.9)	8 (10.5)	<b>6.24</b>	<b>0.01</b>	<b>0.30</b>	<b>0.12–0.79</b>
<i>Allele</i>						
[G]	103 (53.6)	103 (67.8)	<b>7.04</b>	<b>0.008</b>	<b>1.82</b>	<b>1.17–2.83</b>
[T]	89 (46.4)	49 (32.2)			<b>0.55</b>	<b>0.35–0.86</b>
<i>Dominant model</i>						
[GG] + [GT] vs. [TT]	75 (78.1)	68 (89.5)	<b>3.90</b>	<b>0.05</b>	<b>2.38</b>	<b>0.99–3.73</b>
	21 (21.9)	8 (10.5)			<b>0.42</b>	<b>0.17–1.01</b>
<i>Recessive model</i>						
[TT] + [GT] vs. [GG]	<b>68 (70.8)</b>	<b>41 (53.9)</b>	<b>5.21</b>	<b>0.02</b>	<b>0.48</b>	<b>0.26–0.91</b>
	<b>28 (29.2)</b>	<b>35 (46.1)</b>			<b>2.07</b>	<b>1.10–3.89</b>
<i>CTLA-4</i> g.*642AT(8_33)						
		Whole genotype		<sup>c</sup>	$\chi^2 = 7.52$ , df = 5, <i>p</i> = 0.19	
		Whole allele		<sup>d</sup>	$\chi^2 = 4.86$ , df = 2, <i>p</i> = 0.09	
CT60 ( <i>CTLA-4</i> g.*6230G>A, rs3087243)						
		Whole genotype		<sup>e</sup>	$\chi^2 = 9.87$ , df = 2, <i>p</i> = <b>0.007</b>	
Jo31 ( <i>CTLA-4</i> g.*10223G>T, rs11571302)						
		Whole genotype		<sup>f</sup>	$\chi^2 = 6.82$ , df = 2, <i>p</i> = <b>0.03</b>	

Bold indicates statistical significant association

<sup>^</sup>*p*-value after Bonferroni correction; \**p*-value after Yate’s correction; <sup>~</sup>global *p*-value for genotype



**Table 5** *CTLA-4g.319C>T* (rs5742909), *CTLA-4g.\*642AT(8\_32)*, CT60 (rs3087243, *CTLA-4g.\*6230G>A*) genotype and allele frequencies in GD patients stratified by occurrence of GO and in control group

