CLINICAL STUDY

Number of autoantibodies and HLA genotype, more than high titers of glutamic acid decarboxylase autoantibodies, predict insulin dependence in latent autoimmune diabetes of adults

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Abstract

Objective: In latent autoimmune diabetes of adults (LADA), the progression into insulin-dependent diabetes is usually faster than in type 2 diabetes (T2D) but the factors influencing this progression are not completely known. In this study, we searched for sensitive markers associated with early development of insulin dependence.

Design: The screening of 5568 T2D patients for glutamic acid decarboxylase autoantibodies (GAD65Ab) identified 276 LADA patients (M=131; F=145) and in 251 of them, tyrosine phosphatase-2 (IA-2Ab) and thyroperoxidase autoantibodies (TPOAbs), some clinical features and genotype variation of the main type 1 diabetes (T1D) disease susceptibility loci (HLA-DRB1 and HLA-DQB1) were analyzed.

Results: Four years after the diagnosis of diabetes, high GAD65Ab titer was not significantly associated with faster progression toward insulin deficiency ($P=0.104$). Patients with GAD65Ab and TPOAb or IA-2Ab or triple positivity for both islet and TPOAbs (GAD65Ab/IA-2Ab/TPOAb) showed a significantly faster disease progression ($P=0.002$). Among 104 TPOAb-positive LADA patients, 10 received replacement therapy (L-thyroxine), 43 showed high TSH levels (62.7% developed insulin dependence), and 3 had hyperthyroidism treated with methimazole. Multivariate analysis revealed a significant effect on disease progression only for TPOAb ($P=0.022$), female gender ($P=0.036$), low body mass index (BMI; $P=0.001$), and T1D high/intermediate risk HLA-DRB1/DQB1 genotypes grouped ($P=0.020$).

Conclusions: High GAD65Ab titers *per se* are not a major risk factor for disease progression in LADA, while the number of positive autoantibodies and HLA DRB1-DQB1 genotypes at high risk for T1D are significant predictors. Moreover, clinical characteristics such as low BMI and female gender are more likely to identify patients who will require insulin therapy within 4 years of diagnosis.

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Introduction

About 5–10% of all patients diagnosed with type 2 diabetes (T2D) have markers of β -cells autoimmunity, such as glutamic acid decarboxylase (GAD65Ab) and tyrosine phosphatase-2 autoantibodies (IA-2Ab) (1–4). This form of 'autoimmune' diabetes, defined by Zimmet (5) as 'latent autoimmune diabetes of adults' (LADA), shows clinical features somewhat intermediate between classic early-onset type 1 diabetes (T1D) and T2D, and is characterized by the absence of ketoacidosis and insulin therapy for variable periods of time (6). The progressive destruction of β -cells is usually slower in LADA than in

T1D, and has been reported to be faster in the presence of high GAD65Ab titers (7). The existence of distinct levels of GAD65Ab in subgroups of LADA patients was recently highlighted in a large Italian study, which reported a clear bimodal distribution of GAD65Ab titers as well as the association of higher titers with a clinical phenotype more similar to T1D (8). Although this finding may suggest an intrinsic heterogeneity of LADA, no consistent data about a possible influence of GAD65Ab titers on progression toward insulin dependence was provided in that study. Some authors agree that a high titer of GAD65Ab is associated with a shorter insulin-free period, whereas others do not

support this view (9). However, the presence of multiple β -cell autoantibodies such as GAD65Ab and islet cell antibodies (ICA) (10) or GAD65Ab and IA-2Ab (11) in adult patients diagnosed with T2D is highly correlated with a faster decline of islet function.

The potential of several factors such as C-peptide (12), age at diagnosis, and several lipid parameters (13) to predict progression toward insulin dependence has been investigated previously, but conflicting data have been reported so far due to the limited size of LADA cohorts analyzed and/or ethnic admixture (14). Inconsistent data has also been reported about the prevalence and impact of metabolic syndrome (MetS) in the clinical course of LADA (15). Besides this, some genetic markers, e.g. combinations of specific human leucocyte antigens (HLA) class II DRB1–DQB1 haplotypes and genotypes, have been found to be associated with the risk of progression in T1D. This raises the question whether variation at these loci also modulates the probability that a LADA patient will require insulin therapy sooner.

