

Relation between Disease Phenotype and HLA-DQ Genotype in Diabetic Patients Diagnosed in Early Adulthood

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Abbreviations:

BDR Belgian Diabetes Registry

BMI body mass index

CI confidence interval

GADA glutamate decarboxylase autoantibodies

HbA_{1c} glycosylated hemoglobin

HLA human leukocyte antigen genes

IAA insulin autoantibodies

IA-2A insulinoma-associated antigen 2 antibodies

ICA islet cell antibodies

INS insulin (gene)

JDF Juvenile Diabetes Foundation

OR odds ratio

We investigated inaugural disease phenotype in relation to the presence or absence of diabetes-associated autoantibodies and human leukocyte antigen (HLA) DQ risk genotypes in adult-onset diabetic patients. Blood samples and questionnaires were obtained from 1584 recent-onset Belgian Caucasian patients (age 15-39 yr at diagnosis of primary diabetes) who were recruited by the Belgian Diabetes Registry over an 11-yr period. At clinical diagnosis, antibody-positive patients (n = 1198) were on average younger and had more symptoms, a more acute disease onset, lower body mass index, and random C-peptide levels, but higher insulin needs, glycemia, and prevalence of ketonuria, HLA-DQ, and 5' insulin gene susceptibility genotypes ($P < 0.001$ vs. antibody-negative patients; n = 386). In antibody-positive patients, these characteristics did not differ according to HLA-DQ genotype. However, in antibody-negative subjects, we found that patients were younger ($P = 0.001$); had a lower body mass index ($P < 0.001$), higher insulin needs ($P = 0.014$), and amylasemia ($P = 0.001$); and tended to have a higher glycemia and lower C-peptide in the presence of susceptible HLA-DQ genotypes. Differences according to HLA-DQ genotype subsisted after careful age-matching. In conclusion, we found no relation between initial disease phenotype and HLA-DQ genotype in antibody-positive diabetic young adults. In contrast, antibody-negative patients displayed more type 1-like features when carrying susceptible HLA-DQ genotypes known to promote the development of antibody-positive diabetes. The overrepresentation of these susceptibility genotypes in antibody-negative patients suggests the existence of an immune-mediated disease process with as yet unidentified immune markers in a subgroup of seronegative patients. (*J Clin Endocrinol Metab* 87: 2597-2605, 2002)

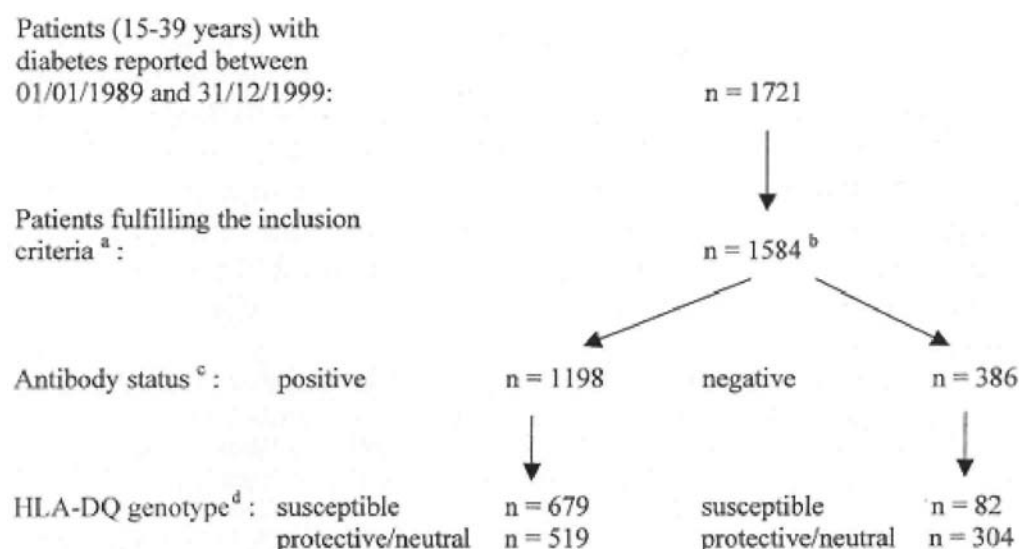
MOST CASES OF type 1 diabetes are believed to arise from the nearly complete destruction of insulin-producing β -cells by immune effector cells^[1]. Although there are indications that the underlying pathological process is often initiated in early childhood, the disease can become clinically manifest at any age, most cases arising after age 15 yr^{[1][2][3][4][5]}. The disease presents along a clinical spectrum ranging from acute-onset ketosis-prone diabetes to latent forms with slow progression to insulin deficiency after initial manifestation^{[6][7][8]}^[9]. Disease phenotype, underlying pathologic changes, and associated biological markers tend to change with age at diagnosis^{[3][9][10][11][12][13][14][15]}. Overall, it becomes increasingly difficult to clinically distinguish type 1

diabetes from other forms of the disease with older age at clinical manifestation^{[3] [4] [6] [9]}. There is thus a need for additional biological markers of adult-onset type 1 diabetes.

