Retrovirus-Like Long-Terminal Repeat DQ-LTR13 and Genetic Susceptibility to Type 1 Diabetes and Autoimmune Addison's Disease

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Controversial data are available on the association between the retrovirus-like long-terminal repeat (LTR) DQ-LTR13 and genetic susceptibility to type 1 diabetes and other autoimmune diseases. We analyzed DNA samples from 315 type 1 diabetic patients, 166 autoimmune Addison's disease (AAD) patients, 1,054 healthy subjects, and 144 families of type 1 diabetic offspring. DQ-LTR13 was more frequent among patients than healthy subjects (P_c < 0.0006), and a preferential transmission of DQB1*0302-LTR13$^+$ from parents to type 1 diabetic offspring was observed. DQ-LTR13 was in linkage disequilibrium (LD) with DQB1*0302 but not DQB1*0201. The presence of DQ-LTR13 increased the odds ratio of DQB1*0302 2.9- to 3.2-fold for type 1 diabetes and AAD. DRB1*0403 was absent in all of the 169 DRB1*04-positive patients but present in 27% (34 of 127) DRB1*04-positive healthy subjects (P_c < 0.001). DQ-LTR13 was detected in 1 of 34 (3%) DRB1*0403-positive healthy subjects and 36 of 93 (39%) individuals carrying another DRB1*04 allele (P_c = 0.002). Multivariate logistic regression analysis revealed that DQ-LTR13 is not independently associated with type 1 diabetes and AAD after correction for DQB1*0302 and DRB1*0403. Conversely, DQB1*0201, DQB1*0302, DRB1*0401, and DRB1*0403 were all significantly associated with disease risk also after correction for DQ-LTR13. We provide conclusive evidence that the genetic association of DQ-LTR13 with type 1 diabetes and AAD is primarily due to a LD with DQB1*0302 and DRB1*0403. *Diabetes* 54:900–905, 2005

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Received for publication 1 September 2004 and accepted in revised form 6 December 2004.

AAD, autoimmune Addison's disease; LD, linkage disequilibrium; LTR, long-terminal repeat.

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Human endocrine autoimmune diseases, which include type 1 diabetes, autoimmune Addison's disease (AAD), thyroid diseases, autoimmune hypophysitis, hypoparathyroidism, and premature ovarian failure, are complex genetic traits with major contribution of HLA gene polymorphism (1,2). HLA-DRB1*03-DQA1*0501-DQB1*0201 is positively associated with most endocrine autoimmune diseases (2,3), while DRB1*04-DQA1*0301-DQB1*0302 is preferentially associated with type 1 diabetes (3), although it has been also found to be associated with AAD (4,5).

The highest genetic risk for childhood type 1 diabetes identified to date is that of the DRB1*03-DQA1*0501-DQB1*0201/DRB1*04-DQA1*0301-DQB1*0302 genotype, which is associated with clinical signs of the disease in 1:25–1:20 subjects (6). Genetic risk conferred by HLA class II haplotypes is modulated by the DRB1*04 subtype (7). DRB1*0403 confers dominant protection from type 1 diabetes even when part of the high-risk DRB1*03-DQA1*0501-DQB1*0201/DRB1*04-DQA1*0301-DQB1*0302 genotype (7–9).

At least 8% of the human genome consists of retrovirus-like elements (10), corresponding to 50–1,000 proviral copies of different full-length human endogenous retrovirus elements per haploid human genome, with up to 25,000 copies of solitary long-terminal repeats (LTRs) distributed on most chromosomes. One LTR (DQ-LTR3), integrated 15 kb upstream of the DQB1 gene, was initially found to be associated with genetic susceptibility for type 1 diabetes (11). However, some DQB1 alleles are characterized by a massive deletion of >5 kb, with absence of LTR3, and DQ-LTR3 is associated with human diseases only because of its presence with some DQB1 alleles (12). Another retrovirus-like element (LTR13) is located much closer to the DQB1 gene (1.3 kb upstream) (13), outside the deletion in the 5′ flanking region. LTR13s are primate specific and do not display sequence similarity to any known retroviral LTRs but have a structure that resembles that of other retroviral LTRs with a potential promoter, polyadenylation signal, and a tandemly repeated 53-bp enhancer-like sequence (14). An association between presence of DQ-LTR13 and risk for both type 1 diabetes and AAD has

