Abstract

Objective: To investigate HLA-DR genotype in association with chronological age or calendar year of disease onset and the time trend of genotype frequencies from 1969 to 2009. Additionally, to examine genotype frequency in relation to B-cell-, islet cell antibodies (ICA)-, autoantibodies to insulin-, insulinoma antigen 2 (IA2)-, glutamic acid decarboxylase-antibody positivity, thyroid antibody positivity, thyroid diseases or coeliac antibody positivity. Genotype associations with gender and ethnicity are also analyzed.

Subjects and methods: HLA-typed children and juveniles (n=1445) aged ≤20 years at disease onset from the German/Austrian DPV-database were examined. $\chi^2$ statistics and mixed hierarchical logistic regression models were used to compare genotype frequencies and establish associations with age at disease manifestation, autoimmune antibodies/diseases, ethnicity and time trend.

Results: Subjects aged <6 years predominantly carried the genotype HLA-DR3/4 (25.2%), whereas in subjects aged >12 years the most prevalent HLA-DR genotype was X/X (18.1%). IA2 positivity was associated with HLA-DR4/X and HLA-DR3/4 positivity ($P=0.011$), and hypothyroidism was linked to HLA-DR4/4 ($P=0.0103$). More females carried the HLA-DR4/4 genotype (18.2 vs 12.7% $P=0.0048$) or were thyroid antibody positive (24.5 vs 14.7% $P=0.0001$). Larger numbers of <6 year olds were coeliac antibody positive than >12 year olds (14.8 vs 9.1% $P=0.0037$). No associations between migration background and B-cell-, thyroid- or coeliac-antibody positivity, and no time trend were found.

Conclusion: HLA-DR genotype associated with age at disease onset, ICA positivity and hypothyroidism; female gender with thyroid antibody positivity and low age of diabetes onset with coeliac antibody positivity.

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Introduction

Worldwide, the incidence of autoimmune type 1 diabetes mellitus (T1DM), resulting from the destruction of insulin-producing β-cells in pancreatic islets, is increasing steadily especially in the age group <20 years (1, 2). This disease often manifests during childhood with a peak between the ages of 10 and 14 years (3). However, T1DM onset in adulthood is also possible (4). Even though genetically determined susceptibility accounts for at least half of the lifetime risk of T1DM manifestation (5), the observed increases in prevalence suggest that environmental factors have a growing impact (6). Hence, a changing ‘diabetogenic’ milieu may modify the impact of risk genes due to gene–environment interplay.

Previous research indicates that progression to disease and age at onset are directly linked to the MHC II on chromosome 6p21.3 (7). In at least 50% of cases, the inheritance of susceptibility and resistance are predicted by specific HLA-DRB1 and HLA-DQB1 alleles (HLA-DR and HLA-DQ genotypes). HLA-DR3 and HLA-DR4 are predisposing genotypes, and susceptibility of heterozygous individuals (HLA-DR3/DR4) is higher than that of any other genetic constellation (8, 9). Individuals with the genotypes HLA-DR3/X, HLA-DR4/X, HLA-DR3/3 and HLA-DR4/4 have a moderate risk of developing disease, while intermediate/‘neutral’ risk is attributed to non-DR3/non-DR4 genotypes (HLA-DRX/X) with the lowest risk of developing disease. Susceptibility may however vary between ethnic groups and geographical regions (10, 11).
In order to examine HLA-DR genotypes over time, a large sample of subjects from the German/Austrian multicentre documentation (DPV) database was investigated from 1969 to 2008. Associations between HLA-DR genotype frequencies, age at disease onset, gender, ethnicity and autoimmune disease markers such as thyroid antibody positivity, coeliac antibody positivity as well as autoimmune diseases such as hypothyroidism or hyperthyroidism (12, 13) were studied. Considering that about 80% of people with T1DM are positive for at least one islet cell antibody (ICA) (14), the frequencies of HLA-DR genotypes were additionally examined in relation to B-cell- (ICA-, autoantibodies to insulin (insulin autoantibodies (IAA))-), insulinoma antigen 2 (IA2)- and glutamic acid decarboxylase (GAD)-) antibody positivity. Antibody associations with age at disease onset and gender were also examined.