The American Diabetes Association and the World Health Organization recently proposed new classification criteria for diabetes in which the presence or absence of diabetes-associated autoantibodies plays a central role^{[7] [8]}. Immunemediated type 1 diabetes (type 1A) is defined as hyperglycemia in the presence of autoantibodies against β -cell antigens^{[7] [8]}. These patients preferentially carry specific HLA-DQA1-DQB1 risk haplotypes (0301-0302 and/or 0501-0201)^{[7] [8]}. In initially noninsulin-requiring adult-onset patients, the presence of autoantibodies has been associated with lower body mass index (BMI), less preserved residual β -cell mass, and more rapid evolution toward insulin dependency^{[6] [16] [17]}. However, a number of diabetic patients, mostly of African or Asian origin, presents with variable degrees of insulin deficiency in the absence of circulating diabetes autoantibodies^{[7] [8]}. A recent Japanese study reported a subgroup of diabetic patients with abrupt clinical onset of diabetes in adulthood without detectable insulinitis or diabetes-associated autoantibodies, but characterized by the presence of hyperamylasemia, hyperlipasemia, and lymphocytic infiltration of the exocrine pancreas^[19]. Whether this subtype occurs in Caucasian populations has so far not been confirmed^{[20] [21]}. The absence of autoantibodies at diagnosis does, however, not exclude the existence of an immunemediated β -cell destruction because we and others have occasionally observed the appearance of diabetes-associated autoantibodies after clinical onset^{[22] [23]}. Likewise, it is conceivable that some patients may have lost autoantibodies before diagnosis or present other immune markers than those currently searched for^[23]. To further improve the etiological classification of adult-onset diabetes, and hence etiology-based therapeutic schemes, it is important to try to relate clinical disease phenotype to biological markers of immune-mediated diabetes also in the absence of autoantibodies.

Therefore, the aims of the present report were: 1) to study the clinical phenotype of adult-onset diabetic patients after stratification for diabetes-associated autoantibodies, and 2) to investigate the possible interaction between the disease phenotype and HLA-DQ genotype in a representative population of adult-onset diabetic patients stratified according to the presence or absence of autoantibodies.

Figure 1. Number of included diabetic patients aged 15-39 yr, according to antibody status and HLA-DQ genotype.^a Absence of gestational or secondary diabetes; Caucasian ethnicity; availability of demographic data and blood samples for DNA, autoantibody, and chemical analysis within 18 months after diagnosis; and Belgian residency for at least 6 months before diagnosis.^b From 1488 of 1584 included patients (94%), a standard questionnaire was available with epidemiological, clinical, and locally determined biological data.^c Positivity for ICA, GADA, and/or IA-2A; negativity for ICA, GAD A, and IA-2A.^d For the definition of HLA-DQ susceptibility genotypes, see Subjects and Methods.



SUBJECTS AND METHODS

Subjects

Between January 1989 and December 1999, 1721 patients (age 15-39 yr) were consecutively recruited by the Belgian Diabetes Registry (BDR), a national data and sample bank for recent-onset diabetic patients and their first-degree relatives under age 40^[3]. Registration of newly diagnosed diabetic patients (all types of diabetes) into the BDR is based on the voluntary reporting by pediatricians and endocrinologists participating in the BDR. Diabetes was diagnosed according to the criteria of the National Diabetes Data Group^[24]. Comparison with a subregion (Antwerp district) with nearly complete case ascertainment (94%) has demonstrated that the larger Belgian group of patients, with incomplete ascertainment (presently 55%), is representative for the Belgian diabetic patients with diagnosis before age 40 yr^[25]. Of the 1721 patients, 1584 were included after verification of the following criteria: 1) absence of gestational or secondary diabetes; 2) Caucasian ethnicity; 3) availability of demographic data and samples for DNA, autoantibody, and chemical analysis taken within the first 18 months after diagnosis; and 4) Belgian residency for at least 6 months before diagnosis (Fig. 1). From 1488 included patients (94%), a standard questionnaire with epidemiological, clinical, demographic, and local biological data was available. Patients (n = 1584) were stratified according to the presence (immune-mediated type 1 diabetic patients) or absence (patients without evidence of immune-mediated type 1 diabetes) of autoantibodies at diagnosis or during follow-up^{[7] [8]}. A total of 1198 subjects (76%) showed positivity for at least one type of autoantibody [islet cell cytoplasmic antibodies (ICA), glutamate decarboxylase antibodies (GADA), or insulinoma-associated antigen 2 antibodies (IA-2A)] at first sampling and/or in any later available blood sample (Fig. 1). Because patients could be included until 18 months after diagnosis, insulin antibodies were not considered in this analysis. It has, moreover, been shown that less than 1% of the diabetic patients diagnosed in young adulthood are positive for insulin autoantibodies (IAA) at clinical onset in the absence of ICA and GADA^[26]. Additional stratification was performed according to the presence or absence of HLA-DQ susceptibility genotypes (Fig. 1). At diagnosis, patients were tentatively classified as type 1, type 2, or unclassifiable diabetes by their treating physicians who were unaware of the antibody or HLA-DQ status at that time.

The study was approved by the ethical committee of the BDR and the universities participating in its scientific projects. Informed consent was obtained from each subject and from the parents in case of minors in accordance with the Helsinki Declaration.

Antibody assays

ICA were measured with an indirect immunofluorescence assay using cryosections of fresh-frozen human blood group O pancreas for substrate^[11]. The results were expressed in Juvenile Diabetes Foundation (JDF) units. Autoantibodies against GADA and IA-2A were determined by liquid phase radio-binding assay using recombinant human ³⁵S-labeled intact glutamate decarboxylase (65 kDa isoform) and the ³⁵S-labeled intracellular domain of insulinoma-associated antigen 2 for tracer, respectively^[27]. Cut-off values for positivity for ICA (>12 JDF units), GADA (>2.6% tracer bound), and IA-2A (>0.4% tracer bound) were established to secure 99% diagnostic specificity after omission of outlying values based on the analysis of 789 healthy control subjects^[23]. The antibody assays performed repeatedly well in successive external quality control programs (Immunology of Diabetes Workshops, Proficiency Testing of the University of Florida, Gainesville, FL, and of the Research Institute for Children, New Orleans, LA). In the latter program, our three assays achieved 100% diagnostic sensitivity, specificity, consistency, and validity. In the combinatorial islet autoantibody workshop assay, sensitivity adjusted for 99% specificity amounted to 73% for ICA and 85% for GADA (IA-2A was not yet available in our laboratory at the time of the workshop, *i.e.* 1995)^[28].

