CLINICAL STUDY

Genotypes in relation to phenotypic appearance and exposure to environmental factors in Graves' hyperthyroidism

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Abstract

Background: Genetic polymorphisms and environmental factors are both involved in the pathogenesis of Graves' disease, but their interaction and effect on Graves' phenotypes have scarcely been investigated.

Objective: To test the hypothesis that subjects with susceptibility genotypes develop more severe Graves' hyperthyroidism at a younger age and after less exposure to environmental factors, with attention to gender differences.

Study design: A prospective observational multicenter study in 205 adult Caucasian patients with untreated first episode of Graves' hyperthyroidism.

Methods: Evaluation of genotypes (HLA DRB1*03, DQA1*05, DQB1*02; CTLA4 49A/G, CT60 A/G; PTPN22 C/T) in relation to phenotypes (age, sex, severity (clinical, biochemical, and immunological)) of hyperthyroidism and environmental factors (smoking, stress questionnaires).

Results: G-alleles in CTLA4 single nucleotide polymorphisms were dose-dependently associated with younger age at the time of diagnosis and less exposure to daily hassles. In gender-specific analysis, this association is enhanced in men and attenuated in women. Males (but not females) in HLA linkage disequilibrium had more severe (biochemical and immunological) hyperthyroidism and a tendency to younger age at diagnosis, compared with those not in linkage disequilibrium.

Conclusion: Graves' hyperthyroidism occurs at a younger age with less exposure to environmental factors in subjects carrying susceptibility genotypes. The impact of genotypes seems to be greater in males than in females.

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Introduction

Graves' hyperthyroidism can be considered as a complex multifactorial disease, in which autoimmunity against thyroid antigens develops against a particular genetic background provoked by environmental factors (1, 2). Good quantitative evidence that genetic factors play an important role in the etiology of Graves' hyperthyroidism is derived from twin studies, showing significantly higher probandwise concordance rates in monozygotic twins than in dizygotic twins (3, 4, 5). Using structural equation modeling, the heritability of Graves' disease has been reported to be 79% (95% CI, 38–90%) (6). It means the relative impact of genetic factors is around 75%; consequently, the relative contribution of environmental factors would be around 25%.

Genes shown to confer susceptibility to Graves' disease can be divided into thyroid-specific genes (*TSHR*, *TG*) and immune regulatory genes (*HLA*, *CTLA4*, *PTPN22*, *CD40*, *FOXP3*, *IL2RA*, *FCRL3*).

Known environmental factors that may induce Graves' disease are smoking, stress, iodine intake, pregnancy, and drugs such as interferon and highly active anti-retroviral therapy.

So far, genetic and environmental factors contributing to Graves' disease have been studied mostly separately from each other. The aims of the present study in patients with newly diagnosed Graves' hyperthyroidism were therefore as follows. First, we evaluated whether specific genotypes are associated with differences in the clinical presentation of Graves' hyperthyroidism. We hypothesized that subjects with genetic susceptibility to Graves' disease might be younger at the time of diagnosis and presenting with more severe disease. Second, we evaluated the relationship between specific genotypes and exposure to environmental factors. We hypothesized that subjects with genetic susceptibility to Graves' disease might require less exposure to environmental factors for contracting Graves' hyperthyroidism. Special attention was given to possible differences in gender; in view of the

strong female preponderance in autoimmune thyroid disease, the development of Graves' disease in males might require a much heavier load of either susceptibility genes or exposure to environmental factors.

Both hypotheses were tested in a prospective observational study of consecutive patients with untreated first episode of Graves' hyperthyroidism. Genotypes and phenotypes were assessed as well as exposure to a number of environmental factors.

Materials and methods

Patients

We included consecutive untreated patients with a first episode of Graves' hyperthyroidism in a prospective, multicenter, observational study. Patients were included from one university and eight city hospitals in The Netherlands. Inclusion criteria were: biochemical hyperthyroidism (TSH <0.4 mU/l; thyroxine (free T₄ (fT₄)) >23 pmol/l; and/or triiodothyronine (T₃) >2.7 nmol/l) and a diffuse homogeneous uptake on thyroid scintigraphy (^{99m}Tc-pertechnetate). Exclusion criteria were: relapse of Graves' disease; no written informed consent; no understanding of the Dutch language; and alcohol or drug abuse. As described previously, 263 patients were assembled satisfying the inclusion and exclusion criteria (7). For the present study, we excluded 51 patients because of non-Caucasian origin and seven patients because no DNA samples were obtained, leaving a study population of 205 patients.