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TABLE 1
Frequencies of DQ-LTR13 genotypes and HLA-DRB1, -DQA1, and -DQB1 alleles/haplotypes in type 1 diabetic or AAD patients and healthy control subjects

	Healthy control subjects (n = 1,056)	Type 1 diabetic patients (n = 315)			AAD patients				
					APS II/IV (n = 78)	Isolated (n = 88)	Total (n = 166)		
		n (%)	P_c	OR (95%CI)	[n (%)]	[n (%)]	n (%)	P_c	OR (95% CI)
DQ-LTR13 genotypes									
DQ-LTR13$^{-/-}$	874 (83)	216 (69)	<0.0002	0.45 (0.34–0.61)	54 (69)	64 (73)	118 (71)	0.001	0.51 (0.35–0.74)
DQ-LTR13$^{+/-}$	170 (16)	94 (30)	<0.0002	2.22 (1.66–2.97)	23 (29)	22 (25)	45 (27)	0.002	1.94 (1.33–2.83)
DQ-LTR13$^{+/+}$	12 (1.1)	5 (1.6)	NS	1.40 (0.49–4.01)	1 (1.3)	2 (2)	3 (1.8)	NS	1.60 (0.45–5.74)
HLA class II-LTR13 extended haplotypes									
DQA1*0301-DQB1*0302-LTR13$^+$	22 (2)	69 (22)	<0.006	13.2 (8.0–21.7)	15 (19)	18 (20)	33 (20)	<0.006	11.7 (6.60–20.6)
DQA1*0301-DQB1*0302-LTR13$^-$	64 (6)	21 (7)	NS	1.11 (0.66–1.84)	3 (3)	4 (5)	7 (4)	NS	0.68 (0.31–1.52)
DQA1*0501-DQB1*0201-LTR13$^+$	34 (3)	38 (12)	<0.006	4.12 (2.55–6.67)	12 (15)	17 (19)	29 (17)	<0.006	6.36 (3.76–10.8)
DQA1*0501-DQB1*0201-LTR13$^-$	107 (10)	112 (36)	<0.006	4.89 (3.61–6.64)	28 (36)	37 (42)	65 (39)	<0.006	5.71 (3.94–8.27)

NS, not significant.

preliminarily been reported (15,16), and it has been hypothesized that DQ-LTR13 might play a role in the pathogenesis of human autoimmunity (17). However, it is still unclear to what extent this association results from linkage disequilibrium (LD) with DRB1 and DQB1 alleles. Pascual et al. (12,18) reported that DQ-LTR13 was invariably associated with DQB1*0302, *0303, and *0402 in the Spanish population, while Krach et al. (19) reported the presence of DQ-LTR13 in German and Belgian subjects negative for these three alleles.

With the aim of testing whether DQ-LTR13 is independently associated with endocrine autoimmune diseases and/or modulates genetic risk conferred by DQB1 alleles, we analyzed a large set of genomic DNA samples from type 1 diabetic patients, AAD patients, and healthy control subjects from continental Italy, as well as complete families of type 1 diabetic children. Both type 1 diabetes and AAD were studied because of common HLA associations, similarities in pathogenesis, and previous reports on the role of DQ-LTR13 in genetic susceptibility for these diseases (15,16).

To address the problem of the association of DQ-LTR13 with DQB1 alleles in the Italian population, we first studied 200 healthy control subjects completely genotyped for HLA-DR and DQ and found 30 positive for DQ-LTR13 (15%). In subjects carrying DQ-LTR13, *0301 was the most frequent HLA-DQB1 allele (detected in 15 subjects [50%]), in line with its frequent occurrence in the Italian population (46% of our healthy control subjects). DQB1*0302 was found in five (17%) and DQB1*0303 in three (10%) DQ-LTR13–positive individuals. No DQ-LTR13–positive subjects were positive for DQB1*0402. A total of eight subjects (27%) were negative for DQB1*0301, *0302, and *0303, and, in this subgroup, *0201 was the most frequent DQB1 allele (detected in five subjects). In three DQ-LTR13–positive subjects, the DQB1*0501-*0602, DQB1*0502-*0503, and DQB1*0501-*0604 genotypes were detected. Thus, in continental Italy, DQ-LTR13 is not exclusively associated with DQB1*0302, *0303, or *0402 but can be found to be associated with DQB1*0201,*0301, and other DQB1 alleles.