Genetic analysis

DNA was extracted from 5 ml potassium EDTA-blood by proteinase-K digestion of SDS-treated white blood cells and subsequent phenol extraction or salt precipitation. The second exons of the *DQA1* and *DQB1* alleles were simultaneously amplified by PCR using primer sets that were described previously^[29]. Amplified DNA was dot-blotted on nylon membranes, fixed by UV irradiation, and hybridized to a panel of 15 different digoxigenin-deoxyuridine triphosphate-labeled allele-specific oligonucleotide probes. After posthybridization washings, the membranes were incubated with antidigoxigenin antibody conjugated to alkaline phosphatase, and probe signals were visualized by chemiluminescent detection^[30].

On the basis of the comparison of HLA-DQA1-DQB1 genotypes in 1866 antibody-positive recent-onset diabetic patients aged under 40 yr at diagnosis (recruited in Belgium between January 1989 and March 1999) and 750

nondiabetic Belgian residents, the following genotypes were found to confer significant susceptibility for type 1 diabetes in the Belgian population^[31] : DQA1*0501-DQB1*0201/DQA1*0301-DQB1*0302 [odds ratio (OR), 21.3; 95% confidence interval (CI), 12.2-38.0], homozygosity for DQA1*0301-DQB1*0302 (OR, 8.2; 95% CI, 3.2-22.8), or DQA1*0501-DQB1*0201 (OR, 5.0; 95% CI, 2.8-8.8) and DQA1*0301-DQB1*0302 in combination with one of the neutral DQA1*-DQB1* haplotypes (0100-0501/0604/0605, 0102-0201, 0102-0502, 0301-0201, 0301-0301, 0301-0303, 0301-0401, 0401-0402 or 0501-0302) (OR, 4.3; 95% CI, 3.0-6.4). Overall, in subjects carrying one of the above susceptibility genotypes, the OR (95% CI) for type 1 diabetes amounted to 15.3 (11.6-20.1)^[31].

The variable number of tandem repeats polymorphism upstream from the insulin gene (INS) transcription initiation site (5INS) was detected by assessment of a diallelic adenine/thymidine polymorphism at position -23 of the INS gene which is in nearly complete linkage disequilibrium with the 5'INS variable number of tandem repeats. Genotypes were assessed by conventional PCR restriction fragment length polymorphism analysis with restriction enzyme HphI (Amersham Pharmacia Biotech, Uppsala, Sweden)^[31] or by a 5'nuclease (TaqMan) assay with fluorogenic probes^[30] on the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA).

Other biological markers

Random C-peptide levels were determined on the serum samples collected for the analysis of diabetes-associated autoantibodies. The C-peptide assay was performed with a commercial kit (¹²⁵I-human C-peptide, guinea pig antihuman C-peptide serum; Linco Research Inc., St. Charles, MO) that has its lower detection limit at 20 pmol/liter^[33]. The same serum samples were analyzed for glucose and total activities of amylase and lipase using a Vitros 950 IC analyser and dedicated Vitros slides (Ortho Clinical Diagnostics, Rochester, NY) according to the manufacturer's specifications^[34].

Statistical analysis

Differences between groups were assessed by Mann-Whitney *U* test for continuous variables and by χ^2 test with Yates' correction or Fisher's exact test, whenever appropriate, for categorical variables. Multivariate analysis was performed by forward stepwise logistic regression with the genotype as the dependent variable and age, BMI, amylase activity, and random C-peptide levels as independent variables. C-peptide was log-transformed before multivariate analysis. All statistical tests were performed two-tailed by SPSS for Windows 10.0 (SPSS, Inc., Chicago, IL) for personal computers and were considered significant at *P* value less than 0.05 or, in case of *k* independent tests, at *P* value less than 0.05/ *k* (Bonferroni correction)^[35].

TABLE 1 — Demographic and clinical characteristics of 1584 diabetic patients (all phenotypes) aged 15-39 yr at diagnosis

Characteristics	Antibody-positive patients (n = 1198)	Antibody-negative patients (n = 386)	<i>P</i> value ^a
Age at diagnosis (yr) ^b	26 (20-31)	32 (26-36)	<0.001
Male-to-female ratio [no. of patients (ratio)]	717/481 (1.49)	259/127 (2.04)	0.013
Presence of one or more symptoms ^c [no. of patients (%)]	1099/1140 (96)	306/347 (88)	<0.001
Duration of symptoms ^d (wk) ^b	4 (2-8)	6 (2-12)	<0.001
BMI ^e (kg/m ²) ^b	21 (19-23)	25 (22-30)	<0.001
Daily insulin dose after stabilization ^f (U/kg) ^b	0.47 (0.31-0.67)	0.34 (0.10-0.53)	<0.001
Initial classification as type 1 by treating physician [no. of patients (%)]	1139/1198 (95)	237/386 (61)	<0.001

^a Mann-Whitney *U* test was used for continuous variables and χ^2 test for categorical variables; the threshold for statistical significance is *P* < 0.05/7 or *P* < 0.007 (Bonferroni adjustment).

^b Median (interquartile range).

^c Polyuria, polydipsia, weight loss, and/or fatigue.

^d Data were available for 963 antibody -positive and 302 antibody -negative patients.

^e Data were available for 1036 antibody -positive and 327 antibody -negative patients.

^f Data were available for 877 antibody -positive and 298 antibody -negative patients.

RESULTS

Clinical and biological characteristics according to autoantibody status

At diagnosis, antibody -positive patients (n = 1198) differed significantly in terms of clinical presentation and biological characteristics from antibody-negative patients (n = 386) (Fig. 1). Overall, antibody -positive subjects were younger (26 vs. 32 yr; $P < 0.001$), and they more frequently presented clinical symptoms of diabetes such as polyuria, polydipsia, fatigue, or weight loss (96 vs. 88%; $P < 0.001$) with an overall shorter duration of the prodromal phase (4 vs. 6 wk; $P < 0.001$); they were leaner at diagnosis (BMI, 21 vs. 25 kg/m²; $P < 0.001$), had higher insulin requirements after stabilization (0.47 vs. 0.34 U/kg·d; $P < 0.001$), and were initially more often classified as type 1 patients by their treating physician (95 vs. 61%; $P < 0.001$) (Table 1). At diagnosis, 96% of the antibody-positive patients were treated with insulin, compared with 76% of the antibody-negative patients ($P < 0.001$). The reported male-to-female excess^[14] tended to be more pronounced in the absence of antibodies (Table 1).

