Low frequency of HLA DRB1*03 – DQB1*02 and DQB1*0302 haplotypes in Romania is consistent with the country's low incidence of Type I diabetes

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Abstract

Aims/hypothesis. Our study aimed to determine the association of HLA class II *HLA-DQB1* alleles with Type I (insulin-dependent) diabetes mellitus and the frequencies of these alleles in the Romanian population, which has one of the lowest incidences of Type I diabetes in children aged 0–14 years in Europe at 3–4 cases per 100 000 person-years.
Methods. We used the sequence specific primer-polymerase chain reaction (PCR-SSP) technique to type *HLA-DQB1* alleles, the *HLA-DRB1* alleles DRB1*03 and one single nucleotide polymorphism (SNP) in the insulin gene (*INS*). We studied 204 Type I diabetic Romanian families, 196 of which were simplex with 70.3 % of subjects diagnosed under 14 years of age. Data was analysed using a modified version of the Transmission Disequilibrium Test, the Transmission Disequilibrium Test itself, and the affected family-based control method.
Results. We found, as expected, the strong positive DQB1*02-DRB1*03 and DQB1*0302, and negative

DQB1*0602, HLA class II allele associations with Type I diabetes in these Romanian families. However, using the affected family-based control method, we found relatively low population frequencies of DQB1*02-DRB1*03 and DQB1*0302 alleles in Romania (15.8 %) compared with Sardinia (31.3 %), a high incidence European region (35 cases per 100 000 person-years in children aged 0–14 years). The *INS* locus had a strong effect in this data set with 80.5 % transmission of the susceptible *INS* allele from parents to affected siblings (relative risk = 4.1).
Conclusion/interpretation. Part of the explanation for the low incidence of Type I diabetes in Romania could be the lower frequency of the DRB1*03 – DQB1*02 and DQB1*0302 susceptibility haplotypes in this country. [Diabetologia (2001) 44 [Suppl 3]: B 60–B 66]

Keywords Diabetes, *HLA DQB1, INS-VNTR*, genetics, AFBAC frequency, Romania.

Type I (insulin-dependent) diabetes mellitus or primary insulin dependent diabetes mellitus (IDDM) is

a common chronic autoimmune disease resulting from a T lymphocyte-mediated destruction of insulin-secreting pancreatic beta cells in genetically predisposed individuals. The almost complete destruction of beta cells results in total insulin dependency. The incidence of Type I diabetes in children and adolescents varies widely between different ethnic groups and even within ethnic groups from 3 – 45 cases per 100 000 person-years in Caucasians with a maximum of 45 cases per 100 000 person-years in 1996 in Finnish children younger than 14 years [1–4]. One of the lowest incidence rates of Type I diabetes in children in Europe is in the Romanian population

with less than 4 cases per 100 000 person-years [4,5]. The role of genes in Type I diabetes is essential but the penetrance of susceptibility or resistance alleles is greatly influenced by environmental factors which have not yet been identified. Diet and infection are likely sources [6,7,8,9,10].

Two susceptibility genes for Type I diabetes have been identified. The first is *IDDM 1*, encoded within the HLA region of the Major Histocompatibility Complex (MHC) on chromosome 6p21, and mapped to the *HLA-DRB1, -DQB1* and *-DQA1* loci [11–16]. Allelic variation of the exon 2 sequences of these genes probably accounts for the vast majority of the effect of *IDDM 1*. The second is *IDDM 2*, which has been mapped to the insulin gene region on chromosome 11p15 [17,18]. The *IDDM 2* is probably the variable number of tandem repeats (VNTR) locus 5' of the insulin gene [19]. The association of a third region, *IDDM 12*, close to the *CTLA4* locus on chromosome 2q, has been established, at least in some populations but the polymorphism responsible for this association has not yet been identified [20,21]. Other regions of the human genome have been linked to Type I diabetes but none of these putative disease genes has been identified [22,23,24].