We then expanded our analysis to determine the frequencies of high-risk alleles and haplotypes in 1,056 healthy control subjects, 315 type 1 diabetic patients, and 166 AAD patients. DQ-LTR13 was detected in 32% of type 1 diabetic patients and 28% of AAD patients compared with 17% of healthy control subjects ($P < 0.0001$ and $P = 0.0005$, respectively) (Table 1).

A permutation test using the EM algorithm showed that DQ-LTR13 is in LD with DQA1*0301-DQB1*0302 (exact P and χ^2 $P < 0.0001$) but not with DRB1*03-DQA1*0501-DQB1*0201. None of the tested variables showed significant deviations from Hardy-Weinberg equilibrium. Coexistence of DRB1*04-DQA1*0301-DQB1*0302 and DQ-LTR13 was demonstrated in 22% of type 1 diabetic patients and 20% of AAD patients compared with only 2% of healthy control subjects (Table 1). Frequency of DQ-LTR13 among DQA1*0301-DQB1*0302–positive individuals increased significantly from 26% (22 of 86) in healthy control subjects to 77% (69 of 90) in type 1 diabetic and 82% (33 of 40) in AAD patients ($P < 0.0001$). Accordingly, presence of DQ-LTR13 increased the odds ratio (OR) of DQA1*0301-DQB1*0302

TABLE 2
Combined transmissions of DQ-LTR13 with selected DQA1-DQB1 haplotypes in Italian families with a type 1 diabetic offspring

	Transmitted haplotypes	Nontransmitted haplotypes	P_{TDT}	P (LTR13$^+$ vs. LTR13$^-$)
n	192	192		
DQA1*0301-DQB1*0302-LTR13$^+$	36	4	0.0003	0.004
DQA1*0301-DQB1*0302-LTR13$^-$	29	19	0.41	
DQA1*0501-DQB1*0201- LTR13$^+$	4	4	1.00	0.71
DQA1*0501-DQB1*0201- LTR13$^-$	47	29	0.19	
X-LTR13$^+$	23	9	0.12	<0.0001
X-LTR13$^-$	53	127	<0.0001	

No parent carrying the X-X genotype was included in the analysis. X, not DQA1*0301-DQB1*0302, not DQA1*0501-DQB1*0201.

2.9- to 3.2-fold. Frequencies of simultaneous presence of DRB1*03-DQA1*0501-DQB1*0201 and DQ-LTR13 were similar to those predicted by random association (Table 1).

A total of 192 parents of type 1 diabetic offspring were found to be heterozygous for HLA-DR-DQ-LTR haplotypes. DRB1*04-DQA1*0301-DQB1*0302 was more often transmitted to affected offspring than expected, only in the presence of DQ-LTR13 (P_{TDT} = 0.0003; TDT, transmission distortion test) (Table 2). No significant transmission was observed for DQA1*0301-DQB1*0302-LTR13$^-$, DRB1*03-DQA1*0501-DQB1*0201-LTR13$^-$, and X-LTR13$^+$, in agreement with the results of the German-Belgian study (15).

A major genetic factor influencing susceptibility for type 1 diabetes is DRB1*0403, which has not been taken into consideration in previous works on DQ-LTR13 (15,16,18, 19). We analyzed the frequency of different DRB1*04 subtypes and the distribution of DQ-LTR13 among these subtypes (Table 3). DRB1*0403 was absent in type 1 diabetic and AAD patients but was the most frequent DRB1*04 allele in healthy control subjects (27% of DRB1*04-positive individuals) (P < 0.0001). This high frequency of *0403 in healthy control subjects was somewhat unexpected, as much lower frequencies have been observed in other European countries (7–9). The high prevalence of *0403 among DRB1*04-positive healthy control subjects, along with the low prevalence of high-risk class II haplotypes, can in part explain the lower incidence of type 1 diabetes in continental Italy compared with North European countries (20).

DRB1*0404 was not significantly increased in Italian AAD patients (Table 3), which is at variance with previous reports from the U.S. (4) and Norway (5). DRB1*0404 was not skewed toward non-DQB1*0302 alleles in our population, as it was found in 5 of 40 (12%) DQB1*0302-positive and 2 of 16 (12%) non-DQB1*0302 AAD patients. Similarly, DRB1*0404 was detected in 15 of 86 (17%) DQB1*0302-positive and 8 of 41 (19%) non-DQB1*0302 healthy control subjects. Differences in DRB1*0403 frequency in the general population may be responsible for the discrepancy between our study and other studies from different geographical areas.