Compared with antibody-negative patients, antibody-positive subjects showed lower random C-peptide values (0.62 vs. 1.39 µg/liter; $P < 0.001$), despite higher random glycemia levels (350 vs. 294 mg/dl; $P < 0.001$), and had a higher prevalence of ketonuria (74 vs. 47%; $P < 0.001$), HLA-DQ (57 vs. 21%), and 5'INS (70 vs. 54%) risk markers ($P < 0.001$) (Table 2). The prevalence of susceptible HLA-DQ genotypes, but not of 5'INS I/I, was higher in antibody-negative patients than in control subjects (21 vs. 9%, $P < 0.001$; and 54 vs. 47%, $P > 0.05$, respectively) (Table 2). Both the high-risk heterozygous HLA-DQ genotype 0301-0302/0501-0201 and the presence of two susceptibility haplotypes (0301-0302 and/or 0501-0201 in homozygous or heterozygous combination) were significantly more prevalent in antibody -negative patients than in nondiabetic control subjects (7.5 vs. 1.9%, $P < 0.001$; and 14.8 vs. 4.5%, $P < 0.001$, respectively). Glycosylated hemoglobin (HbA_{1c}) levels tended to be higher in the presence of autoantibodies ($P = 0.038$) (Table 2). Serum amylase and lipase activities did not differ according to antibody status (Table 2). During follow-up [mean (range), 25 (2-60) months], antibody-positive subjects (n = 677) persistently showed lower BMI, higher insulin requirements, and lower random C-peptide levels than antibody-negative patients (n = 149; $P < 0.001$; data not shown). Also during follow-up, antibody-positive patients were more frequently treated with insulin (98 vs. 81%; $P < 0.001$). Regardless of HLA-DQ status, the presence of first-degree relatives with type 1 and/or type 2 diabetes was more frequently reported in antibody-negative patients than in antibody-positive subjects (34 vs. 20%; $P < 0.001$).

TABLE 2 — *Biological characteristics of 1584 diabetic patients aged 15-39 yr at diagnosis*

Characteristics	Antibody-positive patients (n = 1198)	Antibody-negative patients (n = 386)	P value ^a
Ketonuria [no. of patients (%)]	678/911 (74)	130/276 (47)	<0.001
Random glycemia ^c (mg/dl) ^b	350 (271-469)	294 (207-371)	<0.001
HbA _{1c} ^d — SD from mean of local reference interval ^b	10.4 (6.9-14.5)	9.7 (5.4-14.8)	0.038
Random C-peptide ^e (µg/liter) ^b	0.62 (0.35-1.04)	1.39 (0.80-2.36)	<0.001
HLA DQA1 *-DQB 1 * genotype			
Protective/neutral	519/1198(43)	304/386 (79)	<0.001
Susceptible	679/1198(57)	82/386 (21)	
5'INS I/I genotype	782/1111 (70)	193/357 (54)	<0.001
Amylase ^f (U/liter) ^b	47 (39-56)	46 (38-55)	0.172
Lipase ^f (U/liter) ^b	73 (44-116)	68 (38-113)	0.160

^a Mann-Whitney *U* test was used for continuous variables and χ^2 test for categorical variables; the threshold for statistical significance is $P < 0.05/8$ or $P < 0.006$ (Bonferroni adjustment).

^c Data were available for 1109 antibody-positive and 346 antibody-negative patients.

^b Median (interquartile range).

^d Data were available for 1019 antibody-positive and 326 antibody-negative patients.

^e Data were available for 1041 antibody-positive and 381 antibody-negative patients.

^f Data were available for 1028 antibody-positive and 377 antibody-negative patients.

TABLE 3 — Demographic and clinical characteristics of 1198 antibody -positive diabetic patients aged 15-39 yr according to HLA-DQ genotype

Characteristics	HLA-DQ genotype		P value ^a
	Susceptible (n = 679)	Protective/neutral (n = 519)	
Age at diagnosis (yr) ^b	25 (20-31)	27 (20-32)	0.098
Male-to-female ratio [no. of patients (ratio)]	388/291 (1.33)	329/190 (1.73)	0.033
Presence of one or more symptoms ^c [no. of patients (%)]	625/646 (97)	474/494 (96)	0.578
Duration of symptoms ^d (wk) ^b	4 (2-8)	4 (2-8)	0.992
BMI ^e (kg/m ²) ^b	21 (19-23)	21 (19-24)	0.199
Daily insulin dose after stabilization ^f (U/kg) ^b	0.48 (0.32-0.67)	0.46 (0.31-0.68)	0.875
Initial classification as type 1 by treating physician [no. of patients (%)]	647/679 (95)	492/519 (95)	0.800

^a Mann-Whitney *U* test was used for continuous variables and χ^2 test for categorical variables; the threshold for statistical significance is $P < 0.05/7$ or $P < 0.007$ (Bonferroni adjustment).

^b Median (interquartile range).

^c Polyuria, polydipsia, weight loss, and/or fatigue.

^d Data were available for 546 patients with susceptible and 417 patients with protective/neutral genotypes.

^e Data were available for 592 patients with susceptible and 444 patients with protective/neutral genotypes.

^f Data were available for 504 patients with susceptible and 373 patients with protective/neutral genotypes.

CLINICAL AND BIOLOGICAL CHARACTERISTICS ACCORDING TO HLA-DQ GENOTYPE

Antibody-positive patients.