Clinical parameters were recorded including goiter size (8), the existence of Graves' orbitopathy and pretibial myxedema, and the Hyperthyroid Symptom Scale (HSS) (9). Participants were asked to complete questionnaires asking about smoking habits and stress at the time of diagnosis as published previously (7, 10). Smoking habits were assessed as current smokers, ex-smokers, and never smokers. The Dutch questionnaire on recently experienced stressful life events counts the total number of major life events experienced in the last 12 months (checklist of 60 possible events); the amount of pleasantness and unpleasantness with each experienced life event is rated on a scale of 0 (meaning no (un)pleasantness) to 4 (meaning a huge amount of (un)pleasantness), and the total amount of pleasantness and unpleasantness is calculated (maximum score 240 for each) (11). The Dutch Everyday Problem Checklist is a validated version of the Daily Hassles Scale, and consists of 114 items concerning daily hassles in the last 2 months; it also measures the intensity of each hassle on a scale from 0 to 3, yielding the total intensity of daily hassles (maximum score 342) (12, 13). The Positive and Negative Affect Schedule (PANAS) measures the current mood in terms of positive and negative affect; it consists of 22 mood states (11 positive, 11 negative) rated on a scale from 1 (not at all) to 5

(a lot), yielding the tendency to report positive and negative feelings both on a scale from 11 to 55 (14). The study was approved by the Institutional Review Board of the Academic Medical Center of Amsterdam and the eight other participating centers. All patients gave written informed consent.

Laboratory measurements

Non-fasting venous blood samples were taken for thyroid function tests and thyroid autoantibody measurements before treatment was started; storage was at -20 °C until assay. Serum T₃ and T₄ were measured with in-house RIAs (15). T₃ uptake was determined by a no-extraction, solid-phase ¹²⁵I-RIA (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). Free T₄ index and free T₃ index were calculated by multiplying T₃ uptake with T₄ and T₃ respectively (reference ranges 70–140 and 3.3–8.2 respectively). Serum TSH was determined with a fluoroimmunoassay (Delfia hTSH; Perkin Elmer, Turku, Finland; detection limit 0.01 mU/l, reference range 0.4–4.0 mU/l). Serum TSH-binding inhibitory immunoglobulin (TBII) was determined by a second-generation luminescence receptor assay (TRAK human LIA; B.R.A.H.M.S., Berlin, Germany; detection limit 1.0 IU/l, reference range <2.0 IU/l). Autoantibodies against thyroid peroxidase (TPO-Ab) were analyzed by anti-TPO LIA (B.R.A.H.M.S.; detection limit 30 kU/l, reference range <60 kU/l). All measurements were performed at the laboratory of the Academic Medical Center of Amsterdam.

Genotyping

Genomic DNA was isolated from peripheral blood leukocytes using the MagNA Pure apparatus and the MagNA Pure LC DNA Isolation Kit (Roche Biochemicals) according to the manufacturer's protocol. Genomic amplifications were obtained by the PCR using the TGradient Thermocycler (Biometra, Göttingen, Germany). CTLA4 49A/G (rs231775) and CTLA4 CT60 (rs3087243) single nucleotide polymorphisms (SNPs) were detected using primers as described before (16). The SNP in PTPN22 (rs2476601) was detected using previously described primers (17). The PCR was performed in a volume of 50 µl containing 1.5 mM MgCl₂, 0.5 mM dNTPs, 50 ng genomic DNA, 0.2 µM of each primer, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The PCR products were separated by a 2% agarose gel electrophoresis and identified by ethidium bromide staining. The same primers were used for PCR amplification and sequencing. Cyclic sequencing reactions were performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), and the sequencing products purified using the Agencourt CleanSEQ Kit (GC Biotech, Schiedam, The Netherlands).

Sequencing was performed using the Hitachi 3730 DNA Analyzer (Applied Biosystems). Sequence analysis was done using Sequencing Analysis 5.1 and CodonCode Aligner 5.2.1 programs.

HLA subtypes (DQB1*02, DQA1*05, DRB1*03) were identified on a LightCycler 480 (Roche Molecular Diagnostics) using the LightCycler 480 High Resolution Melting Master (Roche Molecular Diagnostics) according to the manufacturer's protocol. Primer sequences were as described before for DQB1*02 (18), DQA1*05 (19), and DRB1*03 (18, 20).

Statistical analysis

χ^2 tests, *t*-tests, and non-parametric tests were used for comparison of proportions, means, and medians between HLA-positive and -negative patients and PTPN22 heterozygote polymorphism and wild type. The same tests were used for comparison between A-alleles and G-alleles in male and female patients for the CTLA4 49A/G and CTLA4 CT60 polymorphisms. Trend analysis between the allele frequencies of the CTLA4 49A/G and CTLA4 CT60 polymorphisms for age, serum TBII, serum TPO-Ab, and different stress scores was performed using the Jonckheere–Terpstra test. A comparison of serum free T₃ index and free T₄ index and a comparison of proportions between these groups was done using linear regression analysis and χ^2 tests respectively. *P* values <0.05 indicated statistical significance. Statistical analyses were carried out using the SPSS statistical package for Windows, version 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