DQ-LTR13 was detected in 1 of 34 (2.9%) DRB1*0403-positive healthy control subjects, a frequency that was lower than expected, as 36 of 93 (39%) subjects carrying another DRB1*04 allele were positive for this element (P_c = 0.002). This could not be explained by a low frequency of DQB1*0302, as 25 of 34 (74%) DRB1*0403-positive healthy control subjects were carrying DQA1*0301-DQB1*0302. The reasons why DQ-LTR13 is preferentially associated with some HLA haplotypes are not completely elucidated. We speculate that DRB1*0403 is in LD with a DQB1*0302 allele not carrying this element.

The low frequency of DQ-LTR13 in DRB1*0403-positive individuals is an important novel finding and raises the possibility that the association of DQ-LTR13 with type 1 diabetes and AAD is secondary to the negative association of DRB1*0403 with human diseases. To address this question, we performed multivariate logistic regression analysis. When DRB1*0403 was not included in the model, DQA1*0501-DQB1*0201 (P < 0.001, OR 5.91 [95% CI 4.40–7.94]), DQA1*0301-DQB1*0302 (P < 0.001, 3.91 [2.69–5.69]), and DQ-LTR13 (P = 0.014, 1.52 [1.09–2.13]) were all independently associated with type 1 diabetes after correction for age and sex. However, inclusion of DRB1*0403 substantially modified the outcome of logistic regression, as DQ-LTR13 no longer entered the model (Table 4). Inclusion of DRB1*0401 confirmed the secondary association of DQ-LTR13 with type 1 diabetes and showed the independent association of DRB1*0401 with type 1 diabetes. Similar results were observed with AAD patients (Table 4). Analysis of OR associated with DQ-LTR13 revealed

TABLE 3
Phenotypic frequencies of DRB1*04 alleles and DQ-LTR13 in type 1 diabetic patients, AAD patients, and healthy control subjects positive for DRB1*04

	Healthy control subjects (n = 127) [n (%)]*	Type 1 diabetic patients (n = 113)†		AAD patients (n = 56)‡	
		n (%)	P_c	n (%)	P_c
*0401	27 (21)	36 (31)	NS	15 (27)	NS
LTR13$^+$	14/27 (52)	26/36 (72)	NS	12/15 (80)	NS
*0402	31 (24)	45 (38)	NS	20 (36)	NS
LTR13$^+$	10/31 (32)	25/45 (56)	NS	15/20 (75)	NS
*0403	34 (27)	0	<0.001	0	<0.001
LTR13$^+$	1/34 (3)§	ND		ND	
*0404	23 (18)	21 (18)	NS	7 (12)	NS
LTR13$^+$	8/23 (35)	11/21 (52)	NS	4/7 (57)	NS
*0405	7 (6)	7 (6)	NS	7 (12)	NS
LTR13$^+$	2/7 (29)	4/7 (57)	NS	5/7 (71)	NS
Others	8 (6)	8 (7)	NS	8 (14)	NS
LTR13$^+$	2/8 (25)	5/8 (62)	NS	4/8 (50)	NS

*Including three subjects carrying the DRB1*04-*04 genotype (*0401-*0402, *0402-*0403, and *0403-*0404 genotypes); †including four patients carrying the DRB1*04-*04 genotype (*0401-*0402, *0401-*0402, *0401-*0404, and *0402-*0404 genotypes); ‡including one patient carrying the DRB1*04-*04 genotype (*0401-*0402 genotype); §P_c = 0.002 vs. non-0403 HS; P_c corrected P value for number of subtype and LTR13 subgroups versus healthy control subjects. ND, not determinable; NS, not significant.

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TABLE 4
Results of multivariate logistic regression analysis

Factors	Estimate	SE	Wald χ^2	P	OR (95% CI)
Type 1 diabetes					
DQA1*0501-DQB1*0201	1.786	0.153	136.0	<0.001	5.97 (4.42–8.05)
DQA1*0301-DQB1*0302	1.534	0.225	46.6	<0.001	4.64 (2.98–7.20)
DRB1*0401	0.743	0.351	4.48	0.034	2.10 (1.06–4.18)
DRB1*0403*	<−3.270	ND	>9.71	<0.002	<0.03 (0.004–0.29)
DQ-LTR13	0.206	0.181	1.29	0.256	1.23 (0.86–1.75)
AAD					
DQA1*0501-DQB1*0201	2.016	0.186	118.0	<0.001	7.51 (5.22–10.8)
DQA1*0301-DQB1*0302	1.255	0.263	22.7	<0.001	3.51 (2.09–5.88)
DRB1*0403*	<−2.473	ND	>5.34	<0.02	<0.08 (0.01–0.69)
DQ-LTR13	0.156	0.226	0.48	0.490	1.17 (0.75–1.82)