There were no significant differences in clinical (Table 3) or biological (Table 4) characteristics according to HLA-DQ genotype in antibody-positive subjects, except for a trend toward older age at onset ($P = 0.098$) (Table 3) and higher male-to-female ratio ($P = 0.033$) (Table 3), random glycemia ($P = 0.022$), HbA_{1c} levels ($P = 0.046$), and prevalence of the 5'INS I/I genotype ($P = 0.038$) (Table 4) in the absence of HLA-DQ susceptibility genotypes. Furthermore, during follow-up, we found no differences in functional residual β -cell mass, frequency of insulin treatment, or insulin needs for similar glycemic control after stratification for HLA-DQ risk (susceptible, $n = 380$; protective/neutral, $n = 297$; data not shown).

Antibody-negative patients.

In contrast to antibody -positive subjects, antibody -negative patients did markedly differ in terms of clinical presentation and biological markers at diagnosis according to the presence or absence of susceptible HLA-DQ genotypes. Subjects carrying susceptible genotypes were overall younger (28 vs. 33 yr; $P = 0.001$), had a lower BMI (23 vs. 26 kg/m²; $P < 0.001$), and tended to have higher insulin requirements after stabilization (0.41 vs. 0.32 U/kg·d; $P = 0.014$) (Table 5) to achieve the same degree of glycemic equilibration as reflected by similar HbA_{1c} levels (Table 6). These patients were significantly more often classified as suffering from type 1 diabetes by their treating physician compared with subjects carrying protective or neutral HLA-DQ genotypes (78% vs. 57%; $P = 0.001$) (Table 5). There was a tendency toward lower random C-peptide values (1.13 vs. 1.51 μ g/liter; $P = 0.034$) in subjects with susceptible genotypes (Table 6). Amylase activity, but not lipase activity, was overall higher ($P = 0.001$) in subjects with the susceptible genotypes. However, this was not reflected in a higher prevalence of amylase activity above the upper limit of reference values (cut off, 113 U/liter).

TABLE 4 — Biological characteristics of 1198 antibody-positive diabetic patients aged 15-39 yr according to HLA-DQ genotype

Characteristics	HLA-DQ genotype		P value ^a
	Susceptible (n = 679)	Protective/neutral (n = 519)	
Ketonuria [no. of patients (%)]	380/514 (74)	298/397 (75)	0.755
Random glycemia ^c (mg/dl) ^b	343 (270-450)	364 (277-499)	0.022
HbA _{1c} ^d (SD from mean of local reference interval) ^b	10.0 (6.9-14.2)	10.9 (7.0-15.4)	0.046
Random C-peptide ^e (μg/liter) ^b	0.62 (0.37-1.02)	0.61 (0.34-1.06)	0.938
5'INS I/I genotype [no. of patients (%)]	428/631 (68)	354/480 (74)	0.038
Amylase ^f (U/liter) ^b	47 (39-56)	47 (39-56)	0.780
Lipase ^f (U/liter) ^b	73 (43-114)	76 (46-116)	0.192

^a Mann-Whitney *U* test was used for continuous variables and χ^2 test for categorical variables; the threshold for statistical significance is $P < 0.05/7$ or $P < 0.007$ (Bonferroni adjustment).

^c Data were available for 635 patients with susceptible and 474 patients with protective/neutral genotypes.

^b Median (interquartile range).

^d Data were available for 584 patients with susceptible and 435 patients with protective/neutral genotypes.

^e Data were available for 586 patients with susceptible and 455 patients with protective/neutral genotypes.

^f Data were available for 578 patients with susceptible and 450 patients with protective/neutral genotypes.

TABLE 5 — Demographic and clinical characteristics of 386 autoantibody -negative diabetic patients aged 15-39 yr according to HLA-DQ genotype

Characteristics	HLA-DQ genotype		P
	Susceptible (n = 82)	Protective/neutral (n = 304)	
Age at diagnosis (yr) ^b	28 (22-35)	33 (27-36)	0.001
Male-to-female ratio [no. of patients (ratio)]	57/25 (2.28)	202/102 (1.98)	0.695
Presence of one or more symptoms ^c [no. of patients (%)]	65/74 (88)	241/273 (88)	1.000
Duration of symptoms ^d (wk) ^b	4 (2-9)	6 (2-16)	0.147
BMI ^e (kg/m ²) ^b	23 (20-27)	26 (22-30)	<0.001
Daily insulin dose after stabilization ^f (U/kg) ^b	0.41 (0.21-0.66)	0.32 (0.00-0.50)	0.014
Initial classification as type 1 by treating physician [no. of patients (%)]	64/82 (78)	173/304 (57)	0.001

^a Mann-Whitney *U* test was used for continuous variables and χ^2 test for categorical variables; the threshold for statistical significance is $P < 0.05/7$ or $P < 0.007$ (Bonferroni adjustment).

^b Median (interquartile range).

^c Polyuria, polydipsia, weight loss, and/or fatigue.

^d Data were available for 65 patients with susceptible and 237 patients with protective/neutral genotypes.

^e Data were available for 67 patients with susceptible and 260 patients with protective/neutral genotypes.

^f Data were available for 58 patients with susceptible and 240 patients with protective/neutral genotypes.