Thus, the aim of this work is to examine associations between: age at disease onset and HLA-DR genotype; gender, ethnicity and HLA-DR genotype; ICA positivity and HLA-DR genotypes; thyroid antibody positivity, hypothyroidism, hyperthyroidism and HLA-DR genotypes; coeliac antibody positivity and HLA-DR genotypes; age at disease onset, sex, thyroid- and coeliac-antibody positivity; changes in HLA-DR genotype frequencies from 1969 to 2008 (linear time trend).

### Subjects and methods

The multicentre DPV-database which collates data of patients treated for diabetes from 304 DPV-centres (outpatient or inpatient health facilities, university teaching hospitals, general hospitals, specialized diabetes treatment centres or general practitioners offices) in Germany or Austria was used to identify the study cohort. Participating centres transmit anonymous, standardized, prospective data twice a year to the respective centres for correction every 6 months (15). Sixty-three DPV-centres contributed genotyped patients to this study. The sample consisted of 1445 HLA-typed T1DM patients, selection criterion: disease onset at age ≤ 20 years. The cohort included subjects with a migration background. Migration background was defined as father or mother born outside Germany or Austria respectively.

### Genotypes

Risk of T1DM based on HLA-DR genotypes can be categorized as follows: highly predisposing, predisposing, intermediate (neutral), protective or very protective (10). Genotypes were categorized as follows: HLA-DR3/4 (DRB1*03-DQA1*0501-DQB1*0201/-DRB1*04 -DQA1*0301-DQB1*0302) (highly predisposing);

Table 1 Classification of genotypes in the study cohort.

<table>
<thead>
<tr>
<th>DRB1–DQB1 haplotypes</th>
<th>DR3</th>
<th>DR4</th>
<th>DRX</th>
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<tbody>
<tr>
<td>DRB1 03</td>
<td></td>
<td></td>
<td>Neither 03 nor 04</td>
</tr>
<tr>
<td>DQA1 0501</td>
<td>0301</td>
<td></td>
<td>Neither 0501 nor 0301</td>
</tr>
<tr>
<td>DQB1 0201</td>
<td>0302</td>
<td></td>
<td>Neither 0201 nor 0302</td>
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<tr>
<td>HLA-DR3/4</td>
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<td>HLA-DR4/4</td>
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<td>HLA-DR4/X</td>
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<tr>
<td>HLA-DRX/X</td>
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</table>

HLA-DR genotypes: for the purpose of simplicity, genotypes were summarized as follows: HLA-DR3/4, DR4/4, DR4/X, DR3/3, DR3/X and DRX/X. Haplotype present (+); haplotype absent (−).

HLA-DR4/4, HLA-DR3/3, HLA-DR4/X. HLA-DR3/X (predisposing); and non-DR3 and non-DR4 genotypes (HLA-DRX/X) encompassed intermediate/“neutral”/protective genotypes. Table 1 shows the classification of genotypes in this DPV-study sample.

### Genotyping of HLA-DRB1 and DQB1

In the 1970s and 1980s, serological typing was used to establish HLA-DR and DQ alleles for subjects in the DPV-database. In the 1990s, HLA typing was performed using polymerase chain amplification with sequence-specific priming methods. Comparability of results from different laboratories was ensured through the use of standard assays for HLA-typing and the official nomenclature for factors of the HLA System, 1991.

### Autoantibodies

**B-cell antibodies** The following β-cell autoantibodies were analyzed: anti-IAA (measured at diagnosis), anti-GAD, anti-ICA and anti-I2A antibodies. The following cut-off values were defined in order to classify positivity: IAA2 (≥ 0.75 Units/ml); IAA at diagnosis (≥ 1 Unit); ICA (≥ 5 JDF Units); GAD (≥ 0.9 Units/ml).

**Thyroid antibodies** Anti-thyroperoxidase (anti-TPO) antibodies and anti-thyroglobulin (anti-TG) antibodies were measured using commercially available RIAs at different laboratories. For both anti-TPO and anti-TG, positivity was assumed if a titre exceeding 100 U/ml was measured. Each anti-TPO-assay was calibrated using the MRC 66/387 anti-TPO standard.