Characteristics of the study population

The mean age of the 205 patients was 43.5 years (range 16.4–80.0 years). There were 55 males and 150 females. Free T₃ indices were 7.0 ± 3.8 and free T₄ indices were 333 ± 128 (mean $\pm$ s.d.), serum TBII concentrations were 8.9 (5.0–18.6 IU/l), and TPO-Ab were 420 (40–2610 kU/l) (median values with interquartile ranges). Goiter size was grade 0 in 67%, grade I in 18%, grade II in 11%, and grade III in 4% of patients. Graves' orbitopathy was present in 20% and pretibial myxedema in 5%. The hyperthyroid symptom score was 15.1 ± 7.1 (mean $\pm$ s.d.). None of the patients was pregnant, but 67% of women had been pregnant in the past. Oral contraceptives were used by 27%. There were 38% current smokers, 29% ex-smokers, and 33% never smokers. The prevalence of particular genotypes was as follows: HLA DRB1*03, 63%; HLA DQA1*05, 73%; HLA DQB1*02, 61.5%; CTLA4 49A/G, heterozygotes 49.5%, homozygotes 13%; CTLA4 CT60, heterozygotes 51%, homozygotes 32.5%; PTPN22 C/T, heterozygotes 28%, homozygotes 0%.

Table 1 HLA polymorphisms DRB1*03, DQA1*05, and DQB1*02 in relation to sex, age, and disease severity in Caucasian patients with untreated first episode of Graves' hyperthyroidism. Values for age, TBII, and TPO-Ab are in median $\pm$ interquartile range (range between the 25th and 75th percentiles); values for free T₃ index and free T₄ index are given in mean $\pm$ s.d.

	DRB1*03 positive (n=129)	DRB1*03 negative (n=76)	<i>P</i> value	DQA1*05 positive (n=149)	DQA1*05 negative (n=56)	<i>P</i> value	DQB1*02 positive (n=126)	DQB1*02 negative (n=79)	<i>P</i> value	HLA-LD (n=105)	No HLA-LD (n=100)	<i>P</i> value
Male gender	24.0%	25.0%	0.88	25.5%	21.4%	0.54	26.2%	21.5%	0.45	26.7%	22.0%	0.44
Age (years)	43.3 (32.4–53.3)	44.5 (34.5–53.2)	0.66	43.9 (32.4–53.5)	42.3 (34.1–52.1)	0.85	42.7 (31.5–53.0)	44.5 (34.8–54.3)	0.39	42.7 (31.5–53.0)	44.5 (34.8–54.3)	0.64
Free T ₃ index	7.2 (4.1)	6.5 (3.0)	0.28	7.2 (4.1)	6.4 (2.8)	0.21	7.3 (4.2)	6.4 (2.8)	0.13	7.3 (4.3)	6.6 (3.0)	0.23
Free T ₄ index	337 (134)	323 (116)	0.50	337 (133)	320 (114)	0.43	341 (133)	319 (119)	0.26	337 (133)	328 (124)	0.64
TBII (IU/l)	9.5 (5.4–21.6)	7.9 (4.2–12.5)	0.03	8.6 (4.6–19.4)	10.3 (5.5–14.8)	0.73	9.3 (5.3–21.4)	8.0 (4.8–15.8)	0.24	9.4 (5.4–22.3)	8.0 (4.8–14.6)	0.12
TPO-Ab (kU/l)	560 (70–2705)	240 (30–2530)	0.24	420 (40–2560)	450 (42–2902)	0.86	385 (40–2042)	570 (60 to >3000)	0.29	435 (52–2425)	380 (40–2790)	0.84

HLA-LD, HLA linkage disequilibrium.

Table 2 Gender-specific analysis of age at diagnosis, disease severity, and environmental exposure according to the presence of HLA linkage disequilibrium (HLA-LD) in Caucasian patients with untreated first episode of Graves' hyperthyroidism. Values for age, TBII, TPO-Ab, and daily hassles are in median + interquartile range (range between the 25th and 75th percentiles); values for free T₃ index and free T₄ index are given in mean + s.d.

	Male			Female		
	HLA-LD (n=28)	No HLA-LD (n=22)	P value	HLA-LD (n=78)	No HLA-LD (n=77)	P value
Age (years)	41.5 (32.2–55.8)	45.5 (39.0–56.0)	0.38	43.0 (30.0–52.0)	43.5 (31.8–53.0)	0.87
Free T ₃ index	8.9 (6.2)	6.0 (2.2)	0.035	6.7 (3.3)	6.8 (3.2)	0.86
Free T ₄ index	368 (140)	294 (93)	0.051	325 (129)	338 (130)	0.57
TBII (IU/l)	10.0 (6.0–24.2)	8.2 (4.0–17.4)	0.26	9.3 (5.1–21.4)	8.0 (4.7–14.5)	0.29
TPO-Ab (kU/l)	725 (170–2956)	90 (<30 to 1980)	0.02	355 (40–1600)	540 (80–2970)	0.13
Current smokers	35%	57%	0.12	36%	37%	0.87
Daily hassles						
Total number of daily hassles	18 (13–28)	26 (9–35)	0.62	20 (10–32)	20 (11–32)	0.79
Intensity per hassle	1.4 (0.9–1.6)	1.7 (1.3–1.9)	0.02	1.5 (1.2–1.8)	1.6 (1.2–2.0)	0.28
Total intensity of all hassles	27 (11–40)	36 (11–60)	0.35	30 (14–56)	29 (16–58)	0.98

HLA-LD, HLA linkage disequilibrium.