Stepwise forward logistic regression analysis confirmed that in antibody-negative patients with onset in young adulthood, the presence of HLA-DQ susceptibility genes is associated with younger age at diagnosis ($P = 0.014$; OR, 1.052; 95% CI, 1.010-1.095) and independently of age also with BMI ($P = 0.017$; OR, 1.071; 95% CI, 1.013-1.133) and amylase activity ($P = 0.051$; OR, 0.983; 95% CI, 0.967-1.000). Likewise, when all 82 antibody-negative patients with HLA-DQ susceptibility genotypes were one by one matched to an antibody-negative patient of exactly the same age carrying a neutral or protective HLA-DQ genotype, thereby dissipating the age difference between both groups (median age, 28 yr; interquartile range, 22-35 yr, in both groups), the trend toward lower BMI (23 vs. 25 kg/m²; $P = 0.026$), more frequent classification as type 1 diabetes (78 vs. 60%; $P = 0.018$), lower random C-peptide values (1.12 vs. 1.65 μg/liter; $P = 0.009$), and higher amylase activity (50 vs. 47 U/liter; $P = 0.05$) in the presence of HLA-DQ susceptibility genotypes persisted (Table 7). Furthermore, after a median follow-up of 25 months (range, 20-60 months), antibody-negative patients still presented a lower BMI ($P = 0.001$) and tended to have higher insulin needs ($P = 0.073$) to achieve similar glycemic control in the

presence of HLA-DQ risk genotypes ($n = 29$) than in their absence ($n = 120$; data not shown). Neither at diagnosis nor after follow-up was there a significant difference in the frequency of insulin treatment between patients with or without HLA-DQ risk genotypes.

CLINICAL AND BIOLOGICAL CHARACTERISTICS ACCORDING TO OTHER VARIABLES

Antibody-negative patients with HbA_{1c} values above percentile 50 (*i.e.* HbA_{1c} \geq 10.3 SD above mean of local reference interval) presented symptoms more frequently ($P = 0.004$) and had a higher prevalence of ketonuria ($P < 0.001$), higher insulin requirements ($P < 0.001$), higher glycemia at diagnosis ($P < 0.001$), and lower random C-peptide levels ($P < 0.001$) compared with antibody-negative patients with HbA_{1c} values below percentile 50 (data not shown). Similar findings were observed in antibody-positive patients (data not shown) or when comparing patients with HbA_{1c} below percentile 33 and above percentile 66 (data not shown). Regardless of antibody and HLA-DQ status, there was no difference in the prevalence of the 5'INS I/I risk genotype according to BMI (patients with BMI $>$ percentile 50 vs. BMI $<$ percentile 50, or patients with BMI $>$ percentile 66 vs. BMI $<$ percentile 33) or in BMI according to 5'INS status (data not shown).

TABLE 6 — Biological characteristics of 386 autoantibody-negative diabetic patients aged 15-39 yr according to HLA-DQ genotype

Characteristics	HLA-DQ genotype		P value ^a
	Susceptible (n = 82)	Protective/neutral (n = 304)	
Ketonuria [no. of patients (%)]	33/64 (52)	97/212 (46)	0.501
Random glycemia ^c (mg/dl) ^b	309 (207-415)	291 (207-365)	0.130
HbA _{1c} ^d (SD from mean of local reference interval) ^b	8.6 (4.4-14.7)	9.7 (5.4-14.8)	0.651
Random C-peptide ^e (μg/liter)	1.13 (0.59-2.11)	1.51 (0.88-2.38)	0.034
5'INS I/I genotype [no. of patients (%)]	46/76 (61)	147/281 (52)	0.252
Amylase ^f (U/liter) ^b	50 (44-57)	44 (37-54)	0.001
Lipase ^f (U/liter) ^b	70 (38-113)	67 (37-114)	0.627

^a Mann-Whitney *U* test was used for continuous variables and χ^2 test for categorical variables; the threshold for statistical significance is $P < 0.05/7$ or $P < 0.007$ (Bonferroni adjustment).

^c Data were available for 71 patients with susceptible and 275 patients with protective/neutral genotypes.

^b Median (interquartile range).

^d Data were available for 67 patients with susceptible and 259 patients with protective/neutral genotypes.

^e Data were available for 81 patients with susceptible and 300 patients with protective/neutral genotypes.

^f Data were available for 80 patients with susceptible and 297 patients with protective/neutral genotypes.

TABLE 7 — Clinical and biological characteristics of 164 age-matched autoantibody-negative diabetic patients aged 15-39 yr according to HLA-DQ genotype

Characteristics	HLA-DQ genotype		P
	Susceptible	Protective/neutral	
	(n = 82)	(n = 82)	
BMI ^c (kg/m ²) ^b	23 (20-27)	25 (22-29)	0.026
Daily insulin dose after stabilization ^d (U/kg) ^b	0.41 (0.21-0.66)	0.36 (0.15-0.49)	0.195
Initial classification as type 1 by treating physician [no. of patients (percent)]	64/82 (78)	49/82 (60)	0.018
Random C-peptide ^e (μg/liter)	1.12 (0.59-2.11)	1.65 (0.99-2.50)	0.009
Amylase ^f (U/liter) ^b	50 (44-57)	47 (37-53)	0.050

^a Mann-Whitney *U* test was used for continuous variables and χ^2 test for categorical variables; the threshold for statistical significance is $P < 0.05/5$ or $P < 0.010$ (Bonferroni adjustment).

^c Data were available for 67 patients with susceptible and 70 patients with protective/neutral genotypes.

^b Median (interquartile range).

^d Data were available for 58 patients with susceptible and 67 patients with protective/neutral genotypes.

^e Data were available for 81 patients with susceptible and 81 patients with protective/neutral genotypes.

^f Data were available for 80 patients with susceptible and 82 patients with protective/neutral genotypes.

DISCUSSION

Adult-onset diabetes presents along a clinical spectrum ranging from acute ketosis-prone onset to latent forms diagnosed by the presence of chronic complications^{[1] [2] [3] [4] [5] [6] [7] [8] [9]}. It is often difficult to classify patients at diagnosis solely on clinical grounds^[8]. Therefore, both disease classification and etiology-driven therapy would benefit from the optimal use of biological markers related to the underlying pathological process at diagnosis.