**Coeliac-specific antibodies** Positivity for coeliac antibodies was defined by gliadin IgG and IgA values above 25 U/l. Given that a coeliac disease diagnosis based on IgG anti-gliadin antibody positivity is unspecific, a positive titre of IgA autoantibodies directed against tissue transglutaminase (anti-tTG antibodies) or endomysium antibodies (EMA) higher than 10 U indicated that coeliac disease could be suspected.
**Statistical analysis**

Data analysis was carried out using the Statistical Analysis Software (SAS, Cary, NC, USA). \( \chi^2 \) statistics were used to compute genotype frequencies in relation to gender, age category of disease onset (<6, 6–<12 and >12 years), migration background, disease manifestation year, B-cell antibody positivity, ICA positivity, thyroid antibody positivity, hypothyroidism, hyperthyroidism and coeliac antibody positivity. Mixed hierarchical logistic regression models were used to examine associations between genotype and age at disease onset adjusted for sex, manifestation year and treatment centre (random effect). Genotype frequency was also analyzed in association with thyroid- and coeliac-antibody positivity adjusted for age at disease onset, sex, manifestation year and treatment centre (random effect). Adjusted means of covariates are presented in Figs 1–4. The time trend was investigated as follows: i) comparing genotype frequency in the periods 1969–1989 and 1999–2009, and ii) comparing genotype frequency from 1969 to 2009 in 5-year intervals. The period from 1969 to 1978 was summarized in one (10 years) interval, due to the low number of HLA-typed cases documented during these years. Logistic regression models were used to assess genotype frequency over time in both categories, adjusted for age at disease onset, sex and treatment centre.

**Results**

**Study population**

The sample consisted of HLA-typed TIDM patients (\( n = 1445 \)), aged \( \leq 20 \) years at disease onset. Fifty per cent of patients in the cohort were diagnosed after 1992, while 24.6% were diagnosed after 2000. There were only minimal differences between HLA-typed patients and non-HLA-typed patients (\( n = 46287 \)) in the database. Male gender was 50.7 vs 51.8%, and age at disease onset was 8.3 vs 8.7 years for HLA-typed and non-HLA-typed subjects respectively. In the study cohort, 485 patients were <6 years old, 647 patients were 6–<12 years old and 313 patients were 12–<20 years old. Table 2 shows demographic variables pertaining to the study cohort. There were 7.7% patients with migration background in the study sample.

**Frequency of genotypes**

In the study sample, the moderate-risk genotypes (HLA-DR4/X (29.0%), HLA-DR4/4 (15.8%), HLA-DR3/X (10.7%) and HLA-DR3/3 (8.5%)) constituted...
the most abundant category, together accounting for 64% of all genotypes. The second most abundant category was the high-risk genotype, HLA-DR3/4, with 24.8%. The lowest risk category, HLA-DRX/X, was carried by 11.1% of the sample. The high-risk group HLA-DR3/4 consisted of 145 subjects <6 years old, 153 patients 6–12 years old and 61 patients 12–≤20 years old. In the low-risk group, HLA-DRX/X, 43 subjects were <6 years old, 68 subjects were between 6 and 12 years old and 50 subjects were >12 years old. The frequencies of genotypes analyzed are presented in Table 3.

**Age at disease onset and HLA-DR genotypes**

Age at disease onset was significantly related to HLA-DR genotype after adjusting for sex and manifestation year in the study sample (P = 0.0067). A closer look at age/genotype associations within the three categories, i.e. <6 years (n=485), 6–≤12 years (n=647) and >12 years (n=313) revealed that both HLA-DR3/4 and HLA-DRX/X genotypes were significantly associated with age at disease manifestation. In total, 25.2% of subjects in the age category <6 years, 20.1% in the age category 6–≤12 years and 16.4% in the age category >12 years carried the HLA-DR3/4 genotype. Analogously, the HLA-DRX/X genotype was carried by 18.1% of subjects in the age category >12 years, while the 6–≤12 years and <6 years age categories consisted of 12 and 10.3% of subjects with HLA-DRX/X respectively. HLA-DR3/4 was associated with the lowest age at disease onset (6.8 ± 0.3 years), while HLA-DRX/X was linked to the highest age at disease onset (8.2 ± 0.4 years) in the study sample (Fig. 5). Significant differences in age at disease onset for subjects with HLA-DR3/4 and HLA-DRX/X genotypes are illustrated in Fig. 1. The moderate-risk genotypes (HLA-DR3/3, HLA-DR3/X, and HLA-DR4/X, HLA-DR4/4) showed no significant associations with age category at disease manifestation.