With the aim to inquire these LADA-related issues as well as to identify more sensitive markers associated with early development of insulin dependence – which may enable the physician to stratify the risk for insulin dependence in this form of diabetes – we retrospectively evaluated the clinical course during 4 years of observation of a large cohort of LADA patients from the ethnically homogeneous population of Sardinia (2).

Patients and methods

Patients

At the end of 2005, we started a multicentric study of T2D patients (age range 35–70 years) referred to five Diabetic Units of the island of Sardinia, Italy, to screen for the presence of pancreatic islet autoantibodies. Out of 5568 T2D patients, 276 (4.9%) patients were GAD65Ab positive, and 251 of them gave their consent to participate in the study, which was approved by the local ethics committee. The duration of diabetes in these patients dated back from <8 months to no longer than 5 years. None of the patients showed evidence of ketoacidosis and/or significant weight loss, nor had insulin therapy for at least 8 months from diagnosis. All patients were of Sardinian origin for at least two generations, and none of them were affected by severe liver and/or renal disease. Age and gender were recorded at diagnosis, whereas other clinical parameters including height, weight, body mass index (BMI), abdominal circumference, fasting glucose, creatinine, lipid profile (total and high-density lipoprotein (HDL) cholesterol, and triglycerides), uric acid, HbA1c, systolic, and diastolic blood pressure were collected from the clinical records provided by the referring Diabetic Units for a period between 12 and 18 weeks after the diagnosis of diabetes and the start of treatment to

ensure adequate metabolic control. C-peptide was measured only in a limited number of patients whose sera had been available at diagnosis. All study participants were tested for MetS according to the International Diabetes Federation (IDF) worldwide consensus (16).

Autoimmunity assessment

All GAD65Ab-positive sera were also tested for IA-2Ab as well as other organ-specific autoantibodies such as anti-transglutaminase antibodies (tTGABs) and anti-thyroperoxidase antibodies (TPOABs). The results have been entered into ordinal variables taking into consideration single (only GAD65Ab), double (GAD65Ab + another autoantibody), or triple positivity (GAD65Ab + two additional autoantibodies). In 2510 GAD65Ab-negative T2D patients, randomly selected from the 5568 T2D patients already screened, TPOAb and tTGAb were screened as well. TSH plasma level was measured by IRMA (TSH-US IRMA CT, Radim S.p.A., Rome, Italy) in all LADA patients as well as in a random sample of 251 GAD65Ab-negative T2D patients.

Both β -cell antibodies were measured using a radio-binding assay with *in vitro*-translated [3 S]methionine-labeled GAD65 or IA-2 (17). Results were expressed as an index defined as the ratio between the difference of counts per minute between sample and standard control (18). The % intra-assay coefficient of variation of GAD65Ab and IA-2Ab for control samples designated at 35 anti-GAD65 units was 5.7 and 10% respectively (3). All GAD65Ab results were double checked by an independent laboratory (University of Perugia, Italy), using an RIA that uses 125 I-radiolabeled human GAD65 (RSR Ltd, Cardiff, UK), with a 100% concordance of positive/negative results and a high correlation of antibody titers between the two assays ($r=0.936$; $P=0.03$). tTGAb and TPOAb were tested by RIA using commercial kits (Medipan, Berlin, Germany). During the insulin-free period, most patients were treated with nutritional therapy and, when necessary, with hypoglycemic agents. The progression toward β -cell failure was estimated through time (in months) between diagnosis and the start of the insulin therapy as soon as patients showed postprandial glucose levels above 180 mg/dl despite maximum allowed dose of hypoglycemic drugs, significant body weight loss in spite of the consumption of the usual diet, or the appearance of ketosis.