In the present study, antibody-positive patients with primary diabetes diagnosed in adulthood were on average leaner and showed an earlier, more acute, and more severe clinical presentation, a less preserved β -cell mass, higher insulin requirements after stabilization, and a higher frequency of HLA-DQ and 5'INS risk genotypes than antibody-negative patients, in agreement with previous observations^{[4] [16] [17] [18] [19]}. Both HLA-DQ risk genotypes and a more severe metabolic decompensation occur more frequently in childhood than in adult-onset immune-mediated diabetes^{[3] [10] [12] [25] [26]}. We therefore wondered whether the overall less severe clinical presentation in adult-onset type 1 diabetes was primarily associated with the lower prevalence of HLA-DQ risk markers or rather with older age at diagnosis *per se*. Hence, we investigated phenotype-genotype relations in antibody-positive and antibody-negative diabetic patients diagnosed in (early) adulthood.

The presence of high-risk HLA-DQ genotypes is not a necessary condition for the development of immune-mediated diabetes because 43% of the antibody-positive diabetic patients lacked such risk markers. The prevalence of the high-risk HLA-DQ3/DR4 genotype in our population of childhood and adult-onset diabetic patients is similar to the prevalence reported in other studies^{[10] [12] [25] [39]}. Especially in the absence of susceptible HLA-DQ alleles, other genetic risk factors might be needed as suggested by the fact that the prevalence of the 5'INS I/I susceptibility genotype tended to be higher in antibody-positive subjects carrying neutral or protective HLA-DQ genotypes, as shown previously^[30]. In the presence of autoantibodies, we observed no differences in clinical or biological characteristics according to HLA-DQ risk; this is at variance with earlier Scandinavian reports of a milder disease onset in HLA-DQ3-positive type 1 patients^[41] and of a more severe presentation in association with an increased HLA-DQB1 linked genetic risk load in diabetic children^[42]. However, in these studies all patients were under age 20, and the different genotype groups had not been matched for age or antibody status, two important confounders. In line with our findings, a British study^[39] concluded that the HLA-DQ genotype background may not have a major role in disease severity in terms of insulin requirements once autoimmunity has been established in adult-onset diabetes.

In antibody-negative patients, HLA-DQ markers of immunemediated type 1 diabetes were overrepresented compared with the background population as reported previously^[39]. Antibody-negative subjects displayed more type 1-like features in the presence of HLA-DQ risk than in their absence; they developed hyperglycemia at a younger age in the presence of genetic markers associated with earlier disease onset in immune-mediated type 1 diabetes^{[10] [11] [12]}, were leaner overall, and tended to have less preserved C-peptide levels and higher insulin needs. These trends persisted even after adjustment for age at diagnosis and during follow-up. Not surprisingly, antibody-negative patients were more frequently classified as having type 1 diabetes in the presence of HLA-DQ susceptibility genotypes than in their absence by physicians unaware of HLA and antibody status at that time. In carriers of HLA-DQ risk genotypes, the clinical and biological characteristics are suggesting the existence of an immune-mediated β -cell destructive process despite the absence of diabetes-associated autoantibodies. In insulin-dependent diabetes, antibodies indeed sometimes appear only months to years after diagnosis^{[22] [23]}. It is conceivable that certain patients have seroconverted to antibody negativity in the preclinical phase or present other immune markers than those currently searched for^{[23] [43] [44]}. On the basis of previous observations, it can be safely concluded that in the age categories studied there are very few, if any, recent-onset diabetic patients negative for the diabetes-associated autoantibodies measured in the present study (ICA, GADA, and IA-2A) but positive for IAA (<1%)^[26] or for antibodies against a 38-kDa glycosylated islet cell membrane-associated protein (0%)^[43]. At variance with a smaller study in Italian patients diagnosed in the age range 30-54 yr^[38], but in line with the study of Pietropaolo *et al.*^[45], our results suggest that genetic risk markers known to promote the clinical onset of antibody -positive diabetes^{[1] [31]} may indicate the presence of an immune-mediated disease process with yet unknown immune markers in patients with a type 1-like phenotype, but negative for the presently used antibody markers.

A subgroup of Japanese insulinopenic antibody -negative patients with acute diabetes onset in adulthood, low HbA_{1c} levels, and elevated serum activities of pancreatic enzymes at diagnosis has recently been described^[19]. Neither the present large study nor preliminary reports^{[20] [21] [46]} could document the frequent occurrence of this type of idiopathic type 1 diabetes in Caucasians. In our Belgian antibody-negative Caucasian patients, a more severe metabolic decompensation, higher insulin needs, and less preserved β -cell mass were associated with high

rather than low HbA_{1c} levels, which is clearly discordant with the Japanese findings^[19] but in line with a preliminary European report^[20]. Furthermore, we did not identify subjects with elevated amylasemia or lipasemia among the subjects lacking genetic and antibody markers of immunemediated type 1 diabetes. Rather, amylase activity was lowest in antibody-negative subjects without HLA-DQ risk genotypes. It is unknown whether these lower amylase levels reflect increased enzyme clearance, decreased amylase synthesis due to relative acinar insulinopenia, or less leakage of enzymes from acinar cells^[34].

We have observed a higher reported prevalence of familial diabetes in antibody-negative subjects compared with antibody-positive patients regardless of HLA-DQ genotype. It may be warranted to search for mutations associated with maturity onset diabetes of the young and mitochondrial diabetes because these inherited forms of diabetes are likely to be more prevalent in antibody-negative subjects with a familial history of diabetes and lacking HLA-DQ risk genotypes^{[44][47][48]}.

The present report confirms the marked male-over-female excess in adult-onset diabetes^[14]. This excess tended to be more pronounced in antibody-negative patients compared with antibody -positive subjects. It is conceivable that in male subjects with already decreased β -cell function and/or mass increased BMI, a prominent risk factor for type 2 diabetes^[7], and the more visceral distribution of body fat may preferentially accelerate the clinical onset of diabetes as a consequence of higher metabolic demands due to increased insulin resistance^[14]. Alternatively, one may wonder whether a higher BMI may (in part) reflect a better ability to (partially) restore β -cell mass (and hence C-peptide and insulin levels) when confronted with ongoing β -cell destruction and/or peripheral insulin resistance^[49]. In the light of a recent report on the association between the 5'INS I/I and juvenile obesity, we failed to detect a possible relation between BMI and 5'INS genotype^[50].