The incidence of Type I diabetes in Romania is one of the lowest in Europe [4,5]. Until now, no typing for Type I diabetes susceptibility genes in the Romanian population has been undertaken. Our study aimed to evaluate the *IDDM 1* (*HLA-DQB1* alleles and an allele of *DRB1*, DRB1*03) and *IDDM 2* (*INS* –23 *Hph*I Single Nucleotide Polymorphism (SNP)) status in a large group of Type I diabetic Romanian patients. Using a family study approach, we typed 204 Romanian Type I diabetic families by employing a single-protocol method of genotyping for multiple HLA and non-HLA polymorphisms under identical conditions, with the SSP-PCR technique [25,26,27]. To compare results, we used data from another group of families with Type I diabetes from Sardinia [28], a "hot spot" in incidence on the European map [1,2].

Subjects and methods

Families. In this study 204 Romanian Type I diabetic families from all over Romania (Bucharest region and 12 other districts) were genotyped. Informed consent was obtained from all subjects involved before collection of blood for DNA extraction. The study was approved by the local ethics committee. Families were identified in the Bucharest "N.C. Paulescu" Institute outpatient department and in the diabetes centres of the other 12 Romanian districts. The selection of diabetic subjects was made according to the guidelines of EURODIAB epidemiological study protocol, that is those cases that had an abrupt onset with diabetic ketoacidosis or required insulin treatment with first insulin dose within the first month from diagnosis were diagnosed as having Type I diabetes [2]. Families

were selected if both parents were alive and willing to participate in the study. Most families (196) were simplex families (i.e. only one child was affected) but 3 were multiplex families (i.e. at least 2 siblings were affected) and 5 families had a parent/sibling pair affected. A total of 144 families were four-member families and the remaining 60 were three-member families. Overall the study group consisted of 756 individuals with 49.1 % (371) being males and 50.9 % (385) females. The patient cohort consisted of 212 Type I diabetic patients (106 males and 106 females) with the onset of disease between 9 months and 43 years of age. The mean age at the onset of disease was 12.1 ± 6.7 years. In most patients the onset of diabetes was before 17 years of age (166; 78.3 %) and the vast majority (149 out of 212; 70.3 %) had the onset before 14 years of age, consistent with the EURODIAB selection criteria [2]. Some of the subjects were therefore included in the EURODIAB epidemiological study between 1988–1997. There were 544 unaffected subjects (parents and unaffected siblings), 265 males (48.7 %) 279 females (51.3 %).

The 257 Sardinian families were all single subject families, except for 13 who had a parent affected and 10 who had a second sibling affected [28]. In the Sardinian families HLA typing was performed as described previously [28].

Selection of genes. In this study all individuals were typed for class II *HLA-DQB1* alleles at high resolution and for the *DRB1* allele DRB1*03. For the *HLA-DQB1* locus, we typed for HLA *DQ2* (DQB1*02), *DQ4* (DQB1*04), *DQ5* (DQB1*05), *DQ6* (DQB1*0601, *0602, *0603, *0604–9), *DQ7* (DQB1*0301, *0304), *DQ8* (DQB1*0302) and *DQ9* (DQB1*0303, *03 032) alleles. The DRB1*03 allele was typed to permit assignment of DQB1*0201 alleles to the DR3 haplotype, because the DRB1*03 and DQB1*0201 alleles are in almost complete linkage disequilibrium in the white European populations. Of note, the DQB1*02 – DRX assignment is an allele that is not the DRB1*03 haplotype but *DR7* or some other allele. In most European countries DQB1*02 alleles are usually found on the *DR3* or *DR7* haplotypes. We also typed one SNP of *INS* (chromosome 11p15): the –23 *Hph*I A/T substitution [19]. The VNTR, an array of 14–15 bp oligonucleotide sequences, has over 30 alleles but they cluster in two main length classes, denoted class I for up to 45 repeats and class III for over 200 repeats. The class I allele is in almost complete linkage disequilibrium with the "A" allele of the SNP at the –23 *Hph*I site of *INS*, and homozygosity of this class (or the –23 *INS* SNP) is associated with an odds ratio of 2–3 for Type I diabetes in white European populations [19,29].