HLA genotyping

HLA class II genotyping of the HLA-DRB1, DQA1, and DQB1 genes was performed by dot-blot analysis with sequence-specific oligonucleotide probes as previously reported (19). The HLA class II genotypes were ranked as high, intermediate, or low risk for T1D (19). The high-risk category included persons with two high-risk haplotypes; the intermediate risk category was

constituted mainly by individuals carrying genotypes given by the combinations of one high risk and one permissive-neutral haplotype while the low risk category encompassed genotypes constituted by two copies of negatively associated haplotypes or combinations of negatively associated and neutral haplotypes.

Statistical analysis

SPSS statistical software (version 16.0, Chicago, IL, USA) was used to perform most analyses. Power analysis was performed with EpiCalc 2000 version 1.02. Results were expressed as means $\pm$ s.d. or frequencies. Statistical differences between groups of quantitative variables were tested using nonparametric Mann–Whitney *U* test. Differences in frequencies were tested by the χ^2 test with Yates' correction or exact Fisher test, whenever appropriate. Patients were divided into two groups according to the GAD65Ab mode titers (high- or low anti-GAD65Ab titers based on a cut-off index 0.5 corresponding to the sample median). Receiving operating curve (ROC) analysis was also performed to evaluate the overall performance of GAD65Ab titer in discriminating patients who required insulin therapy and to find an appropriate cut-off. Kaplan–Meier (KM) curves were plotted, and log-rank test was performed to identify possible markers capable to influence the progression into insulin dependence. A multivariate Cox proportional hazard model was also constructed and, apart from gender and age at diagnosis, other variables such as number and titer of autoantibodies, and the HLA-associated genetic risk, were included in the model. Hazard ratios (HRs) were computed, and the log-likelihood ratio test was used for model testing.

Results

Autoantibodies and clinical parameters in patients and controls

The screening for GAD65Ab in 5568 T2D patients diagnosed between mid-2000 and the end of 2005 revealed the presence of 276 GAD65Ab-positive subjects (4.9%). The number of males and females were 131 (47.4%) and 145 (52.6%) respectively, with a F/M ratio of 1.1. Among GAD65Ab-negative subjects, the number of males and females were 1160 (46%) and 1350 (54%) respectively. In total, 251 patients out of 276 gave their informed consent for further investigation. The patients who refused to participate in the study showed antibody titers and phenotypic characteristics very similar to those who eventually continued the study. The clinical characteristics of patients who participated in the study, subdivided by GAD65Ab titers, are reported in Table 1 compared with those of 2510 GAD65Ab-negative T2D patients. Most of the clinical parameters in the LADA cohort as a whole showed average values distinctly different from T2D, especially BMI, waist circumference, blood pressure, the frequency of MetS, and TPOAb positivity. The distribution of GAD65Ab titers among all LADA patients showed two modes with a peak around the log-index value of -2.0 and another around $+1.0$ respectively (Fig. 1). The number of patients with high (index ≥ 0.5) and low (index < 0.5) GAD65Ab titer was 127 (51%) and 124 (49%) respectively; 53 patients exhibited also positivity for IA-2Ab (21% of the total) and 104 (41%) were positive for TPOAb. Only one female patient resulted positive for serum tTGAb (0.4%). The comparison of phenotypic characteristics between high and low GAD65Ab titer groups in LADA patients showed

Table 1 Clinical and laboratory variables (mean $\pm$ s.d.) in latent autoimmune diabetes of adults (LADA) patients (subdivided according to GAD65Ab titer) and GAD65-negative type 2 diabetes (T2D) controls.