	Control group I n (%)	GD patients without GO II n (%)	GD patients with GO III n (%)	<i>p</i> <sub>global</sub> <sup>#</sup>	$\chi^2$	<i>p</i>	Odds ratio	95 % confidence interval	
<i>CTLA-4g.319C&gt;T</i> (rs5742909)									
<i>Genotype</i>									
[CC]	309 (80.3)	23 (82.1)	105 (72.9)		<i>Referent</i>				
[CT]	74 (19.2)	4 (14.3)	36 (25.0)		I:II	0.89	0.35	0.65	0.26–1.60
					I:III	2.40	0.12	1.43	0.91–2.23
					II:III	0.91*	0.34*	1.97	0.64–6.09
[TT]	2 (0.5)	1 (3.6)	3 (2.1)		I:II	0.60*	0.44	8.13	0.71–93.74
					I:III	1.55*	0.21*	4.41	0.73–26.78
					II:III	0.09*	0.76*	0.66	0.07–6.61
<i>Allele</i>									
[C]	692 (89.9)	50 (89.3)	246 (85.4)		I:II	2.28	0.13	0.56	0.27–1.20
					I:III	<b>4.13</b>	<b>0.04</b>	<b>0.66</b>	<b>0.44–0.99</b>
					II:III	0.58	0.44	0.70	0.28–1.74
[T]	78 (10.1)	6 (10.7)	42 (14.6)		I:II	2.28	0.13	1.77	0.84–3.77
					I:III	<b>4.13</b>	<b>0.04</b>	<b>1.51</b>	<b>1.01–2.27</b>
					II:III	0.58	0.44	1.42	0.57–3.53
<i>Dominant model</i>									
[CC] + [CT] vs. [TT]	383 (99.5)	27 (96.4)	141 (97.9)		I:II	0.47*	0.49*	0.14	0.01–1.61
					I:III	1.32*	0.25*	0.25	0.04–1.48
					II:III	0.04*	0.84*	1.74	0.17–17.37
	2 (0.5)	1 (3.6)	3 (2.1)		I:II	0.47*	0.49*	7.09	0.62–80.72
					I:III	1.32*	0.25*	4.07	0.67–24.64
					II:III	0.04*	0.84*	0.57	0.06–5.73
<i>Recessive model</i>									
[TT] + [CT] vs. [CC]	76 (19.7)	5 (17.9)	39 (24.8)		I:II	0.00*	1.00*	0.88	0.33–2.40
					I:III	3.32*	0.07*	0.66	0.42–1.03
					II:III	0.62*	0.43*	1.71	0.61–4.81
	309 (80.3)	23 (82.1)	105 (72.9)		I:II	0.00*	1.00*	1.13	0.42–3.07
					I:III	3.32*	0.07*	1.51	0.97–2.36
					II:III	0.62*	0.43*	0.59	0.21–1.65
<i>CTLA-4g.*642AT(8_33)</i>									
<i>Genotype<sup>c</sup></i>									
[AT <sub>&lt;16</sub> ]/[AT <sub>&lt;16</sub> ]	99 (25.4)	4 (14.3)	27 (18.8)		<i>Referent</i>				
[AT <sub>&lt;16</sub> ]/[AT <sub>16–21</sub> ]	104 (26.7)	12 (42.9)	40 (27.8)		I:II	2.48*	0.12*	2.86	0.89–9.15
					I:III	1.45	0.23	1.41	0.81–2.47
					II:III	0.72*	0.40*	0.49	0.14–1.69
[AT <sub>&lt;16</sub> ]/[AT <sub>&gt;21</sub> ]	64 (16.5)	2 (7.1)	20 (13.9)		I:II	0.02*	1.00*	0.77	0.14–4.35
					I:III	0.16	0.69	1.15	0.59–2.21
					II:III	0.00*	1.00*	1.48	0.25–8.90
[AT <sub>16–21</sub> ]/[AT <sub>16–21</sub> ]	50 (12.9)	2 (7.1)	33 (22.9)		I:II	0.19*	0.67*	0.99	0.18–5.59
					I:III	<b>8.22</b>	<b>0.004</b>	<b>2.42</b>	<b>1.31–4.46</b>
					II:III	0.34*	0.56*	2.44	0.42–14.38
[AT <sub>16–21</sub> ]/[AT <sub>&gt;21</sub> ]	54 (13.9)	6 (21.4)	24 (16.7)		I:II	1.52*	0.22*	2.75	0.74–10.17
					I:III	2.24	0.13	1.63	0.86–3.10
					II:III	0.16*	0.69*	0.59	0.15–2.35
[AT <sub>&gt;21</sub> ]/[AT <sub>&gt;21</sub> ]	18 (4.6)	2 (7.1)	0 (0.0)		I:II	0.35*	0.55*	2.75	0.47–16.15
					I:III	3.45*	0.06*	–	–
					II:III	<b>4.62*</b>	<b>0.03*</b>	–	–
<i>Allele<sup>d</sup></i>									
[AT <sub>&lt;16</sub> ]	366 (47.0)	22 (39.3)	114 (39.6)	ns	<i>Referent</i>				
[AT <sub>16–21</sub> ]	258 (33.2)	22 (39.3)	130 (45.1)		I:II	1.26	0.26	1.42	0.77–2.62
					I:III	<b>10.10</b>	<b>0.001</b>	<b>1.62</b>	<b>1.20–2.18</b>
					II:III	0.16	0.69	1.14	0.60–2.17
[AT <sub>&gt;21</sub> ]	154 (19.8)	12 (21.4)	44 (15.3)		I:II	0.49	0.48	1.30	0.63–2.69
					I:III	0.18	0.67	0.92	0.62–1.36
					II:III	0.75	0.39	0.71	0.32–1.55