HLA genotypes

The HLA polymorphisms DRB1*03, DQA1*05, and DQB1*02 were not associated with gender, age, biochemical severity (free T₃ and free T₄ indices), or immunological severity (TBII and TPO-Ab), with the single exception of a slightly higher TBII in DRB1*03-positive patients (Table 1). Neither were there associations with the clinical severity of Graves' hyperthyroidism (goiter size, Graves' orbitopathy, pretibial myxedema, hyperthyroid symptom score),

nor with smoking and scores on the three stress questionnaires (data not shown). The HLA genotypes were in strong linkage disequilibrium ($P < 0.01$), but no differences in any parameter were observed between patients with and without HLA linkage disequilibrium (Table 1). When this analysis was done separately for each gender, the outcome was the same in females (no differences between the 78 women with and the 77 women without HLA linkage disequilibrium) but slightly different in males (Table 2). Men with HLA linkage disequilibrium had more severe biochemical

Table 3 Relationship between the CTLA4 49A/G polymorphism and sex, age, disease severity, and environmental exposure in Caucasian patients with untreated first episode of Graves' hyperthyroidism. Values for age, TSH, TBII, TPO-Ab, and stress scores are in median + interquartile range (range between the 25th and 75th percentiles); values for HSS-score, free T₃ index, and free T₄ index are given in mean + s.d.

	A/A wild type (n=76)	A/G heterozygote (n=100)	G/G homozygote (n=25)	P value
Male gender	25%	23%	31%	0.74
Age (years)	47.5 (38.0–57.0)	41.0 (31.0–50.8)	40.5 (29.8–46.2)	<0.01
Free T ₃ index	6.7 (2.7)	7.2 (4.5)	7.2 (3.6)	0.47
Free T ₄ index	331 (124)	331 (134)	344 (131)	0.76
TBII (IU/l)	7.7 (4.5–14.1)	9.9 (5.4–18.8)	10.4 (3.5–24.0)	0.10
TPO-Ab (kU/l)	365 (40–2652)	395 (48–2260)	1310 (105–2903)	0.34
Current smokers	36%	42%	36%	0.71
Stress scores				
PANAS				
Tendency to report negative feelings	22 (17–29)	22 (17–28)	24 (16–28)	0.65
Tendency to report positive feelings	37 (32–41)	37 (33–40)	38 (34–42)	0.36
Daily hassles				
Total number of daily hassles	21 (14–35)	20 (10–30)	15 (6–28)	0.08
Intensity per hassle	1.7 (1.3–2.0)	1.6 (1.2–1.8)	1.4 (0.8–1.7)	<0.01
Total intensity of all hassles	38 (20–60)	28 (14–54)	17 (6–46)	0.02
Recent life events				
Total number of life events	8 (5–14)	9 (5–14)	6 (4–12)	0.44
Number of unpleasant events	5 (3–9)	5 (2–8)	3 (1–6)	0.13
Number of pleasant events	2 (1–5)	2 (1–5)	3 (1–5)	0.80
Amount of total unpleasantness	16 (7–33)	15 (7–28)	11 (4–19)	0.17
Amount of total pleasantness	9 (4–17)	10 (4–17)	8 (4–20)	0.97

Table 4 Gender-specific analysis of age at diagnosis, disease severity, and environmental exposure according to the presence of A- and G-alleles in SNPs CTLA4 49A/G and CT60 A/G in Caucasian patients with untreated first episode of Graves' hyperthyroidism. Values for age, TBII, TPO-Ab, and stress scores are in median + interquartile range (range between the 25th and 75th percentiles); values for free T₃ index and free T₄ index are given in mean + S.D.