Variables	Low titer GAD65Ab	High titer GAD65Ab	LADA	GAD65Ab negative T2D
Number of cases (%)	124 (49)	127 (51)	251	2510
Mean age at diagnosis (years)	55.4 $\pm$ 11.7	53.4 $\pm$ 10.9	54.3 $\pm$ 11.2	57.7 $\pm$ 10.1 [†]
Mean age at recruitment (years)	56.0 $\pm$ 12.8	54.5 $\pm$ 10.4	55.2 $\pm$ 11.6	58.1 $\pm$ 11.9
Gender (M:F)	59:65	58:69 [‡]	117:134	1160:1350 [‡]
Body mass index (kg/m ²)	28.8 $\pm$ 4.9	26.9 $\pm$ 4.6*	27.7 $\pm$ 4.8	30.8 $\pm$ 6.1 [†]
Waist circumference (cm)	97.5 $\pm$ 11.8	94.9 $\pm$ 10.1	96.0 $\pm$ 10.8	101.1 $\pm$ 12.1 [†]
Total cholesterol (mg/dl)	206.9 $\pm$ 44.5	213.5 $\pm$ 43.8	210.9 $\pm$ 44.0	220.9 $\pm$ 54.4 [†]
HDL cholesterol (mg/dl)	55.1 $\pm$ 14.0	55.8 $\pm$ 18.1	55.5 $\pm$ 16.5	48.3 $\pm$ 21.4 [†]
Triglycerides (mg/dl)	118.2 $\pm$ 76.0	117.6 $\pm$ 65.1	117.7 $\pm$ 69.3	123.2 $\pm$ 108.2
Fasting glucose (mg/dl)	134.3 $\pm$ 28.1	136.8 $\pm$ 31.4	135.2 $\pm$ 29.5*	138.8 $\pm$ 27.4
HbA1c (%)	7.3 $\pm$ 2.7	6.5 $\pm$ 1.9	6.9 $\pm$ 2.4*	7.1 $\pm$ 1.8
Uric acid (mg/dl)	5.2 $\pm$ 3.3	4.7 $\pm$ 1.7	4.9 $\pm$ 2.1*	5.3 $\pm$ 2.8
Metabolic syndrome (%)	59 (47)	47 (37) [‡]	106 (42)	2184 (87) [§]
Systolic blood pressure (mmHg)	135.3 $\pm$ 20.4	133.8 $\pm$ 18.8	134.3 $\pm$ 19.4	141.4 $\pm$ 7.9 [†]
Diastolic blood pressure (mmHg)	82.3 $\pm$ 10.9	81.3 $\pm$ 9.6	81.7 $\pm$ 10.1	87.3 $\pm$ 12.6 [†]
TPOAb positive (%)	42 (34)	62 (49)	104 (41)	290 (11) [§]

*High versus low GAD65Ab titer ($P=0.008$, Mann–Whitney *U* test). [†]LADA versus GAD65Ab-negative T2D ($P<0.001$, Mann–Whitney *U* test). [‡]NS (Fisher's exact test). [§] $P<0.0001$ (Fisher's exact test). ^{||} $P=0.020$ (Fisher's exact test).

Figure 1 Bimodal distribution of $\log_e$ (GAD65Ab index) among 251 LADA patients.

a significant difference only in BMI ($P=0.008$) and in the frequency of TPOAb positivity ($P=0.001$; Table 1).

Among the 104 TPOAb-positive LADA patients, ten received replacement therapy (L-thyroxine, L-T₄), and three patients were diagnosed with hyperthyroidism requiring methimazole treatment. In other 43 patients, TSH values above the cut-off limit of 4.0 mU/l were detected (41.3%), whereas this percentage was as low as 4.5% in GAD65Ab-negative T2D patients. These patients had mild biochemical hypothyroidism, and did not receive L-T₄ treatment.

Progression to insulin dependence

The proportion of LADA patients who required insulin therapy within 4 years of diagnosis was 37.4%. Table 2 shows the phenotype of LADA patients according to their progression into insulin dependence. HDL cholesterol levels and the frequency of TPOAb positivity were significantly higher, whereas BMI and waist circumference were significantly lower in patients who progressed into insulin dependence. Four out of ten LADA patients who were treated with substitutive hormonal therapy, two out of three patients with hyperthyroidism, and 27 out of 43 (62.7%) with high TSH levels developed insulin dependence within 4 years. Moreover, 16 LADA patients out of 61 (26%) with TSH levels <4.0 mU/l progressed toward insulin dependence. All other variables were similar in both groups.