*Parameters for this allele could only be estimated because of the absence of DRB1*0403 among type 1 diabetic and AAD patients. ND, not determinable.

a reduction of genetic risk by 51–55% (in AAD and type 1 diabetes, respectively) when DQB1*0302 was included and by 78–80% (in type 1 diabetes and AAD, respectively) when DRB1*0403 was also included. Thus, at least 80% of genetic association between DQ-LTR13 and endocrine autoimmune diseases is explained by LD with DRB1 and DQB1 alleles.

In conclusion, although the risk conferred by DQB1*0302 is increased by the presence of DQ-LTR13, presumably because of the absence of DRB1*0403 in subjects positive for DQ-LTR13, this element did not provide any independent genetic risk in our study, and association of DQ-LTR13 with both type 1 diabetes and AAD appears to be secondary to LD with the DQB1 and DRB1 alleles. Although a minimal genetic contribution of DQ-LTR13 cannot be completely excluded, it is unlikely that this retrovirus-like element plays a relevant independent role in genetic susceptibility for type 1 diabetes or other endocrine autoimmune diseases.

RESEARCH DESIGN AND METHODS

Genomic DNA was obtained from 315 type 1 diabetic patients, 166 AAD patients, and 1,056 healthy control subjects. Type 1 diabetic patients (median age at diagnosis 13 years [range 1–49], M:F ratio 1.37) were consecutively recruited between 1 January 1993 and 31 March 2004 and were all residents of Umbria (central Italy). Diagnosis was made according to National Diabetes Data Group criteria (21). Islet cell autoantibodies were detected in >95% of type 1 diabetic patients. Our type 1 diabetic group included 85% of incident cases in Umbria during the period 1993–2003 who were <30 years of age. Type 1 diabetic case subjects who were >30 years of age at disease onset were consecutively recruited at the Department of Internal Medicine, Perugia, Italy. No type 1 diabetic patients were positive for adrenal autoantibodies.

For transmission analysis, 144 families of type 1 diabetic children were also studied. In each family, genomic DNA samples from the diabetic proband (median age at diagnosis 9 years [range 1–19], M:F ratio 1.29) and both parents were analyzed.

Between January 1998 and April 2004, the Italian Addison Network (22) enrolled 316 patients with primary adrenal insufficiency. According to a recent update of diagnostic criteria, based on autoantibody levels, imaging data, and biochemical parameters (22), 217 cases (69%) were classified as AAD. Of these 217 cases, DNA was available for 166. All 166 patients included in this study (median age at diagnosis 34 years [range 8–73], M:F ratio 0.63) were resident in central-northern Italy. Of these 166 patients, 78 (47%) had other autoimmune diseases. More specifically, 24 AAD patients (14%) also had type 1 diabetes. No APS I patient was included in our study.

Blood samples collected between March 1994 and February 2004 were available for 1,056 unrelated healthy control subjects (median age 32 years [range 4–63], M:F ratio 1.16) with no family history of endocrine autoimmune diseases. All subjects gave their informed consent for the study.

HLA-DR-DQ genotyping. HLA-DR-DQ genotyping was performed by SSO-dot blot analysis with modifications of a previously described method (23) using sequence-specific oligonucleotides that were 3′ end labeled with digoxigenin (Roche Diagnostics, Monza, Italy). A chemiluminescent signal, generated by using alkaline phosphatase–labeled anti-digoxigenin (Roche) and CSPD (Roche), was measured in a microplate scintillation/luminescence counter (TopCount NXT; Packard Instrument, Meriden, CT). Each membrane contained 10 control samples with known HLA genotype. HLA-DRB1*04 subtyping was performed by PCR-SSP (sequence-specific primer) according to Zetterquist and Olerup (24).