**Table 5** continued

	Control group I n (%)	GD patients without GO II n (%)	GD patients with GO III n (%)	$p_{\text{global}}^{\#}$	$\chi^2$	$p$	Odds ratio	95 % confidence interval
<i>Dominant model</i>								
[AT <sub>&lt;16</sub> ]/[AT <sub>16-21</sub> ] + [AT <sub>&lt;16</sub> ]/[AT <sub>&gt;21</sub> ] + [AT <sub>16-21</sub> ]/[AT <sub>16-21</sub> ] vs. others	203 (53.6)	20 (71.4)	97 (67.4)		I:II 3.36	0.07	2.17	0.93–5.04
					I:III <b>8.12</b>	<b>0.004</b>	<b>1.79</b>	<b>1.20–2.68</b>
					II:III 0.18	0.67	0.83	0.34–2.01
	176 (46.4)	8 (28.6)	47 (32.6)		I:II 3.36	0.07	0.46	0.20–1.07
					I:III <b>8.12</b>	<b>0.004</b>	<b>0.56</b>	<b>0.37–0.84</b>
					II:III 0.18	0.67	1.21	0.50–2.95
CT60 ( <i>CTLA-4</i> )(g.*6230G>A, rs3087243)								
<i>Genotype<sup>e</sup></i>								
[GG]	133 (34.3)	9 (32.1)	65 (45.1)		<i>Referent</i>			
[GA]	187 (48.2)	17 (60.7)	63 (43.8)		I:II 0.48	0.49	1.34	0.58–3.11
					I:III 2.79	0.09	0.69	0.46–1.04
					II:III 2.23	0.13	0.51	0.21–1.24
[AA]	68 (17.5)	2 (7.1)	16 (11.1)		I:II 0.56*	0.46*	0.43	0.09–2.07
					I:III <b>5.47</b>	<b>0.02</b>	<b>0.48</b>	<b>0.26–0.90</b>
					II:III 0.08*	0.78*	1.11	0.22–5.64
<i>Allele</i>								
[G]	453 (58.4)	35 (62.5)	193 (67.0)		I:II 0.37	0.55	1.19	0.68–2.08
					I:III <b>6.57</b>	<b>0.01</b>	<b>1.45</b>	<b>1.09–1.93</b>
					II:III 0.43	0.51	1.22	0.67–2.21
[A]	323 (41.6)	21 (37.5)	95 (33.0)		I:II 0.37	0.55	0.84	0.48–1.47
					I:III <b>6.57</b>	<b>0.01</b>	<b>0.69</b>	<b>0.52–0.92</b>
					II:III 0.43	0.51	0.82	0.45–1.49
<i>Dominant model</i>								
[GG] + [GA] vs. [AA]	320 (82.5)	26 (92.9)	128 (88.9)		I:II 1.34*	0.25*	2.76	0.64–11.92
					I:III 3.25	0.07	1.70	0.95–3.04
					II:III 0.08*	0.77*	0.62	0.13–2.84
	68 (17.5)	2 (7.1)	16 (11.1)		I:II 1.34*	0.25*	0.36	0.08–1.56
					I:III 3.25	0.07	0.59	0.33–1.05
					II:III 0.08*	0.77*	1.63	0.35–7.50
<i>Recessive model</i>								
[AA] + [GA] vs. [GG]	255 (65.7)	19 (67.9)	79 (54.9)		I:II 0.06	0.80	1.11	0.49–2.52
					I:III <b>5.14</b>	<b>0.02</b>	<b>0.64</b>	<b>0.43–0.94</b>
					II:III 1.62	0.20	0.58	0.24–1.36
	134 (34.4)	9 (32.1)	65 (45.1)		I:II 0.06	0.80	0.90	0.40–2.05
					I:III <b>5.14</b>	<b>0.02</b>	<b>1.57</b>	<b>1.06–2.31</b>
					II:III 1.62	0.20	1.74	0.74–4.10

Bold indicates statistical significant association

*CTLA-4*g.319C>T (rs5742909): GD patients without GO vs. control group:  $\chi^2 = 3.70$ ,  $df = 2$ ,  $p = 0.16$ ; GD patients with GO vs. control group:  $\chi^2 = 5.12$ ,  $df = 2$ ,  $p = 0.08$ ; GD patients without GO vs. GD patients with GO:  $\chi^2 = 1.65$ ,  $df = 2$ ,  $p = 0.44$

*CTLA-4*g.\*642AT(8\_32): Genotype: GD patients without GO vs. control group:  $\chi^2 = 7.25$ ,  $df = 5$ ,  $p = 0.20$ ; GD patients with GO vs. control group:  $\chi^2 = 16.53$ ,  $df = 5$ ,  $p = 0.006$ ; GD patients without GO vs. GD patients with GO:  $\chi^2 = 16.31$ ,  $df = 5$ ,  $p = 0.006$