	CTLA4-49				CTLA4-60			
	Male		Female		Male		Female	
	A-allele (n=61)	G-allele (n=37)	P value	G-allele (n=113)	A-allele (n=30)	G-allele (n=42)	P value	G-allele (n=149)
Age (years)	48.0 (39.1–57.4)	39.0 (32.2–47.4)	<0.01	45.2 (33.7–53.6)	41.7 (30.8–49.7)	41.2 (33.8–53.9)	0.12	44.6 (34.3–53.3)
Free T ₃ index	7.3 (4.7)	8.4 (5.9)	0.31	6.7 (3.0)	6.8 (3.5)	8.0 (5.7)	0.92	6.9 (3.4)
Free T ₄ index	325 (120)	360 (138)	0.21	334 (130)	330 (130)	356 (130)	0.82	341 (142)
TBII (IU/l)	8.0 (6.0–18.4)	10.1 (4.8–22.4)	0.62	8.5 (4.8–14.8)	10.2 (5.3–21.4)	10.9 (6.3–22.9)	0.65	8.1 (4.6–14.5)
TPO-Ab (kU/l)	260 (40–2660)	780 (95–2860)	0.26	420 (40–2350)	680 (50–2170)	340 (108 to >3000)	0.25	450 (40–2170)
Current smokers	46%	44%	0.85	36%	39%	46%	0.30	41%
Stress scores								
PANAS	20 (16–24)	19 (16–27)	0.93	22 (17–30)	14 (18–29)	20 (16–27)	0.88	22 (18–29)
Tendency to report negative feelings								
Tendency to report positive feelings	36 (32–40)	37 (32–39)	0.98	37 (32–41)	38 (33–42)	35 (30–39)	0.53	37 (32–41)
Daily hassles								
Total number of daily hassles	24 (14–32)	16 (7–26)	0.03	20 (12–33)	21 (9–32)	17 (9–28)	0.21	18 (12–28)
Intensity per hassle	1.5 (1.2–1.8)	1.4 (1.0–1.8)	0.58	1.7 (1.2–2.0)	1.5 (1.0–1.8)	1.5 (1.2–1.8)	0.58	1.6 (1.2–2.0)
Total intensity of all hassles	35 (12–48)	22 (8–34)	0.04	32 (18–58)	26 (13–56)	29 (11–46)	0.59	29 (18–49)
Recent life events								
Total number of life events	8 (5–12)	8 (4–11)	0.62	9 (5–15)	9 (5–14)	7 (4–13)	0.88	8 (5–14)
Number of unpleasant events	4 (2–6)	3 (1–6)	0.38	5 (3–10)	5 (3–8)	3 (1–6)	0.80	5 (3–8)
Number of pleasant events	2 (1–4)	2 (1–3)	0.89	2 (1–5)	3 (1–6)	2 (1–3)	0.72	2 (1–5)
Amount of total unpleasantness	10 (6–18)	12 (3–19)	0.58	17 (7–35)	15 (7–29)	10 (6–20)	0.57	16 (8–30)
Amount of total pleasantness	9 (4–14)	9 (4–10)	0.68	10 (4–17)	10 (4–21)	9 (4–12)	0.94	10 (4–18)

and immunological hyperthyroidism by means of higher serum free T_3 index ($P=0.035$), free T_4 index ($P=0.051$), and TPO-Ab ($P=0.02$) compared with men without HLA linkage disequilibrium (Table 2). The number of current smokers was non-significantly lower in the linkage disequilibrium group (35 vs 57%, $P=0.12$). According to the stress scores, the intensity per hassle was lower in men with HLA linkage disequilibrium ($P=0.02$) while the total intensity of all hassles did not differ ($P=0.35$). The data suggest more severe Graves' hyperthyroidism and less daily hassles in males (but not in females) in whom HLA class II genes are in linkage disequilibrium.

CTLA4 genotypes

Table 3 lists the associations with the CTLA4 49A/G polymorphism. Whereas gender distribution was similar between the three genotypes, age at diagnosis of Graves' hyperthyroidism decreased progressively with increasing number of G-alleles. G/G homozygotic patients were 7 years younger when presenting with Graves' hyperthyroidism compared with wild-type patients. Biochemical, immunological, and clinical severity were not related to the number of G-alleles, although there was a non-significant trend towards higher TBII and TPO-Ab concentrations in homozygotes. Goiter size also tended to be larger in the presence of G-alleles (goiter size grade 0 and grades ≥ 1 were 74 and 26% respectively in wild type, 67 and 33% in heterozygotes, and 54 and 46% in homozygotes, P for trend = 0.088). No associations were found with exposure to

environmental factors, except exposure to stress. Trend analysis indicated progressively less daily hassles with increasing number of G-alleles.

We further analyzed the CTLA4 49A/G polymorphism by comparing patients with A-alleles and patients with G-alleles separately for each gender (Table 4). In males, the presence of G-alleles was associated with younger age at the time of diagnosis (9 years difference) and less exposure to daily hassles. In females, the presence of G-alleles was slightly related to younger age at diagnosis of Graves' hyperthyroidism (3.5 years difference, just failing to reach statistical significance with a P value of 0.06), but not to the number of daily hassles or the total intensity of all hassles (only the intensity per hassle was lower).

Table 5 presents the associations with the CTLA4 CT60 polymorphism, which were available in 165 patients. Trend analysis showed younger age at the time of diagnosis with increasing number of G-alleles: homozygotes were 8 years younger when compared with wild-type patients. No other associations were found, neither in biochemical, immunological, or clinical severity of Graves' hyperthyroidism, nor in gender and exposure to environmental factors. Table 4 contrasts patients with A-alleles and patients with G-alleles according to gender. Male patients with G-alleles were on average 4.7 years younger at the time of diagnosis, but the difference in age with A-allele patients was not significant ($P=0.12$). Female patients with G-alleles were also non-significantly 2.7 years younger at the time of diagnosis ($P=0.12$). No other associations were observed, neither in males nor in females.