Detection of DQ-LTR13. Presence/absence of DQ-LTR13 was tested by a nested PCR approach as described by Pani et al. (16). External primers (5′-GGTCAGAAGTAATGTTTGCC-3′ and 5′-TAATGGTTATAAAGCAATTAGAAC-3′) were used to generate a 1,057-bp fragment in the presence or a 51-bp fragment in the absence of DQ-LTR13. The results were confirmed by using a pair of internal primers (5′-AGTAATGTTTGCCAGTCTGTAG-3′ and 5′-AATTAGAACAATGCCTGGTGTG-3′) that generates a 1,035-bp fragment. A third PCR with primers 5′-CCAGTCTCAGGTGCTCTAGAA-3′ and 5′-AGAAGCATTTCCTAGGTCCTGA-3′ generated a 1,530-bp fragment in the presence and a 532-bp fragment in the absence of DQ-LTR13. PCR fragments were separated in a 1.5% agarose gel and stained with ethidium bromide.

Statistical analysis. Differences in allele/haplotype/genotype frequencies between patients and healthy control subjects were tested by the χ^2 method. Yates' correction or Fisher's exact test was used when necessary. Probability values were corrected (P_c) for the number of comparisons according to the number of HLA-DRB1, -DQA1, and -DQB1 alleles or haplotypes observed or for the degree of freedom for DQ-LTR13 allele- and genotype-wise comparisons. Association of the dichotomous variables presence/absence of DQA1*0501-DQB1*0201, DRB1*0401, DRB1*0403, DQA1*0301-DQB1*0302, and DQ-LTR13 with endocrine autoimmune diseases, and dependence on other variables such as sex and age at diagnosis, was tested by multivariate logistic regression analysis using SPSS for Windows (SPSS, Chicago, IL). The transmission distortion test (25) was used to detect preferential transmission of DQA1-DQB1-LTR13–extended haplotypes to type 1 diabetic offspring. The χ^2 test was used to compare different subgroups and observed transmissions to random expected transmissions of 50%. Pairwise LD was tested by permutation test using the EM algorithm. Deviations from Hardy-Weinberg equilibrium were tested by comparison of observed and expected genotype frequencies. A P (or P_c) value <0.05 was considered significant in all tests.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Ministry of University and Scientific Research project “Morbo di Addison: forme cliniche, eziopatogenesi e storia naturale,” PRIN 20001063439 (to C.B. and A.F.), and funds from the Svenska Institutet, Stockholm, Sweden (to G.G.).

The following members of the Italian Addison Network contributed to the collection of data and blood samples from patients with primary adrenal insufficiency: B. Ambrosi (Milan), A. Angeli (Turin), G. Arnaldi (Ancona), E. Arvat (Turin), A. Baccarelli (Milan), L. Barbetta (Milan), P. Beck-Peccoz (Milan), A. Bellastella (Naples), A. Bizzarro (Naples), M. Boscaro (Ancona), F. Cavagnini (Milan), C. Dal Prà (Padova), A. De Bellis (Naples), F. Dotta (Rome),

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E. Ghigo (Turin), S. Laureti (Perugia), F. Loré (Siena), M. Mannelli (Florence), F. Mantero (Padova), G. Mantovani (Milan), P. Paccotti (Turin), F. Pecori-Gilardi (Milan), R. Perniola (Lecce), F. Santeusanio (Perugia), M. Terzolo (Turin), C. Tiberti (Rome), P. Toja (Milan), M. Torlontano (S. Giovanni Rotondo), V. Toscano (Rome), V. Trischitta (S. Giovanni Rotondo), R. Zanchetta (Padova).

The following members of the Umbria Type 1 Diabetes Registry contributed to the collection of data and blood samples from patients with type 1 diabetes: A. Angeli (Gubbio), E. Baiocchi (Assisi), D. Belladonna (Todi), R. Bellanti (Città di Castello), G. Berioli (Spoleto), M. Bracaccia (Orvieto), G. Campanelli (Città di Castello), G. Campolo (Todi), C. Cicioni (Terni), S. Coaccioli (Terni), A. Coletti (Gualdo Tadino), M. Cozzari (Cascia), G. Di Matteo (Perugia), G. Divizia (Spoleto), Ad. Falorni (Perugia), G. Fracassi (Marsciano), A. Fragasso (Foligno), A. Frascarelli (Assisi), S. Gagliardo (Narni), G. Giannico (Marsciano), A. Lilli (Gubbio), E. Madeo (Città della Pieve), G. Mancini (Orvieto), R. Marcacci (Castiglione del Lago), C. Marino (Gubbio), M. Napolitano (Foligno), G. Pennoni (Gualdo Tadino), E. Picchio (Perugia), M.L. Picchio (Gualdo Tadino), S. Pocciati (Foligno), F. Santeusanio (Perugia), M. Scattoni (Narni), E. Vignai (Città della Pieve).

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