DNA extraction. DNA was extracted from fresh peripheral EDTA anti-coagulated blood. We left 10 ml of EDTA anti-coagulated blood to settle on the bench for 1 h and then the plasma was discarded or stored at –80ºC. The remaining blood was mixed with Lysis buffer (109.5 g of Sucrose, 10 g of Triton-X-100, 1.02 ml of 4.9 mol/l $MgCl_2$, 1 ml of 1.0 mol/l TRIS-HCl, pH 7.4 up to 1 l with double distilled water) up to a total volume of 40 ml. The mixture was centrifuged for 15 min at 2500 rev/min at room temperature and the supernatant was discarded. The pellet was re-suspended in 20 ml Lysis Buffer by vigorously mixing the tube to ensure the pellet was completely homogenised before being centrifuged for 15 min at 2500 rev/min. After discarding the supernatant, DNA was extracted from this white blood cell pellet by adding to the pellet consecutively 3.5 ml of 6 mol/l guanidine hydrochloride, then 0.25 ml of 7.5 mol/l ammonium acetate and finally 0.25 ml of 20 % sodium N-lauroyl-sarcosinate. The tube was vortexed after each step until the pellet had dissolved. The pellet was incubated with 50 μl of 10 mg/ml Proteinase K (BDH 390373P) at 37 ^{0}C

over night. The tube was left to cool to room temperature and then 2 ml of chloroform were added and the tube vortexed thoroughly followed by centrifugation for 3 min at 2500 rev/min. The upper layer (DNA phase) was collected and added to 10 ml of absolute ethanol and chilled at –20°C for 1 h. The tube was gently inverted to precipitate the DNA and then centrifuged for 15 min at 3000 rev/min at room temperature to pellet the DNA. Finally, the DNA pellet was washed in 4 ml of 70% ethanol and centrifuged at 3000 rev/min for 5 min. The ethanol was poured off and the pellet left to dry for 1–2 h at room temperature. We added 300 μl of TE buffer pH 7.5 (1 ml of 1 mol/l TRIS, pH7.5, 20 μl of 0.5 mol/l EDTA, up to 100 ml with double distilled water) to the pellet which was then placed at 4°C until fully re-suspended. The DNA was quantified using PicoGreen double stranded DNA (dsDNA) Quantitation Reagent (Molecular Probes).

Primer design. All listed primers are 5' to 3'. PCR primers for *HLA-DQB1* allele analysis were as previously described [25,27]. The sequences of the primers used for typing of the –23 *Hph*I A/T polymorphism were (Genebank accession number J00265): T allele forward primer CCTCAgCCCTgCCTgTCT (annealing position 2384–2401); reverse primer gACgTgACCAAgAgAACTTC (annealing position 2761–2742), 100 ng/PCR amplification, 378 bp amplicon; A allele forward primer CCTCAgCCCTgCCTgTCA (annealing position 2384–2401); reverse primer gACgTgACCAAgAgAACTTC (annealing position 2761–2742), 100 ng/PCR amplification, 378 bp amplicon. For both alleles we used an internal control to validate amplification: *HLA-DRB1* intron 3 amplicon of 796 bp, forward primer TgCCAAgTggAgCACCCAA with annealing position exon 3 amino acid 173–179; reverse primer gCATCTTgCTCTgTTgCAgAT with annealing position in exon 4 amino acid 193–200, both 12 ng/PCR amplification. The primers used for DRB1*03 alleles amplification were: *DR3* forward primer 1 TACTTCCATAACCAGGAGGAGA, *DR3* forward primer 2 TTCCATAACCAGGAGGAGTC, *DR3* reverse primer 1 GTCCACCCGGCCCCGCT. We used the same primers for the internal control product as for *INS-Hph*I typing.