Table 5 Relationship between the CTLA4 CT60 A/G polymorphism and sex, age, disease severity, and environmental exposure in Caucasian patients with untreated first episode of Graves' hyperthyroidism. Values for age, TBII, TPO-Ab, and stress scores are in median + interquartile range (range between the 25th and 75th percentiles); values for free T_3 index and free T_4 index are given in mean + s.d.

	A/A wild type ($n=27$)	A/G heterozygote ($n=85$)	G/G homozygote ($n=53$)	<i>P</i> value
Male gender	18%	23%	22%	0.79
Age (years)	46.0 (38.0–57.0)	43.0 (33.0–51.5)	38.0 (30.0–51.0)	0.04
Free T_3 index	7.3 (3.3)	7.0 (4.6)	7.1 (3.2)	0.88
Free T_4 index	343 (146)	339 (144)	339 (105)	0.91
TBII (IU/l)	7.2 (4.2–10.5)	9.8 (5.4–19.4)	10.1 (4.9–18.4)	0.28
TPO-Ab (kU/l)	520 (<30 to 2870)	260 (40–1820)	565 (108–2955)	0.37
Current smokers	56%	38%	43%	0.48
Stress scores				
PANAS				
Tendency to report negative feelings	23 (20–31)	21 (16–27)	24 (19–30)	0.71
Tendency to report positive feelings	37 (33–41)	36 (33–40)	37 (30–42)	0.83
Daily hassles				
Total number of daily hassles	18 (12–32)	19 (12–28)	26 (9–37)	0.43
Intensity per hassle	1.7 (1.2–2.0)	1.5 (1.2–1.8)	1.6 (1.2–1.9)	0.90
Total intensity of all hassles	33 (18–55)	28 (14–48)	29 (14–69)	0.78
Recent life events				
Total number of life events	11 (6–15)	7 (4–12)	9 (5–17)	0.50
Number of unpleasant events	4 (3–9)	4 (2–7)	5 (3–10)	0.46
Number of pleasant events	3 (2–6)	2 (1–3)	3 (1–6)	0.26
Amount of total unpleasantness	20 (12–35)	13 (6–22)	17 (8–39)	0.79
Amount of total pleasantness	10 (7–24)	8 (4–13)	13 (4–23)	0.56

Table 6 Relationship between the PTPN22 C/T polymorphism and sex, age, disease severity, and environmental exposure in Caucasian patients with untreated first episode of Graves' hyperthyroidism. Values for age, TBII, TPO-Ab, and stress scores are in median + interquartile range (range between the 25th and 75th percentiles); values for free T₃ index and free T₄ index are given in mean + s.d.

	PTPN22 C/C (n=147)	PTPN22 C/T (n=58)	P value
Male gender	24%	24%	0.96
Age (years)	44.0 (33.7–53.2)	41.0 (31.1–53.2)	0.58
Free T ₃ index	6.9 (3.7)	7.0 (3.9)	0.89
Free T ₄ index	341 (136)	313 (104)	0.18
TBII (IU/l)	8.9 (4.8–18.5)	9.0 (5.4–18.9)	0.86
TPO-Ab (kU/l)	330 (40–1795)	710 (58 to >3000)	0.12
Current smokers	38%	39%	0.94
Stress scores			
PANAS			
Tendency to report negative feelings	22 (17–29)	21 (17–27)	0.41
Tendency to report positive feelings	36 (32–40)	40 (35–42)	<0.01
Daily hassles			
Total number of daily hassles	20 (10–32)	21 (12–30)	0.73
Intensity per hassle	1.6 (1.2–1.9)	1.6 (1.2–1.8)	0.64
Total intensity of all hassles	29 (14–56)	29 (13–49)	0.91
Recent life events			
Total number of life events	8 (5–14)	9 (5–12)	0.78
Number of unpleasant events	5 (3–9)	4 (2–7)	0.35
Number of pleasant events	2 (1–4)	3 (1–6)	0.07
Amount of total unpleasantness	15 (7–31)	14 (8–25)	0.68
Amount of total pleasantness	9 (4–14)	11 (5–23)	0.06

PTPN22 genotypes

Table 6 compares patients with wild-type PTPN22 C/C and patients with PTPN22 C/T (heterozygotes). No differences between the two groups were observed, with the exception that heterozygotes had a greater tendency to report positive feelings ($P=0.008$) and had experienced a greater number of pleasant events ($P=0.07$) and a higher amount of total pleasantness ($P=0.06$). The greater tendency to report positive feelings in the presence of a T-allele was stronger in males ($P=0.009$) than in females ($P=0.081$).