**Gender, ethnicity and HLA-DR genotype**

In the study cohort, there were more males than females with the HLA-DR3/3 (56.1 vs 43.9%), HLA-DR3/4 (53.5 vs 46.5%), HLA-DR4/X (54.9 vs 46.1%) and HLA-DRX/X (51.6 vs 48.4%) genotypes respectively. Inversely, more females than males had the HLA-DR3/X (55.5 vs 44.5%) and HLA-DR4/4 (59.2 vs 40.8%) genotypes. The significant gender difference in genotype frequency (P = 0.0078) was lost after adjusting for age at disease onset and manifestation year for all genotypes except HLA-DR4/4. In this subgroup, there were more females (18.2%) than males (12.7%), P = 0.0048 (Fig. 2).

Genotype frequencies did not significantly differ between subjects with and without a migration background.

**ICAs and HLA-DR genotypes**

B-cell antibody positivity was tested in 1073 (74.3%) of patients in the study cohort, and 811 (75.6%) were found positive for any B-cell antibody. There was no significant association between HLA-DR genotypes and ICA positivity in general. HLA-DR genotype associations with individual ICAs (i.e. ICA, IAA, GAD and IA2) revealed a significant association for IA2 only (P = 0.0119). More subjects with HLA-DR3/4 and HLA-DR4/X genotypes were IA2 positive than IA2 negative, i.e. HLA-DR3/4 (57 vs 42) and HLA-DR4/X (68 vs 37) respectively. Conversely, more IA2-negative subjects than IA2-positive subjects carried the HLA-DR3/X genotype (32 vs 15). All other genotypes had comparable frequencies between IA2-positive and IA2-negative subjects.

**Thyroid antibody positivity, thyroid disease and HLA-DR genotype**

Thyroid antibodies were tested in 1182 (81.8%) subjects of the study sample, and 294 (24.9%) were positive. No significant association was found between thyroid antibody positivity and HLA-DR genotype. There was however, an association between HLA-DR
genotype and clinically diagnosed hypothyroidism ($P=0.0103$) but not hyperthyroidism ($P=0.6305$). Among the 30 subjects diagnosed with hypothyroidism, 12 carried the HLA-DR4/4 genotype and 9 carried the HLA-DR4/X genotype. Less than five individuals carrying other genotypes were diagnosed with hypothyroidism in the study sample.

**Coeliac antibody positivity and HLA-DR genotype**

In the study sample, 1103 (76.3%) subjects were examined for coeliac antibodies, and 168 (15.2%) were positive (Table 4); of the 1439 subjects examined, 3.5% had clinically diagnosed coeliac disease. No significant association was found between HLA-DR genotype and coeliac antibody positivity. Thus, among patients of the study sample, coeliac antibody positivity was independent of HLA-DR genetic make-up.

**Immunological phenotypes in association with gender and age at disease onset**

A significant gender difference was found with regard to thyroid antibody positivity. More females (24.5%) than males (14.7%) remained positive after adjusting for genotype and calendar year of disease manifestation ($P=0.001$). (Fig. 3). There was no significant relationship between age category and B-cell antibody positivity.

**HLA-DR genotypes: time trend from 1969 to 2009**

Before adjusting for age at disease onset and sex, differences in genotype frequencies between 5-year intervals from 1969 to 2009 were significant ($P=0.001$). This was also true when genotype frequencies within the periods 1969–1989 and 1999–2009 were compared ($P=0.0056$). However, after adjusting for age at disease onset and sex, significance was lost for all genotypes for all intervals examined. Thus, no significant linear time trend persisted for any genotype over the 40 years of investigation in the study sample.
gender on the disease phenotype (20).

and hypothyroidism, suggesting a direct influence of the

assumed. Female gender dominance was also

determining susceptibility to T1DM than previously

HLA-DR genotypes have stronger gender associations

Nevertheless, this result may be an indication that

HLA-DR4/4 genotype observed for this DPV-cohort.