PCR amplification and electrophoresis. PCR amplification and electrophoresis for *HLA-DQB1* and *INS-Hph*I alleles was performed as previously described [25,26,27]. Genomic DNA was amplified in 13 μg reactions. For each PCR, the reaction mix consisted of base buffer (67 mmol TRIS base pH 8.8, 16.6 mmol ammonium sulphate; 0.01% v/v Tween); 2 mmol magnesium chloride; 200 mmol of each of dATP, dGTP, dCTP and dTTP; 0.1875 units of *Taq* polymerase (Advanced Biotechnology, London, UK) and between 40–60 ng DNA. DNA was briefly mixed with the buffer [26] and 8 μl of DNA/buffer mix was aliquoted onto 5 μl primer mix. All the reactions were performed under 10 μl mineral oil (Sigma, Dorset, UK) in 96 well PCR plates (Advanced Biotechnology). The DNA samples were amplified in MJ Research PTC-200 or GeneAmp 9600 PCR system (Perkin-Elmer Corporation, Calif., USA) thermal cyclers. The PCR plates were previously sealed with thermo-well sealers (Costar, Buckinghamshire, UK) and dipped in mineral oil in order to improve plate-to-block contact during amplification. The cycling parameters were 1 min at 96°C followed by 5 cycles of 96°C for 25 s, 70°C for 45 s and 72°C for 45 s, followed by 21 cycles of 96°C for 25 s, 65°C for 50 s and 72°C for 45 s, followed by 4 cycles of 96°C for 25 s, 55°C for 60 s and 72°C for 120 s. After PCR, 5 μl of loading buffer (0.25% Orange G, 30% v/v glycerol and 0.5xTBE buffer – TRIS/Boric acid/EDTA) was added to each reaction mix. Subsequently, PCR products were electrophoresed through 1% agarose gels containing 0.5 μg/ml ethidium bromide in

0.5xTBE buffer (89 mmol TRIS base, 89 mmol boric acid, 2 mmol EDTA, pH 8.0) for 20–30 min at 15 V/cm. Following electrophoresis, the products were visualised with UV illumination and the gel photographed with a Polaroid camera. Gel interpretation was simple and done according to the presence or absence of an amplicon. For validation of PCR amplification, all reactions included an internal control, a 796 or 256 bp product depending on the HLA amplicon size, as described previously [25,26,27].

The HLA-DRB1*03 allele was also typed using PCR-SSP under the following assay conditions: Genomic DNA was amplified in 13 μl reactions each comprising of 3 μl primer mix (2500 ng forward primer 1 and 2, 2500 ng reverse primer 1 and 750 ng of each of the internal control primers in a total volume of 300 μl made up to 3 μl with double distilled water) and 5 μl of 2 mmol/l TDMH (2.6 X buffer (Bioline) and 0.48 mmol of each of dATP, dGTP, dCTP and dTTP, and 5.2 mmol MgCl$_2$ in a total volume of 1 ml made up with double distilled water) and 0.06 μl BioTaq (Bioline) added to 5 μl DNA at 4 ng/μl. The reactions were performed in 96 well PCR plates (AB Gene, Surrey, UK) and plates were sealed with 0.2 ml micro-Caps (AB Gene, Surrey, UK). The DNA samples were amplified in MJ Research PTC-225 thermal cyclers. The cycling parameters were 1 min at 96°C followed by 5 cycles of 96°C for 20 s, 70°C for 45 s and 72°C for 25 s, followed by 21 cycles of 96°C for 25 s, 65°C for 50 s and 72°C for 30 s, followed by 4 cycles of 96°C for 30 s, 55°C for 60 s and 72°C for 120 s. After PCR, 5 μl of loading buffer was added to each reaction mix and the PCR products were electrophoresed through a 3% v/v 1.5% agarose and 1.5% Nusieve gel in 1xTBE buffer until bands in the 100–300 bp range were clearly resolvable. The products were visualised with UV illumination and the gel photographed. The gel was scored for the presence or absence of a 151 bp amplicon. For validation of PCR amplification, all reactions included the 796 bp internal control product.