Discussion

The investigated genetic variants in *HLA*, *CTLA4*, and *PTPN22* all carry a risk for Graves' disease (21, 22). The variants cause abnormalities in antigen presentation and T-cell activation in the immunological synapse, thus playing a significant role in the etiology of Graves' disease (23). The observed frequencies of genetic variants in our population of patients with Graves' hyperthyroidism are in good agreement with figures reported in other studies in adult Caucasian patients with Graves' disease. The corresponding frequencies in the present study and in the literature are respectively: for *HLA*, DRB1*0304 63% and 47–48%, DQB1*02 61% and 58–59%, DQA1*0501 73% and 63–67%, haplotype DRB1*304–DQB1*02–DQA1*0501 51% and 47% (24, 25); for *CTLA4* 49A/G, AA 37.8% and 27–39%, AG 49.8% and 45–59%, GG 12.4% and 14–25%, A-alleles 63% and 52–57%, G-alleles 37% and 44–48% (25, 26, 27, 28, 29); for *CTLA4* CT60, AA 16.4% and 13–17%, AG 51.5% and 46–53%, GG 32.1% and

30–41%, A-alleles 42% and 36–44%, G-alleles 58% and 56–64% (28, 29); for PTPN22 C/T, CC 72% and 65–74%, CT 28% and 25–31%, TT 0% and 1–4%, C-alleles 86% and 81–86%, T-alleles 14% and 14–19% (17, 30).

The well-known linkage disequilibrium between Graves' disease and genes in the *HLA* class II region, also observed in our study, is due to the extended haplotype DRB1*304–DQB1*02–DQA1*501 (24). It indicates a lack of independent associations of each allele with Graves' disease; the primary susceptibility gene, however, might well be DRB1*03 (23). The two SNPs *CTLA4* 49A/G and *CTLA4* 60CT are in the same haplotype block and also in linkage disequilibrium (28, 29, 31, 32). The occurrence of SNPs in *CTLA4* is independent of the presence of DRB1*03 (28). Likewise, the frequency of the PTPN22 C/T variant is independent of DRB1*03 and *CTLA4* 49A/G (30). Thus, the genetic variants in *HLA*, *CTLA4*, and *PTPN22* are independent risk factors for Graves' disease (16).

Genotypes in relation to phenotypes

We observed a significant younger age at the time of diagnosis of Graves' hyperthyroidism in patients with SNPs in *CTLA4*, both in 49A/G and 60CTA/G genotypes. The G-allele of both SNPs increases the odds for Graves' disease (31), and a dose–response effect for the G-allele was found by trend analysis: G/G homozygotes were the youngest. We did not find a relationship between the age of onset and the genetic variants in *HLA* class II and *PTPN22*. Our data are at variance with a Polish study in adult Caucasian patients with Graves' hyperthyroidism, which observed no relationship between the age of onset and *HLA* variants or *CTLA4* 49A/G but patients with

G-alleles of the *PTPN22* SNP were 10 years younger (30). The age difference in our cohort is in the same range: 7 years for the difference between homozygotes and the wild type of *CTLA4* 49A/G, and 8 years for that of *CTLA4* CT60. These values are in the same order of magnitude when the age of onset is related to a family history of thyroid disease (33). A positive family history is associated with a younger age of onset when compared with no family history (difference: 6 years in males and 5 years in females) (34). Interestingly, there are no significant differences between the frequencies of HLA class II polymorphisms and the susceptibility alleles of *CTLA4* 49A/G and CT60 SNPs in the familial and sporadic cases of Graves' disease, suggesting that additional genes must contribute to the aggregation of Graves' disease within families (16).

The distribution of gender was similar in patients with or without the investigated genetic variants, in agreement with previous studies (30, 32). We also did not observe a relationship between genotypes and biochemical, immunological, or clinical severity of Graves' hyperthyroidism, with the single exception that males in HLA linkage disequilibrium had more severe (biochemical and immunological) hyperthyroidism than males without HLA linkage disequilibrium; this was not observed in females. We could not find comparative data on this topic in the literature. There are just a few studies reporting the absence of a relationship between HLA class II, *CTLA4*, or *PTPN22* variants and the presence of Graves' ophthalmopathy (27, 30, 32, 35). In summary, phenotypic appearance of Graves' hyperthyroidism in our study did not differ between the genotypes, but the age at diagnosis was significantly younger in carriers of G-alleles in *CTLA4* polymorphisms.

Genotypes in relation to environmental factors

The proportion of current smokers did not differ between patients with or without the genetic variants in HLA, *CTLA4*, or *PTPN22*. In contrast, the polymorphism *CTLA4* 49A/G (but none of the other genotypes) was quantitatively related to stress exposure. The total number of daily hassles, the intensity per hassle, and the total intensity of daily hassles were all lower in carriers of the G-allele of this polymorphism in a dose-dependent manner. The *CTLA4* 49A/G SNP was not related to mood (PANAS scales) or to recent life events. According to psychological studies, everyday exposure to daily hassles in general constitutes a greater burden of stressors than incidental exposure to major life events (12, 36). The observation of less daily hassles is not biased by mood changes in our study (current mood state may influence the perception and thereby the scores of daily hassles and recent life events) (12, 36). Less daily hassles in carriers of G-alleles of *CTLA4* 49A/G can also not be explained by their lower age, as recent studies have shown a progressive decrease in daily hassles with advancing age in healthy subjects

with highest scores occurring in the youngest age groups of <40 years (37). The finding that SNP *CTLA4* 49A/G is associated with younger age at diagnosis and with less exposure to stress supports our preformulated hypothesis that subjects with genetic susceptibility to Graves' disease might require less exposure to environmental factors for contracting Graves' hyperthyroidism. A literature search did not reveal other studies relating genetic variants in HLA, *CTLA4*, and *PTPN22* to environmental factors, apart from the notion that the frequency of tobacco smoking was not related to the SNP in *PTPN22* (30).