*CTLA-4*g.\*642AT(8\_32): Allele: GD patients without GO vs. control group:  $\chi^2 = 1.33$ ,  $df = 2$ ,  $p = 0.51$ ; GD patients with GO vs. control group:  $\chi^2 = 13.19$ ,  $df = 2$ ,  $p = 0.001$ ; GD patients without GO vs. GD patients with GO:  $\chi^2 = 1.45$ ,  $df = 2$ ,  $p = 0.48$

CT60 (*CTLA-4*g.\*6230G>A, rs3087243): GD patients without GO vs. control group:  $\chi^2 = 2.54$ ,  $df = 2$ ,  $p = 0.28$ ; GD patients with GO vs. control group:  $\chi^2 = 6.51$ ,  $df = 2$ ,  $p = 0.04$ ; GD patients without GO vs. GD patients with GO:  $\chi^2 = 2.72$ ,  $df = 2$ ,  $p = 0.26$

OR odds ratio, 95 % CI 95 % confidence intervals

<sup>^</sup> $p$ -value after Bonferroni correction; \* $p$ -value after Yate’s correction

allele was associated with active GO ( $p = 0.002$ , OR = 2.08, 95 % CI: 1.29–3.37;  $p = 0.04$ , OR = 2.05, 95 % CI: 1.04–4.40). A more evident association was observed

when the presence of this allele was considered ( $p = 0.002$ , OR = 2.35, 95 % CI: 1.36–4.05;  $p = 0.01$ , OR = 2.67, 95 % CI: 1.24–5.76) (Supplementary Table 12).

Albeit the *CTLA-4c.49A>G* (rs231775) and Jo31 (rs11571302) SNPs were not related to GO development, they were associated with the GO outcome (Supplementary Table 12). The *CTLA-4c.49A>* [G] allele favored severe disease outcome ( $p=0.04$ , OR = 1.63, 95 % CI: 1.01–2.61), which was confirmed when the recessive model ([GG] + [AG] vs. [AA]) was adopted ( $p=0.03$ , OR = 1.89, 95 % CI: 1.06–3.36;  $p=0.007$ , OR = 2.70, 95 % CI: 1.30–5.61) (Supplementary Table 12).

When GO is present, carriers of Jo31 (rs11571302) [TT] genotype were protected against severe eye symptoms ( $p=0.04$ , OR = 0.37, 95 % CI: 0.14–0.96), which was confirmed in a dominant model ([GG] + [GT] vs. [TT]) ( $p=0.04$ , OR = 0.61, 95 % CI: 0.38–0.98;  $p=0.05$ , OR = 0.43, 95 % CI: 0.18–1.02) (Supplementary Table 12).

#### The course of GO—haplotype associations

A statistically significant difference in haplotype distribution between GD patients regarding GO occurrence and healthy controls was observed (Supplemental Table 13). When severity and activity status were taken into account, different global haplotypes distribution was seen in case of absence of GO symptoms compared to severity and activity status, but they did not reach statistical significance (in both  $p_{\text{corrected}}=0.06$ ) (Supplemental Table 14). Haplotype TCG [AT<sub>16–21</sub>]GG(m) favors not only direct GO development, but also the severe and active GO course ( $p=0.015201$ ;  $p=0.015448$ ;  $p=0.015266$ ) (Supplementary Tables 13 and 14).

Heavy outcome of GO were also promoted by TCG [AT<sub>16–21</sub>]GG(m), TCA[AT<sub><16</sub>]GT(m), and TCA[AT<sub>>21</sub>]AT(m) haplotypes (Supplemental Table 14), while haplotypes CCG[AT<sub>16–21</sub>]GG(m), TCG[AT<sub>>21</sub>]GG(m), TCG[AT<sub><16</sub>]GG(l), TCA[AT<sub><16</sub>]GG(m), TCA[AT<sub><16</sub>]GT(l), and TCA[AT<sub>16–21</sub>]GG(m) were linked to non-severe mild GO outcome. TCA[AT<sub><16</sub>]AT(l) haplotype protected against an active course of eye changes (Supplementary Table 14).

In contrast, haplotypes TCA[AT<sub>>21</sub>]GG(m) and TCG [AT<sub><16</sub>]GG(m) directly favored light GO outcome without clinical eye symptoms ( $p=0.000174$ ;  $p=0.015662$ ) (Supplementary Table 13), which were also confirmed within GO stratified according to activity and severity ( $p=0.038863$ ;  $p=0.030938$ ;  $p=0.012530$ ) (Supplementary Table 14).