Data analysis. Data were initially analysed using the Transmission Disequilibrium Test (TDT) [30]. The TDT tests the possibility that transmission of alleles from heterozygous parents to affected siblings is not the expected 50%. The TDT relative risk (RR) was calculated by dividing the number of times an allele was transmitted by the number of times it was not transmitted. Only alleles that were common enough to occur in 10 or more heterozygous parents were included. An alternative, but related, test is the affected family-based control (AFBAC) approach which assembles controls from the parental alleles not transmitted to affected siblings (or never transmitted in an affected sib-pair family) [31,32]. Allele and haplotype frequencies in these artificial controls are unbiased estimates of the frequencies in the general population from which the families were ascertained [31]. The AFBAC method requires that alleles are in Hardy Weinberg equilibrium (HWE), and does, to some extent, control for the genetic mismatching of patients and control subjects that can occur if control subjects are simply selected from the population as healthy volunteers [31]. All tested *HLA-DQB1* alleles and the two alleles of *INS – 23Hph* locus were in Hardy-Weinberg Equilibrium in the analysed families. All *p* values of less than 0.05 were noted.

The third method we used was a modified version of the extended TDT (ETDT) [33] referred to as the pairwise ETDT (PETDT) [34]. The PETDT estimates a Relative Transmission Probability (RTP) for each *DQB1* allele or haplotype compared to one fixed haplotype or allele. In the present report the *DQB1* allele often associated with the *DR1* haplotype, DQB1*05 was chosen as the reference haplotype. The DQB1*05 allele or haplotype is generally accepted as having a neutral effect in Type I diabetes and hence the application

Table 1. Transmission of *HLA-DQB1* alleles to affected children in Romanian families (TDT results), their AFBAC population frequencies and the results of PETDT analysis

DQB1 allele	PETDT				TDT		AFBAC
	RTP	p	T	NT	%T	p	AFBAC (%)
*0302	6.1	$8.7 \cdot 10^{-10}$	95	16	85.6	$6.5 \cdot 10^{-14}$	7.8
*02 – DR3	4.5	$2.5 \cdot 10^{-9}$	132	31	81	$2.6 \cdot 10^{-15}$	8.0
*0401/*0402	3.6	0.07	7	5	58.3	0.6	1.3
*02 – DRX	1.6	0.2	25	25	50	1	7.0
*05	1	1	53	84	38.7	0.008	27.7
*0604	0.98	0.98	8	12	40	0.4	3.1
*0601	0.3	0.06	5	12	29.4	0.09	3.1
*0301/*0304	0.3	$6.2 \cdot 10^{-5}$	14	96	12.7	$5.4 \cdot 10^{-15}$	25.3
*0603	0.2	0.002	4	29	12.1	$1.3 \cdot 10^{-5}$	7.5
*0303	0.1	0.004	1	17	5.6	0.0002	4.4
*0602	0.04	$4 \cdot 10^{-5}$	1	16	5.9	0.0003	4.1

RTP = Relative Transmission Probability; T = Transmitted; NT = Not transmitted; %T = Transmission percentage; AFBAC (%) = allele frequency estimated by AFBAC method

Table 2. Transmission of *HLA-DQB1* alleles to affected children in Sardinian families (TDT results), their AFBAC population frequencies and the results of PETDT analysis

DQB1 allele	PETDT				TDT		AFBAC
	RTP	p	T	NT	%T	p	AFBAC (%)
*0302	5.7	$1.1 \cdot 10^{-11}$	88	32	73.3	$3.2 \cdot 10^{-7}$	8.7
*02 – DR3	5.6	$4.1 \cdot 10^{-20}$	225	49	82.1	$2.1 \cdot 10^{-26}$	22.6
*02 (*DR4*)	2.6	0.1	6	8	42.9	0.6	1.6
*05	1	1	46	140	24.7	$5.5 \cdot 10^{-12}$	35.3
*02 – DR7	0.7	0.5	6	21	22.2	0.004	4.2
*0301	0.2	$2.9 \cdot 10^{-5}$	9	108	7.7	$5.6 \cdot 10^{-20}$	21.8

RTP = Relative Transmission Probability; T = Transmitted; NT = Not transmitted; %T = Transmission percentage; AFBAC (%) = allele frequency estimated by AFBAC method

of the PETDT allows haplotypes to be distinguished that are significantly more or less predisposing than DQB1*05 haplotypes. The null hypothesis is that the two haplotypes have the same relative risk. The RTP values give a clear indication of which alleles are predisposing and which alleles are protective, rather than neutral. In other words the PETDT ensures that allele are not classified as negatively associated with disease or protective, for example, simply because the other common alleles in the parents were highly susceptible DQB1*02 – DRB1*03 or DQB1*0302 alleles.