evident reason for this female gender dominance for the

has not been previously reported. Currently, there is no

gender dominance of HLA-DR4/4 genotype in diabetes

more females than males carried this genotype. Female

found only within the HLA-DR4/4 subgroup, where

genotypes (HLA-DR3/3, HLA-DR4/4, HLA-DR4/X and

age at disease onset and the moderate-risk

risk HLA-DRX/X genotype (18). Associations between

ficantly later in patients with the intermediate/neutral'

T1DM manifests at lower ages in individuals with the

highly predisposing HLA-DR3/4 genotype and signi-

results confirm the findings of previous studies whereby

remained unchanged over several decades (17, 20) . Additionally, modern lifestyles of excessive

calorie intake (increases in BMI), physical inactivity,

shortened sleep, excessive hygiene or exposure to toxic

substances (23) may have also contributed to this

related increased prevalence of enteroviruses caused

illnesses in winter months than in the summer months

(21, 22). Additionally, modern lifestyles of excessive

calorie intake (increases in BMI), physical inactivity,

hygiene or exposure to toxic substances (23) may have also contributed to this

development. Nevertheless, environmental pressures

and lifestyle may reduce the need for a strong genetic

background for the development of T1DM (24).
An analysis of HLA-DR genotype and autoimmunity revealed that IA2 positivity was significantly associated with HLA-DR3/4 and HLA-DR4/X genotypes. Previous studies have reported associations between IA2 and HLA-DR genotypes with at least one HLA-DRB1*0401 allele (25, 26). Our results confirm these findings, and underline a genotype-dependent association of IA-2 in the aetiology of T1DM. Even though IAA positivity has been associated with homozygosity of the HLA-DR4 genotype and male gender during adolescence (27), no similar association was found in the study sample. No significant associations were also found between HLA-DR genotype and GAD-, ICA- and B-cell-antibody positivity in general.

An association between HLA-DR genotype and autoimmune disorders was found for hypothyroidism, which was predominantly diagnosed in patients with the HLA-DR4/4 and HLA-DR4/X genotypes. This suggests that moderate-risk-associated HLA-DR genotypes also play a role in thyroid disease manifestation. Thyroid antibody positivity was not genotype related but gender (female) related. This significant female gender dominance for thyroid antibody positivity in the study sample has been previously reported (20, 28). Additionally, age at disease onset was significantly linked to coeliac antibody positivity in the sample. Subjects <12 years old were more likely to be positive for coeliac antibodies than older subjects in the study cohort. This age association has also been observed in previous studies (12).

**Conclusion**

This work, based on a large cohort of T1DM patients from two medium-risk European countries (Germany and Austria), shows that a lower age of disease onset (<6 years) is linked to the highly predisposing genotype (HLA-DR3/4), while the intermediate (neutral) genotype, HLA-DRX/X, is associated with higher age at disease manifestation (>12 years). Changes in genotype prevalence did not follow a linear trend across the 40-year period of investigation. Thus, despite the increase in incidence of T1DM in Germany/Austria, the frequency of HLA genotype risk categories has remained unchanged.

**Study limitations and strengths**

The absence of a linear time trend may have been due to the fact that a large number of subjects were examined for changes which are not immediately evident, e.g. environment-attributed influences. Since these were not examined in detail, the validity of the study findings for time trends may have been compromised. This may also explain the fluctuations in genotype frequency observed. Furthermore, it is possible that among patients with adult onset of disease, different genotype associations exist from disease onset during childhood.

Nevertheless, a major strength of this study is that the data used were collected in the standardized, long-term follow-up DPV-documentation process from a large sample. As such, a selection bias was unlikely. Nonetheless, similar studies over comparable periods are needed in order to backup the results of this analysis.

**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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**Key points:**

- Type 1 diabetes mellitus (T1D) is a complex disease involving both genetic and environmental factors.
- The incidence of T1D has increased over the past decades globally.
- The association of specific human leukocyte antigen (HLA) haplotypes with T1D is well-established.
- Environmental factors such as infections, diet, and smoking play a role in the development of T1D.
- Autoimmune responses are a hallmark of T1D, with islet autoantibodies being a key diagnostic tool.

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**Citation:**