Association of Autoimmunity to Autonomic Nervous Structures WithNerve Function in Patients With Type 1 Diabetes: A 16-Year Prospective Study

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Association of autoimmunity to autonomic nervous structures with nerve function in patients with type 1 diabetes: a 16-year prospective study

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ABSTRACT

Objective To prospectively evaluate the association between autoimmunity to autonomic nervous structures and autonomic neuropathy in type 1 diabetes, in relation to clinical variables.

Research Design and Methods A cohort of 112 patients with type 1 diabetes was prospectively followed from adolescence (T0) to approximately four (T4) and sixteen (T16) years later. Standard cardiovascular (CV) tests, neurological examination were performed and related to the presence of circulating antibodies (Ab) to autonomic nervous structures, detected at T0 and T4. Quality of life was assessed by a diabetes-specific questionnaire.

Results Sixty-six patients (59%) of the cohort were re-examined at T16 (age 31.4 ± 2, disease duration 23.4 ± 3.7 years). Nineteen had circulating Ab to autonomic structures. Prevalence of abnormal tests and autonomic symptoms were higher in Ab-positive (68% and 26%, respectively) than Ab-negative patients (32% and 4%) ($p < 0.05$). Among Ab-positive patients, the relative risk of having at least one altered CV test was 5.8 [95% CI 1.55-21.33], and an altered DB test (< 15 beats per minute) was 14.7 [2.48-86.46]. Previous glycemic control was the only other predictor (RR 1.06 [1.002-1.13] per mmol/mol HbA1c increase). Presence of Ab carried over a 68% probability of developing an altered CV test; absence of Ab carried a 91% probability of not having an altered DB test, and an 89% probability of not having an altered Valsalva ratio. Autonomic neuropathy was independently associated with worse quality of life.

Conclusions Circulating Ab to autonomic structures are associated with the development of autonomic dysfunction in young diabetic patients independently of glycaemic control.

Keywords: Type 1 diabetes, diabetic autonomic neuropathy, cardiovascular tests, nervous tissue autoantibodies, quality of life.

Neuropathy is a chronic complication that includes a number of distinct syndromes and autonomic dysfunctions and contributes to increase morbidity and mortality in the diabetic population. In particular, cardiovascular autonomic neuropathy (CAN) is an independent risk factor for mortality in type 1 diabetes and is associated with poor prognosis and poor quality of life (1-3). Cardiovascular autonomic regulation rests upon a balance between sympathetic and parasympathetic innervation of the heart and blood vessels, controlling heart rate and vascular dynamics. CAN encompasses several clinical manifestations, from resting tachycardia to fatal arrhythmia and silent myocardial infarction (4).

The mechanisms responsible for altered neural function in diabetes are not fully understood and it is assumed that multiple, mutually perpetuating, pathogenic mechanisms may concur. These include dysmetabolic injury, neurovascular insufficiency, deficiency of neurotrophic growth factors and essential fatty acids, advanced glycosylation products (5, 6) and autoimmune damage. Independent cross-sectional and prospective (7-13) studies identified circulating autoantibodies (Ab) to autonomic nervous structures and hypothesized that immune determinants may be involved in autonomic nerve damage in type 1 diabetes. Such concept is supported by evidence that other forms of dysautonomia, idiopathic and paraneoplastic, can be immune mediated (14, 15). However, demonstration of a cause-effect relationship between Ab and diabetic autonomic neuropathy awaits confirmation.

We report on a 16-year follow-up study specifically designed to prospectively examine a cohort of patients with type 1 diabetes and aimed at assessing whether the presence of circulating Ab to autonomic nervous structures is associated with increased risk and predictive value of developing CAN. This, in turn, would be highly suggestive of the involvement of autoimmune mechanisms in the pathogenesis of this complication.

Research design and methods

Subjects

In the original design, all patients with type 1 diabetes, older than 11 and in their