The KM curves for LADA patients subdivided according to GAD65Ab mode titers adjusted for age and sex are represented in Fig. 2a where no significant difference (log-rank test: $P=0.104$) was observed. Figure 2b and c show KM curve for patients subdivided by IA-2Ab and TPOAb positivity revealing a significant association between double antibody positivity and a faster insulin dependence (GAD65Ab+/IA-2Ab+) (log-rank test: $P=0.020$) or (GAD65Ab+/TPOAb+) (log-rank test: $P=0.007$). Interestingly, regardless of GAD65Ab titers, LADA patients with triple positivity for both islet and thyroid antibodies (GAD65Ab/IA-2Ab/TPOAb) significantly showed faster disease progression ($P=0.001$).

As reported in Fig. 3a and b, KM analysis showed significant association (log-rank test: $P=0.001$) between double antibody positivity (GAD65Ab+/TPOAb+) with a faster insulin dependence in IA-2Ab-negative patients, whereas the association of double antibody positivity (GAD65Ab+/IA-2Ab+) was not significant in TPOAb-negative patients.

HLA-DRB1 and HLA-DQB1 genotypes that confer either high or intermediate risk to T1D were over-represented among LADA patients who required insulin therapy 4 years or less following diagnosis ($P=0.036$, Fisher's exact test; Table 2), and KM analysis showed association with progression of diabetes (log-rank test: $P=0.013$; Fig. 4).

Cox regression analysis (Table 3) showed that the four most relevant factors for future disease progression were a low BMI ($HR=0.853$; $P=0.001$), HLA-associated genetic risk (1.473 ; $P=0.020$), TPOAb ($HR=2.018$; $P=0.022$), and female gender ($HR=0.520$; $P=0.036$). On the contrary, IA-2Ab ($HR=1.491$; $P=0.236$) positivity and age at diagnosis ($HR=0.981$; $P=0.209$) were not significantly associated. Finally, C-peptide levels measured in a limited number of LADA patients at diagnosis of diabetes did not differ between the two groups and did not influence the progression of diabetes (data not shown). A simple predictive score was constructed on the basis of these four variables giving one point for being a female and additional points for having a BMI in the lower tertile, a positivity for TPOAb

Table 2 Clinical and laboratory variables in latent autoimmune diabetes of adults patients (subdivided according to the progression into insulin dependence within 4 years).

Variables	Insulin independent	Insulin dependent	P value
Number of cases (%)	157 (63)	94 (37)	
Mean age at diagnosis (years)	54.6 ± 10.6	54.1 ± 11.4	NS ^a
Gender (M:F)	81:76	36:58	0.049 ^b
Fasting plasma glucose (mg/dl)	135.2 ± 29.5	138.8 ± 27.4	NS
HbA1c (%)	6.9 ± 2.4	7.1 ± 1.8	NS
Body mass index (kg/m ²)	29.1 ± 5.4	25.8 ± 3.6	0.0001 ^a
Waist circumference (cm)	98.5 ± 9.0	91.4 ± 12.8	0.032 ^a
Total cholesterol (mg/dl)	210.7 ± 47.9	209.2 ± 39.8	NS
HDL cholesterol (mg/dl)	52.0 ± 12.2	57.6 ± 14.9	0.015 ^a
Triglycerides (mg/dl)	117.7 ± 53.4	112.9 ± 85.3	NS
MetS (%)	70 (44)	36 (38)	NS
Systolic blood pressure (mmHg)	134.4 ± 20.8	132.8 ± 16.9	NS
Diastolic blood pressure (mmHg)	82.7 ± 10.5	80.4 ± 9.4	NS
TPO positivity (%)	55 (35)	49 (52)	0.008 ^b
HLA-related risk	66 (42)	53 (56)	0.036 ^b

^aMann-Whitney U test.

^bFisher's exact test.

Figure 2 Kaplan–Meier curve showing the effect of the GAD65Ab titer (a), IA-2Ab (b), and TPOAb (c) for the subsequent development of insulin dependence in LADA cohort.

and a 'high risk' HLA genotype. Half of patients scored <2 , and the other half 2. This scoring system was highly predictive for the subsequent development of insulin deficiency (log-rank test, $P=0.00019$).

This score did not correlate significantly with the GADA levels ($r=0.011$; $P=0.204$).