With 204 families, we had 80% statistical power to detect an odds ratio of 2 or greater at $p = 0.0001$ for a disease locus with minor allele frequency of 0.3 or greater. Because the odds ratios for certain alleles at *HLA* and at *INS* are greater than 2, we were confident this could replicate previous Type I diabetes associations at these loci.

Results

Table 1 shows the TDT, PETDT and AFBAC results for 11 *HLA-DQB1* alleles in 204 Type I diabetic Romanian families. For comparison, in Table 2 we show the results of the same tests for 6 alleles in 257 Sardinian families (the other 5 alleles were too rare in the Sardinian sample to be considered). Compared with DQB1*05, the predisposition of the DQB1*0302 and DQB1*02 – DRB1*03 alleles is the same in this

Romanian sample as in the Sardinian families with RTP values of between 4 and 6. That is, in Romanian and in Sardinian simplex families the *0302 and *02 – DRB1*03 alleles are 4 to 6 times more predisposing than the DQB1*05 allele ($p < 10^{-8}$). These two alleles, *0302 and *02 – DRB1*03 are transmitted 86% and 81% to affected siblings and have odds ratios from the TDT transmission data of 5.9 and 4.3, respectively (Table 1) compared to 73% and 82% transmission in the Sardinian families (Table 2). In the Romanian families, 5 alleles are less predisposing, that is protective, than allele DQB1*05 ($p < 0.005$): *0301/*0304, *0603, *0303, and *0602, all DQ β-chain Asp57 encoding alleles [14]. For example, the RTP value for *0602 was 0.04, in other words, *0602 is 25 times less predisposing in this data set than allele DQB1*05 ($p = 0.00004$). Allele DQB1*0602 is very rare in Sardinians but it is to be noted that the DQB1*0301 allele is equally protective in both populations and about four times less predisposing than DQB1*05 allele (Tables 1 and 2).

Importantly, all alleles, except DQB1*0303, were transmitted to unaffected Romanian siblings at a percentage transmission not very different from the expected 50% (Table 3). For DQB1*0303 the sample size was very small (10 transmitted and 1 not trans-

Table 3. Transmission of *HLA-DQB1* alleles to unaffected siblings in Romanian families (TDT results)

DQB1 allele	TDT			
	T	NT	%T	*p*
*0302	27	40	40.3	0.1
*02 – DR3	58	54	51.8	0.7
*0401/*0402	6	4	60	0.5
*02 – DRX	18	15	54.5	0.6
*05	41	43	48.8	0.8
*0604	7	9	43.8	0.6
*0601	6	2	75	0.2
*0301/*0304	37	40	48.1	0.7
*0603	10	10	50	1
*0303	10	1	90.9	0.007
*0602	6	5	54.5	0.8

T = Transmitted, NT = Not transmitted; %T = Transmission percentage

mitted; *p* = 0.007) and the apparent positive transmission to unaffected siblings will have to be re-evaluated in a larger data set.

Therefore, it appears that the relative susceptibility or protection of *HLA-DQB1* alleles is not different between the two countries, even though they have vastly different population incidences of Type I diabetes. Interesting then are the AFBAC data which indicate that the general population frequencies of the DQB1*0302 and DQB1*02 – DRB1*03 alleles are lower in Romania than in Sardinia, 15.8 % versus 31.3 % (Tables 1 and 2).

Finally, the *INS -23 Hph*I SNP data were analysed and a very strong association of the A allele or, conversely, a negative or protective effect of the T allele was observed in Romanian families (Table 4). At 80.6 % transmission (RR = 4.2) this is the same predisposition as the *HLA-DQB1* alleles *02 – DRB1*03 or *0302. Transmission of the A allele to unaffected siblings was not noticeably different than 50 % (45.8 %). The AFBAC control frequency was 77 % for the A allele, not noticeably different than that observed in other European countries e.g. 72.7 % in the UK and 79.2 % in Sardinia (Table 4), where the percentage transmission of the A allele is lower at 61.9 % and 62.4 %, respectively [19].