adolescence, attending the Paediatric Diabetic Clinic of the University of Turin between 1994 and 1997 were considered for participation in the study. Their clinical characteristics at recruitment (T0) and at first follow-up, approximately 4 years later (T4), were described previously (11, 12). At this third follow up visit, 16.5 ± 1.2 years later (T16), 66 patients out of the original cohort of 112 (59%) were available for re-study. One patient had died, 11 had moved, 27 could not be located and 7 declined to participate. Their clinical characteristics are shown in Table 1. In total, 19 patients had circulating antibodies against autonomous nerve structures, previously detected at T0 (11) and persisting at T4 (12): 8 patients (12%) for vagus nerve, 10 (15%) for cervical ganglia, and 1 patient for both. The antibodies had been assessed by indirect immunofluorescent complement-fixation technique as described (8). Briefly, 5 μm cryostat sections of snap-frozen cervical ganglia and vagus nerve, obtained from adult New Zealand white rabbit, were allowed to air dry, fixed in acetone and incubated with 50 μl of neat test serum for 30 minutes. Slides were then washed in PBS and 50 μl of normal human serum, as a source of complement, were added at 1:5 dilution in PBS for 30 minutes at 37°C. After washing, 50 μl of 1:20 FITC sheep anti-human C3c antiserum were added for a further 30 minutes at room temperature. The slides were washed, mounted in 90% glycerol in PBS and examined by UV microscopy by two independent observers unaware of clinical details. Positive staining of nerve fibres or cytoplasmic staining of ganglion cell bodies, were confirmed by repeated measurements using substrates from a different animal.

Informed consent was obtained and the investigations carried out in conformity with the Declaration of Helsinki.

HbA1c levels (HPLC, IFCC standardised) of the previous year (mean of 3 values) and previous 5 years (mean of available data) were collected to assess long-term metabolic control.

Data on frequency of hypoglycaemic episodes over the previous 5 years were collected by interview, severe hypoglycaemia being defined as needing the assistance of another person. Further information was acquired on the level of physical activity, occupation, schooling level, smoking habit and alcohol consumption.

Assessment of neuropathy

A structured questionnaire, designed according to Dyck (16) was used to identify symptoms related to autonomic, motor and sensory function. Consumption of food and caffeine-containing beverages and smoking was restricted for 2 hours before testing. Heart rate was measured after 10 minutes of supine resting. Autonomic neuropathy was assessed between 8.00 and 12.00 am, by four standard cardiovascular (CV) tests on an automated, computer-integrated system consisting of: heart rate variability upon 6 maximal breaths per minute [deep breathing (DB) test; abnormal < 15 beats per minute, bpm, borderline 15-19 bpm]; lying to standing heart rate change (30/15 ratio; abnormal < 1.17, borderline 1.17-1.27); heart rate change during Valsalva manoeuvre (Valsalva ratio; abnormal < 1.35, borderline 1.35-1.41); postural systolic blood pressure decrease on standing (abnormal > 20 mmHg), as previously described (17). Age-related index values (18) were used to establish abnormality of the tests.

Peripheral somatic neuropathy was assessed by clinical and neurological examination, including deep tendon knee and ankle reflexes and recording of vibratory perception threshold at the tip of the great toe, using a biothesiometer (Biomedical Instruments Co, Newbury, Ohio). The mean of three readings was used.

Quality of life

Diabetes related quality of life was measured in 56 patients by the DQOL questionnaire (19), a diabetes-specific tool designed by the DCCT Research Group and consisting of a 46-item multiple-choice questionnaire exploring 4 primary subscales regarding Satisfaction with Life, Impact, Diabetes-Related Worries and Social/Vocational Worries. Responses are along a Likert scale ranging from 1 to 5 (1=never, 5=all the time for impact, social and diabetes related worries subscales; 1=very satisfied, 5=very unsatisfied). Hence the total score ranges between 46 (highest quality of life) and 230 (lowest quality). The questionnaire had been translated and validated in Italian (20).

Statistical analysis

Values for all cardiovascular tests and for the vibration threshold in the patient groups satisfied the hypothesis of normality, as assessed by the Kolmogorov-Smirnov goodness of fit test. Differences between T0, T4 and T16 data, within and between patient groups, were tested by means of t-tests (paired and unpaired respectively).

Correlations between neurological and clinical parameters were analysed using the Pearson's or Spearman's correlation coefficient, depending on the variable distribution.

Association between presence of nervous tissue autoantibodies and clinical data of autonomic neuropathy (dichotomised as 'presence of at least one altered CV test' vs 'no altered CV tests') was assessed using Fisher's exact test or chi-square test on a 2x2 table. A multivariate logistic regression analysis was used to test the independent effect of the Ab presence on autonomic neuropathy, adjusting the model for glycaemic control, disease duration, exercise level, insulin dose and value at T0 of the corresponding CV test.

The correlation between the DQOL scores, total and for its 4 different dimensions, and presence and severity of autonomic and somatic neuropathy was computed by means of a linear regression model where each of the different DQOL scores, taken one at a time, represented the dependent variable and presence and severity of retinopathy, presence and severity of autonomic and somatic neuropathy the independent ones; the model was adjusted for gender, diabetes duration and frequency of hypoglycaemic episodes.