Furthermore, we observed that the percentage of sulfonyl urea-treated LADA patients in 'insulin-dependent' and 'noninsulin-dependent' subgroups were comparable (28 vs 26%; NS). Besides, markers of metabolic controls, such as fasting glucose and HbA1c, were not significantly different in either groups (Table 2).

Finally, the proportion of LADA patients who met the criteria for MetS was 42% in the whole cohort, while in the low and high GAD65Ab groups were 47 and 37% respectively. A tendency of a lower proportion of patients with MetS was found among those who required insulin early, although the rate of progression did not differ between patients with or without MetS according to KM analysis (log-rank test; $P=0.209$). Actually, a significantly increased waist circumference was observed in patients who did not progress into insulin dependence (Table 2).

Discussion

In our cross-sectional study, we observed a clear bimodal distribution of GAD65Ab values in agreement with the data reported previously (8). Analysing the clinical characteristics in the 'high titer' versus 'low titer' GAD65Ab subgroups, we found the existence of a modest phenotypic heterogeneity since only BMI displayed a significant difference between the two groups, while triglyceride, total and HDL cholesterol levels, and blood pressure were not different, in contrast with what previously reported (13). On the other hand, a wide difference was found between LADA patients as a whole and GAD65Ab(–) T2D patients, as reported by several studies (20, 21). An interesting question is whether high GAD65Ab titers are also capable to predict β -cell exhaustion.

In our study, we analyzed the predictive value of GAD65Ab titers in relation to the progression of the disease into insulin dependence taking advantage of careful clinical observations spanning 4 years. The choice of a 4-year period is due to the need to evaluate the mid-term evolution of the ongoing destructive process against β -cells. Our data revealed a slight but not significant association of GAD65Ab titer with faster progression of diabetes toward insulin deficiency. However, given the limited statistical power of our sample size, this data must be taken cautiously. In our comparisons between high and low GAD65Ab titer groups, as far as the disease progression is concerned, we had only 9% points and a proportion of about 50%, so that the statistical power was 40% for a one-sided test. The poor predictive value of high GAD65Ab levels into disease progression also persisted when several cut-off values were tested. In fact, although ROC analysis identified better discriminating values of cut-offs ≥ 0.5 , Cox analysis failed to reveal any significant

Figure 3 Kaplan-Meier curve showing the effect of IA-2Ab in TPOAb negative (a) and of TPOAb in IA-2Ab negative (b) for the subsequent development of insulin dependence in LADA cohort.

improvement in predictive value of GAD65Ab titer when all other confounding variables were included in the model (data not shown).

Several authors reported a strong predictive power of high GAD65Ab levels in the progression of LADA toward insulin dependence (11, 22). However, in these studies, regression analysis was not performed to control potential confounding variables. Where such analysis was carried out in large cohorts (9), the predictive role of high GAD65Ab levels resulted to be negligible. Furthermore, some studies which reported a positive association between high GAD65Ab titer and progression even after multivariate analysis (7, 10) did not take into account HLA-associated T1D susceptibility markers. Thus, the lack of control for confounders may have produced a spurious association between GAD65Ab titers and disease progression. Higher GAD65Ab levels were reported in patients with the age range of 7–16 years presenting with typical type 1 diabetes (23) carrying HLA-DR3 haplotype in comparison with subjects carrying DR4 haplotypes. Furthermore,

Buzzetti *et al.* (8) showed an increased frequency of HLA-DR3 subjects in high GAD65Ab LADA subgroup. Similarly, in our LADA cohort, HLA-DR3 carriers showed a significantly increased GAD65Ab level in comparison with carriers of HLA-DR4 and other less frequent haplotypes. This suggests that at least in Caucasian populations, subgroups of patients with an increased frequency of HLA-DR3 may have elevated serum level of GAD65Ab regardless of the severity of the underlying autoimmune process and clinical expression of the disease. Accordingly, in our cohort, the frequency of HLA-DR3 carriers was slightly higher in high GAD65Ab subgroup, whereas the overall distribution of HLA susceptibility genotypes did not differ. A study of Kasuga *et al.* (24) in 43 Japanese LADA patients reported both higher GAD65Ab levels and an increased frequency of HLA-DR4 in patients who progressed faster toward insulin dependency. Unfortunately, these findings, which seem to support a role for genetic background on the evolution of the disease, have been obtained in a non-Caucasian ethnic group that shows both HLA Class II haplotype distribution and genetic associations with autoimmune diabetes very different than those of a Sardinian population, and are not easily comparable with our data.