In conclusion, antibody-positive adult-onset patients develop diabetes overall at a younger age and, regardless of HLA-DQ genotype, have a more severe clinical presentation at diagnosis than antibody-negative subjects. In the absence of autoantibodies, patients carrying HLA-DQ-linked risk have on average an earlier disease onset and, regardless of age, show more type 1-like features than subjects lacking HLA-DQ risk genotypes. Genetic risk markers of seropositive type 1 diabetes may thus indicate the existence of an immune-mediated disease process in patients lacking conventional diabetes autoantibodies. Idiopathic type 1 diabetes with acute onset and increased amylase activity is not a frequent phenotype in Caucasians.

Acknowledgments

We thank the Vlaamse Diabetes Vereniging, the Association belge du Diabète, and the following members of the Belgian Diabetes Registry for their invaluable help in recruiting diabetic patients and first-degree relatives and/or in the handling of blood samples: E. Anckaert, P. Arnouts, J.-P. Baeyens, E. Balasse, H. Becq, J. Beirincx, L. Berghmans, W. Bettens, A. Bocquet, A. Bodson, T. Boereboom, R. Bouillon, M. Buysschaert, A. Carlier, M. Cardon, A. Chachati, N. Christophe, S. Claes, I. Claey \ddot{s} , L. Claey \ddot{s} , P. Cochez, M. Coeckelberghs, I. M. Colin, J.-L. Coolens, P. Coremans, F. Coucke, W. Coucke, E. Couturier, R. Craen, S. Daens, C. Daubresse, J. De Cock, P. Decraene, I. De Feyter, R. De Hauwere, J. De Lepeleire, B. Delgrange, C. Delvigne, R. Demaeseneer, P. De Monie, G. De Proft, L. Derdelinckx, P. De Roeck, J. De Schepper, S. De Weer, P. De Winter, L. Dooms, E. Duvivier, L. Emsens, C. Ernould, A. Eykens, A. Fassotte, N. Gaham, K. Garmijn, J. Gérard, C. Gillet, I. Gios, P. Goetstouwers, C. Guiot, A. Haemers, S. Haemers, F. Hay, J. Helmer, C. Herbaut, G. Heremans, V. Immegeers, R. Janssen, P. Jopart, J. Koumans, G. Lamberigts, C. Lauwers, M.-C. Lebrethon, P. Lefèbvre, P. Lemmens, I. Leunckens, T.-T. Lim, C. Litvine, K. Logghe, M. Maes, W. Maes, Y. Maus, R. Meulepas, J. Michiels, J. Mockel, J. Monballyu, G. Moorkens, W. Musch, D. Nicolaij, G. Op de Beeck, F. Peiffer, M.-C. Pelckmans, H. Penninckx, O. Peters, M. Pieron, L. Pleysier, K. Poppe, P. Prims, A. Purnode, G. Raes, C. Righes, D. Rocour-Brumioul, M.-P. Roggemans, R. Rooman, C. Roose, B. Roth, J. Schets, L. Schillemans, I. Schoemaker, S. Schrans, J. Schutyser, O. Segers, J.-C. Sodoyez \ddot{t} , G. Somerst, D. Staessen, M.-P. Stassen, A. Stroobant, P. Taelman, L. Terriere, M. Tersago, J. Teuwen, G. Thenaers, P. Thielemans, G. Thiry-Counson, U. Timmermans, J. Tits, A. Torfs, K. Van Acker, A. Van Baarle, J. Van Boxem, P. Van Crombrugge, L. Van Damme, L. Van de Mierop, P. Van den Bogaert, E. Vandenbussche, B. Van der Henst, M. Vanderijst, H. Vanderstappen, W. Van Dessel, C. Van Deun, P. Van de Wouwer, D. Van Doom, E. Van Fleteren, J. Van Haegenborgh, M. Van Hecke, E. Van Hollebeke, W. Van Horen, S. Van Imschoot, R. Van Landeghem, F. Van Loon, S. Vanneste, D. Van Nimmen, C. Van Parijs, H. Van Ravensteyn, P. Van Rooy, P. Van Vaerenbergh, J. Verbeeck, R. Verbiest, C. Vercammen, E. Vercruyssen, K. ver Elst, A. Vertruyen, A. Verhaegen, H. Verhaegen, J. Vertommen, E. Weber, I. Weemaes, L. Wouters, and G. Wyffels. We are indebted to N. Alaerts, M. Bodson, V. Claessens, A. Demarré, T. Demesmaeker, L. De Pree, S. Exterbille, T. Ghysels, P. Goubert, C. Groven, A. Ivens, D. Kesler, F. Lebleu, G. Schoonjans, and H. Thomas for excellent technical assistance, (\ddot{t} , Deceased.)

JDF standard for ICA determination and cDNA for preparation of ³⁵S-recombinant human GAD65 were donated by Dr. Å. Lernmark (University of Washington, Seattle, WA) and by Dr. A. Falorni (when at the Karolinska Institute, Stockholm, Sweden). Human IA-2ic cDNA was a gift from Dr. M. Christie (King's College School of Medicine and Dentistry, London, UK).

This work was supported by the research council of the Vrije Universiteit Brussels (research fellowship to I.W.), the Belgian Diabetes Association (research fellowship to V.S.), the Belgian Fonds voor Wetenschappelijk Onderzoek (Grants 3-0113-97 and 6-0319-01, and LevenslijnDiabetes project 7.0021.96; postdoctoral research fellowship to C.M.), and by Bayer Corp., Life Scan, Novo Nordisk, Ortho Clinical Diagnostics, and Roche. The Belgian Diabetes Registry is supported financially by grants from the Ministries of Public Health of the Flemish and French Community.

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