Haplotype TCG[AT<sub>>21</sub>]GG(m) was statistically significantly more frequent in healthy persons ( $p=0.003529$ ;  $p=0.029963$ ) (Supplementary Tables 13 and 14).

#### The course of GO—multivariate associations

The unconditional multivariate logistic regression analysis of all studied polymorphisms, including four prone genetic

parameters: presence of [G] allele at *CTLA-4c.49A>G* (rs231775) (genotype [GG] and [AG]), presence of [G] allele at CT60 (rs3087243) (genotype [GG] and [GA]), presence of *CTLA-4g.\*642AT(8\_33)[AT<sub>16–21</sub>]* allele, Jo31 (rs11571302) [GG] genotype and environmental factors: smoking and male gender, showed that presence of *CTLA-4g.\*642AT(8\_33)[AT<sub>16–21</sub>]* allele ( $p=0.04$ , OR = 2.10, 95 % CI: 1.03–4.27) and male gender ( $p=0.00008$ , OR = 4.29, 95 % CI: 2.11–8.72) were independent risk factors for GO ( $\chi^2_2=24.1$ ,  $p=0.00001$ ).

## Discussion

Since a number of studies have established a central role for T cells in GD [2, 5–7], the region containing genes encoding the immune regulatory molecules, CTLA-4, CD28, and ICOS, is good candidate for disease risk factor. Selected for our study polymorphic sites located within genes encoded, these co-stimulatory molecules had to have been reported to be associated with altered immune response and/or susceptibility to autoimmune disease [9, 13, 18–22, 24–26]. Based on the fact that all three genes are close to one another within the chromosomal region 2q33, and that LD was observed between polymorphisms located within these genes, we postulated that the association between GD (and/or its clinical phenotype) and this region might represent the effects of a combination of polymorphic variations of these three genes. We therefore looked for a possible association of studied polymorphisms as one susceptible genetic region defined as a haplotype block with the GD phenotype.

Our study indicated specific haplotypes significantly linked with sporadic GD and haplotypes over-represented in patients with a familial history of thyroid disease. These results led us to the conclusion that patients with sporadic incidence and those with a familial background of GD are characterized by a combination of various markers, and it is therefore possible that they are genetically different. These markers may be predictive factors for the development of GD in families in whom thyroid disease is observed. Although the 2q33 chromosomal region is linked to GD, the association between the two was found to be different in female and male subjects. Our group of patients was too small for stratification by gender at the haplotype level in the context of GO outcome, therefore the possibility that differences in our overall population at the univariate level will also be true in GO subgroups cannot be excluded. To date, no literature concerning such a relationship has been published. In terms of predicting the clinical outcome, two specific haplotypes are possible: one reducing (TTA[AT<sub>16–21</sub>]GT(l)) and another (TCA[AT<sub><16</sub>]AT(m)) increasing the successful outcome of anti-thyroid treatment.

The roles of genetic factors in the GO pathomechanism are not well characterized. Although a genetic predisposition to GD has been confirmed, to increase one's risk of developing the disease, the significance of genotype–phenotype correlations in GD remains speculative, and the results of family-based studies are conflicting. So far—and only at a univariate level—polymorphisms located in *CTLA-4* have been linked to GO risk and outcome predictors. Therefore, we tested the combined impact of the 2q33 region and GO course, and observed statistically significantly different haplotype associations in GO status. In our search for deeper insight, specifically GO course (severity and activity), the differences were more evident. Particularly, one specific haplotype TCG[AT<sub>16–21</sub>]GG(m) favored not only the development of GO but also a severe outcome. The haplotypes TCA[AT<sub>>21</sub>]GG(m) and TCG[AT<sub><16</sub>]GG(m), on the other hand, were directly linked to GD without any eye symptoms. As this study is the first association analysis based on the aforementioned concept, our observations require confirmation by future studies. It must be noted that we were able to collect a relatively large cohort of patients with severe eye symptoms and compare them with subjects with the milder form of GO.

When we used the classical approach—a univariate association model—the association with GD in our southern Polish population was seen only in case of two polymorphisms located within the *CTLA-4* 3'UTR region: CT60 (rs3087243) and g.\*642AT(8\_33). When we looked deeper, taking ancestral background into account, three *CTLA-4* polymorphic variants (g.319C>T (rs5742909) [CC], c.49A>G (rs231775) [G], g.\*642AT(8\_33)[AT<sub>>21</sub>]/[AT<sub>>21</sub>]) were associated with the family burden of GD. This finding may provide some evidence in support of our hypothesis; specifically that the superiority of the association defined as one susceptible *locus* expressed as the haplotype over the individual polymorphism association. As compared to other studies, which describe only univariate analyses [28, 29, 30, 31], our research takes a deeper and more detailed approach. The most GD-essential genetic marker in our study, *CTLA-4*g.\*642AT(8\_33), was also relevant in other populations [2, 18, 25]. Importantly, this marker has functional implications; specifically the long AT-repeat allele is associated with reduced control of T-cell proliferation and thus contributes to the pathogenesis of GD [25].