The other predictive markers of progression into insulin dependence that we have found do not suffer from statistical limitations.

In GAD65Ab-positive patients often other organ-specific markers of autoimmunity such as IA-2Ab and anti-thyroid antibodies are present (25). Our data indicate that irrespective of the level of GAD65Ab titer, the occurrence of IA-2Ab and/or TPOAb is associated with a significantly increased evolution of the disease compared with GAD65Ab-positive patients

Figure 4 Kaplan-Meier curve showing the effect of HLA-associated genetic risk for the subsequent development of insulin dependence in LADA cohort.

Table 3 (a) Univariate statistics using Cox's proportional hazard model, for the variables predicting insulin deficiency within 4 years in 251 latent autoimmune diabetes of adults patients. (b) The results of Cox's proportional hazard model using forward selection on the basis of the likelihood ratio statistic for the test group.

Variable	β	HR	95% Confidence interval for HR	Significance
(a)				
Age at diagnosis	-0.018	0.982	0.953–1.011	0.225
Gender	-0.695	0.499	0.269–0.928	0.028
GAD65Ab	-0.526	0.591	0.307–1.138	0.115
IA-2Ab	0.403	1.497	0.772–2.900	0.232
TPOAb	0.861	2.366	1.200–4.665	0.013
BMI	-0.166	0.847	0.778–0.922	0.001
HLA risk	0.366	1.442	1.029–2.021	0.034
(b)				
Gender	-0.653	0.520	0.283–0.957	0.036
TPOAb	0.702	2.018	1.104–3.686	0.022
BMI	-0.159	0.853	0.786–0.925	0.001
HLA risk	0.387	1.473	1.064–2.040	0.020

with no further autoimmune markers. Therefore, we confirm the results of previous studies, which showed that GAD65Ab together with an additional specific anti- β -cell antibody (ICA) in the same patient is associated with a poorer long-term prognosis, a feature which was reported in relatives of T1D patients (26). This is consistent with the concept that apart from GAD65Ab, the occurrence of both IA-2 Ab and TPOAb seem to identify subgroups of LADA patients who will develop insulin deficiency very soon.

Actually, the effect of IA-2Ab seems to be modulated by the copresence of TPOAb, in fact when only IA-2Ab+TPOAb— patients are considered, the effect on progression is no longer apparent. On the contrary, the predictive value of TPOAb on disease progression is significantly high even in IA-2Ab-negative patients, suggesting a more powerful predictive effect of anti-thyroid antibodies on diabetes evolution. Accordingly, when IA-2Ab and TPOAb have been entered in the Cox model, only TPOAb showed a significant effect on disease progression.

Our findings are in line with data recently reported in a Japanese study, although in a very limited number of LADA patients, about an association between the presence of TPOAb and β -cell failure (27). Although at the moment we do not have any definite explanation of this finding, a possibility that should be considered is that the presence of extrapancreatic autoimmunity markers at diagnosis may reflect a more extended underlying immune process against β -cells. Clearly, further investigations in larger LADA cohorts are necessary to better understand the role of TPOAb in disease progression.

A substantial proportion of LADA patients with anti-thyroid antibodies also displayed a high frequency of subclinical or clinical thyroid disease. Four out of ten patients who were treated with substitutive hormonal therapy ($L-T_4$), two out of three patients affected by hyperthyroidism, and 62.7% of patients with high TSH

levels progressed rapidly into β -cell failure. Additionally, 16 patients with TPOAb positivity and low TSH progressed to insulin dependence. These data indicate that LADA patients should be routinely screened for thyroid autoimmunity as they have an increased risk of clinical thyroid disease as well as to progress faster into β -cell exhaustion. On the contrary, the frequency of positivity for markers of celiac disease was not different from the GAD65Ab-negative population.