Discussion

Romania has one of the lowest incidences of Type I diabetes in children and in adolescents in Europe. Three explanations are possible. Firstly, the Romanian environment such as diet or infection pattern is not permissive for the development of Type I diabetes. Secondly, the main susceptibility haplotypes bearing the most strongly disease-associated *HLA-DQB1* alleles, *0302 and *02 – DRB1*03, are not as predisposing as non-Romanian *0302 and *02 – DRB1*03 haplotypes because the Romanian haplotypes carry alleles at other MHC loci that lower their predisposition to Type I diabetes. Finally, the overall population frequencies of the *0302 and *02 – DRB1*03 haplotypes are lower in Romania than in countries with higher Type I diabetes incidence rates. Our data suggest that the third possibility is part of the explanation because the combined frequency of the *0302 and *02 – DRB1*03 alleles is only approximately half (15.8 %) that of Sardinia, a high incidence country (31.3 %). For Sardinia, the AFBAC value of 31.3 % is almost identical to the HLA alleles frequencies obtained from typing newborn children from Sardinia (F. Cucca, unpublished data). The AFBAC approach is further validated by comparison of AFBAC *HLA-DQB1* allele frequencies from Type I diabetic affected sib-pair families and the allele frequencies from UK organ donors (J. A. Tood, K. Welsh, unpublished data). Interestingly, the AFBAC frequencies of *0302 plus *02 – DRB1*03 in the UK (an intermediate incidence country at approximately 15 cases per100 000 person-years) are higher than in Romania, at 19.3 % (J. A. Tood, unpublished data). Calculating the AFBAC frequencies of *HLA* alleles from several countries would throw light on any possible correlation between incidence and population frequency of *HLA* susceptibility alleles. Previously, a correlation has been found between *HLA-DQB1* alleles and population incidence of disease [35]. The relative frequency of protective alleles could also be a factor together with diet, and the level of infection. The role of diet, not addressed in this study, could well be a factor since the average diet in Sardinia and the UK can be expected, even today, to have a higher animal protein, fat and carbohydrate content than the diet in Romania. It will be interesting to see

Table 4. Transmission of *INS-Hph*I alleles to affected children in Romanian and Sardinian families (TDT results) and their AFBAC population frequencies

	TDT					AFBAC
	*INS-23Hph*I allele	T	NT	%T	*p*	AFBAC (%)
Romania	A	83	20	80.6	$5.4 \cdot 10^{-10}$	77.2
Sardinia	A	63	38	62.4	0.01	79.2

T = Transmitted; T = Not transmitted; %T = Transmission percentage; AFBAC (%) = allele frequency estimated by AFBAC method

if the incidence of Type I diabetes increases as the economy of Romania develops.

The other main finding of our study is the exceptionally strong association of the A allele of the *INS* –23 *Hph*I SNP, which is an accurate marker for the *INS* VNTR locus believed to be the *IDDM 2* locus. In our sample of Romanian families it was as predisposing as the most predisposing of *HLA-DQB1* alleles. This result requires confirmation in a second independent set of Romanian families. The increased penetrance of the protective effect of the T allele, which is thought to be the relevant trait at the *IDDM 2* locus, could be due to differences in the genetic background of the population at HLA or at other genes still to be identified. Or it could be due to changes in the environment that has increased the penetrance of the class III VNTR allele [19].

Another explanation could, however, be the existence of what has been designated as the Very Protective Haplotype (VPH) and the Protective Haplotype (PH), each with a different subset of class III alleles [19]. We could not distinguish the VPH and PH using our methods. If class III alleles belong more often to the VPH group in this Romanian cohort, this could explain the higher transmission of class I alleles to affected offspring from class I/III heterozygous parents. This would make the study of the association of the VNTR class III alleles in Romanian Type II diabetes patients important, as these allele have been associated with predisposition to Type II diabetes, rather than protection from, Type II diabetes [36].
Our results show the potential value of studying the genetics and gene-environment interactions of Type I diabetes in a country with a low disease incidence.

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