Other Polish studies [29, 30, 31] focused mostly on *CTLA-4*c.49A>G (rs231775) and revealed a significant association of this marker with disease; however, we did not. On the other hand, the association of the marker CT60 (rs3087243) observed in our group was not as significant as in another study of Polish patients with GD [29]. Our observation of a significant association of the CT60

(rs3087243) [G] allele aligns with the results of other reports [9, 18, 28], including a large meta-analysis where this polymorphic site strongly increased GD risk [28].

Since the goal of anti-thyroid drug therapy (to restore an euthyroid state) may be achieved in up to 50% of GD patients, defining the genetic predictors of remission could be helpful in clinical practice; therefore, we tested studied markers to forecast the clinical outcome. At a univariate level, CT60 (rs3087243) [AA] and Jo31 (rs11571302) [TT] homozygosity significantly increased the success of anti-thyroid treatment, but multivariate logistic regression analysis only pointed CT60 (rs3087243) as an independent risk factor for disease progression.

Previous studies focusing on the *CTLA-4*c.49A>G (rs231775) polymorphism [32, 33, 34, 35] showed its association with a lower chance of remission after discontinuation of propylthiouracil treatment [32, 33, 34], indicating that GD patients with the *CTLA-4*c.49A>G (rs231775) [GG] genotype may not be the best candidates for anti-thyroid drug therapy. Moreover, GD patients with the [G] allele at this SNP needed to continue anti-thyroid drug treatment for longer periods to achieve remission, indicating that this polymorphic variant may be an indicator of poor prognosis [33, 34]. In contrast, but in line with our results, Kim et al. [35] did not observe such a relationship when it came to *CTLA-4*g.319C>T (rs5742909) and *CTLA-4*c.49A>G (rs231775). So far, no research has assessed the impact of *CD28* and *ICOS* variation on the success of medical treatment, and only variation within *CTLA-4* has been documented to be a prognostic factor for remission of disease after treatment. The observed association is probably due to the functional consequence of studied polymorphic markers.

We found an association between *CTLA-4* polymorphic variations and the development and course of GO on a univariate level. If severity of GO was considered an outcome, associations were observed in the case of markers for *CTLA-4*c.49A>G (rs231775), *CTLA-4*g.\*642AT(8\_33), CT60 (rs3087243), and Jo31 (rs11571302). When looking for a combined effect (genetics and environment), *CTLA-4*g.\*642AT(8\_33) and male gender were found to be independent risk factors for GO. *CTLA-4*c.49A>G (rs231775) was linked to GO course but not development. A large meta-analysis [37] of inconsistent results of studies, focusing on the association between *CTLA-4* gene polymorphisms and GO, revealed no evidence in support of an association between this SNP and eye changes.

Moreover, in the one-marker association analysis no relationship between studied polymorphisms within *CD28* and *ICOS* and GD or the course of the disease was observed in the overall group association analysis. Our results confirmed previous data showing a lack of association between these polymorphic sites and GD [9, 27]. The absence of a

relationship between the *CD28c.17+3T>C* (rs3116496) SNP and the disease was evidenced by the lack of impact on thyroid antibody production [35]; however, stratification by gender showed that females with long alleles at the *ICOSc.1554+4GT(8\_15)* polymorphic site were more prone to GD. This is the first gender-stratified analysis; therefore, corroboration by future studies is necessary.

We, and others, have provided evidence to support the idea that, in a single gene polymorphic variation, changes within *CTLA-4* may be considered to be GD risk factors and factors capable of modifying the clinical course of the disease. We also postulated that because of the location—in one common chromosomal region—and LD, the analyzed *CD28/CTLA-4/ICOS* gene polymorphic variability should be considered at the haplotype level, rather than by the single-marker model. Moreover, since GD and GO are associated with gender and familial autoimmune thyroid history, these factors should also be taken into account when a patient's genetic background is analyzed.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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