Furthermore, on the basis of our multivariate analysis, the frequency of HLA DRB1-DQB1 genotypes that are most strongly associated with T1D risk is also associated with higher risk of insulin requirement within 4 years of diagnosis, and this effect remained even after adjustment of the other variables.

Our data also showed that another marker of disease progression is gender. In fact, although the F/M ratio in LADA patients at diagnosis was close to 1, in line with other reports (28), the evolution of the disease into insulin dependence in our cohort was faster in women. Therefore, we think it may be linked to a different body composition, i.e. a lower muscle mass and increased insulin resistance in women compared with men. These findings are similar to the gender-dependent progression to β -cell failure reported by Lindholm *et al.* (29) in GAD65Ab-positive patients in the age range of 40–59 years requiring insulin during the first year of diabetes as well as by Turner *et al.* (6) in diabetes subjects older than 45 years in the UKPDS cohort. Another independent factor associated with progression of diabetes is a low BMI, again in agreement with previous studies (6, 12, 13). Multivariate analysis showed that its predictive value persisted even after adjustment for all the other variables. In fact, when the full model using a forward stepwise procedure was run, BMI, gender, TPOAb, and HLA genetic risk were all highly significant. Conversely, age at diagnosis, IA-2Ab, and GAD65Ab titer did not appear in the final regression model, and therefore, they cannot be considered independent predictors of progression. Despite higher HDL cholesterol levels in patients who progressed into insulin dependence, it did not prove to be a significant marker of progression in the multivariate analysis. We also evaluated the possibility that sulfonylurea treatment, before starting insulin therapy, could have accelerated disease progression, thus acting as a confounder. Actually, the percentage of LADA patients treated with sulphonylurea, in the two groups with and without β -cell failure, was comparable (26 vs 28%; NS). Therefore, it is unlikely that sulphonylurea treatment may have influenced disease progression in our cohort.

In addition, we do not think that disease progression may have been differentially influenced by glucotoxicity as no significant difference in fasting glucose and HbA1c levels was noticed between insulin-dependent and noninsulin-dependent LADA patients.

Finally, in our study, we addressed also the problem of the influence of MetS on disease evolution, as this

condition is supposed to affect negatively the course of diabetes. In our cohort, 42% of LADA patients had MetS according to IDF criteria, and its frequency was not significantly different between low and high GAD65Ab group. However, its frequency was increased, although not significantly, in patients who did not require insulin therapy. In the paper by Buzzetti *et al.* (8) which adopted the ATPIII criteria instead of IDF to diagnose MetS, the frequency of MetS in LADA patients with low GADA titer was significantly higher in comparison with our patients. The high frequency MetS in that study (8) could be related to the high frequency of hypertriglyceridemia, which was virtually absent in our patients. This might be attributed to a better metabolic control of our patients, as reflected by lower fasting glucose and HbA1c levels. Other authors (9) also reported a MetS frequency much closer to that found in our study.

Interestingly, a significant lower frequency of insulin dependence was observed in patients with higher waist circumference, which is the essential component of MetS according to the IDF criteria. This is probably due to the fact that in patients with the same level of β -cell impairment, insulin resistance associated with MetS was able to increase fasting glucose levels earlier than in patients who did not have it. Therefore, LADA patients who presented with MetS at the time of recruitment probably had a residual islet function better than patients without MetS, although it cannot be ruled out that an excess of fat mass may play a protective role on the autoimmune process itself. Accordingly, evidence from NOD mice with leptin receptor knockout causing hyperleptinemia showed that obesity may delay the onset of insulin dependence (30).

In conclusion, the markers of progression analyzed in this study can help to improve the definition of the risk of LADA patients developing insulin dependence. However, as they explain only 42% of the variability of the insulin-free period, it is advisable to find additional markers to help the physician decide who should be treated appropriately with insulin therapy in LADA.

Declaration of interest

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

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