Genotypes in relation to gender

Subset analysis has been shown to be a powerful method to dissect the roles of 'weak' susceptibility genes in complex diseases: weak susceptibility genes may have a stronger effect in a particular subset of patients (23). Because the median age at diagnosis of Graves' disease is lower in women than in men, and younger age is associated with more severe hyperthyroidism (higher serum FT₄) (34), we performed a subset analysis according to gender.

Comparing women with and without HLA linkage disequilibrium, no differences were found. However, men with HLA linkage disequilibrium had more severe (biochemical and immunological) hyperthyroidism and a lower intensity per hassle. Furthermore, the amount of current smokers in men with HLA linkage disequilibrium was lower (35 vs 57%, $P=0.12$) and their age at diagnosis was 4 years lower than males not in linkage disequilibrium. The difference was not significant most likely due to the small sample size of males.

With regard to *CTLA4* 49A/G, male carriers of a G-allele had a lower age at diagnosis and less daily hassles; their values of free T₄ index, free T₃ index, TBII, and TPO-Ab were – albeit not significantly – higher. These associations were much weaker in female carriers of G-alleles (despite the greater sample size of females) but the trend was in the same direction. Female patients carrying a G-allele were 3.5 years younger at the time of diagnosis compared with A-allele carriers ($P=0.06$).

Evaluation of the SNP *CTLA4* CT60 according to gender did not disclose new findings; the lower age at diagnosis in the presence of G-alleles was non-significantly observed in both sexes ($P=0.12$). For the SNP *PTPN22* C/T, the age at diagnosis in males was 5 years lower in heterozygotes than in the wild type but the difference was not significant, whereas the age in females was not dependent on G-alleles (data not shown).

Gender-specific analysis thus disclosed that the association of *CTLA4* 49A/G with younger age at diagnosis and less daily hassles was maintained in males but lost in females. Furthermore, males in HLA linkage disequilibrium had more severe (biochemical and immunological) hyperthyroidism, and tended to be younger at diagnosis and less exposed to tobacco

smoking and hassles; this was not observed in females. Because the proportion of patients with HLA linkage disequilibrium and that of patients with G-alleles of CTLA4 SNPs did not differ between males and females, the conclusion seems warranted that the impact of susceptibility genes is greater in males than in females. It follows that the role of environmental factors in the pathogenesis of Graves' disease might be relatively greater in females than in males.

Strengths and limitations

The main strength of the present study is its prospective nature, allowing simultaneous assessment in each patient of phenotypic appearance of first episodes of Graves' hyperthyroidism and exposure to environmental factors in relation to genotypes. The study might well be representative for the whole Dutch adult population of Caucasian patients with Graves' hyperthyroidism, because we included consecutive patients originating from nine hospitals throughout The Netherlands; almost all hyperthyroid patients in The Netherlands are treated by hospital-based specialists. The frequencies of the various genotypes are in good accordance with literature data, lending further validity to our study findings.

A limitation of our study is that we did not assess all presently known susceptibility genes, not to speak of the many more unknown genotypes which must be involved in Graves' disease. We restricted ourselves, however, to genotypes that have been widely studied and whose functional significance is to a certain extent understood. The main limitation in our view is the still limited sample size, which is a severe drawback especially in the gender-specific analysis. Trends observed in males might very well become significant with a larger sample size.

Concluding remarks

The number of G-alleles in the SNP CTLA4 49A/G and, to a lesser extent, in CTLA4 CT60 is dose-dependently associated with younger age at diagnosis of Graves' hyperthyroidism and less exposure to stress. This was not observed for polymorphisms of HLA and PTPN22. The divergent effects of the various genetic variants may have emerged not by chance: the attributable fraction (that is a measure of the proportion of the risk conferred) for CTLA4 SNPs in autoimmune thyroid disease is around 25%, one of the highest known for any gene in a complex disorder (31). Thus, any inverse relationship between frequencies of susceptibility alleles and exposure to stress is likely more easily detected in CTLA4 than in other genes. The data support the hypothesis that subjects with genetic susceptibility to Graves' disease might require less exposure to environmental factors to get the disease. Gender-specific analysis was hindered by limited sample size, especially in males. Nevertheless, males with HLA linkage

disequilibrium had more severe hyperthyroidism which was not observed in females, and the association of G-alleles in the SNP CTLA4 49A/G with younger age and less stress was preserved in males but not in females. Thus, the role of susceptibility genes might be greater and the role of environmental factors might be smaller in males compared with females.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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