Data were analysed by the SPSS statistical package (SPSS, Chicago, ILL, USA). *p* values lower than 0.05 were considered significant.

Results

Antibody-positive and negative patients did not differ for clinical characteristics or prevalence of diabetic micro and macrovascular complications at T16 (Table 1). The HbA1c levels of the year preceding T16 (mean of 3 values) correlated with the mean HbA1c of the 5 years preceding T16 ($r = 0.81$, $p < 0.01$).

Of the 56 patients who were administered the DQOL questionnaire, 10 did not report hypoglycaemic episodes over the last 5 years, 11 reported less than 1 episode/month, 9 at least 1/month, 7 reported 1/week and 19 reported more than 1/week. Severe hypoglycaemia was experienced at least once by 15 (27%) patients.

Assessment of neuropathy at T16 and relationship with Ab to autonomic nervous structures

Clinical neurological variables are summarised in Table 2 and Figure 1. Values for DB test, 30:15 ratio and Valsalva ratio had decreased at T16 compared to recruitment at baseline (T0), in both study groups (Figure 1a, b, c). On univariate analysis, at T16, the Ab-positive patients had lower DB test values compared to Ab-negative patients ($p < 0.05$) but the difference was no longer significant after adjusting for HbA1c, diabetes duration and baseline DB value by linear regression ($p = 0.081$) (Figure 1a). Similarly, the 30:15 ratio and Valsalva ratio mean values were lower in the Ab-positive patients, although not significantly, compared to the Ab-negative patients. Using dichotomised data, Ab-positive patients had more abnormal tests (at least 1 abnormal test in 13/19, 68%; >1 abnormal test in 8/19, 42%) than Ab-negative patients (at least 1 abnormal test in 15/47, 32%; >1 abnormal test in 4/47, 8%), $p < 0.005$ and $p < 0.002$, respectively (Table 2).

Seven patients (11%) reported autonomic symptoms at cardiovascular, gastrointestinal, genitourinary or sudomotor level (Table 2). Postural systolic blood pressure drop > 20 mmHg upon standing was detected in 2 patients, while another 5 reported occasional orthostatic symptoms (fainting feeling) with an average systolic blood pressure drop of -11mmHg. Presence of autonomic symptoms was associated with positivity for either anti-vagus nerve Ab or anti-cervical ganglia Ab (5/19, 26%) compared to Ab-negative patients (2/47, 4%) ($p < 0.05$) (Table 2). The patients who reported autonomic symptoms had higher HbA1c values during the previous 5 years (73.4 ± 16 mmol/mol) than asymptomatic ones (59.9 ± 9 mmol/mol) ($p < 0.01$).

Symptoms of peripheral somatic neuropathy were reported by 12 patients (18%) at the feet or hands (Table 2). Carpal tunnel syndrome was diagnosed in 4 of them by electrophysiological assessment. One patient had developed multiple sclerosis. There was a trend to higher prevalence of

symptoms among patients with depressed reflexes (5/19, 26%) compared to those with evocable reflexes (7/47, 15%). Vibratory perception threshold was higher in the patients with depressed reflexes (7.5 ± 3.2 V vs 5.2 ± 1.4 V; $p < 0.02$), and correlated with HbA1c levels over the previous 5 years ($r = 0.27$, $p < 0.05$). Vibratory perception threshold tended to be higher in the patients with (6.2 ± 1.9 V) than without (5.6 ± 1.6 V) somatic symptoms, but the difference was not significant. Presence of somatic neuropathy did not differ according to Ab status and autonomic function. There was no association between neurological variables and presence of microvascular complications or smoking habit.

According to logistic regression analysis, the relative risk for Ab-positive patients of having at least one abnormal CV test at T16 was 5.77 [95% CI 1.56-21.33], and that of having an abnormal DB test was 14.65 [2.48-86.46]. This effect was independent from HbA1c, which was independently responsible for the increase of the relative risk for an altered CV test (RR: 1.06 [95% CI 1.002-1.13] per mmol/mol HbA1c increase) (Table 3).

Progression of neuropathy

Prevalence of at least one abnormal/borderline CV test significantly increased from 5% at T0, to 12% (25%) at T4, without significant differences between Ab-positive and Ab-negative patients (11, 12), to 42% at T16 ($p < 0.05$ vs T0 and T4), with higher prevalence among the Ab-positive ($p < 0.005$ vs Ab-negative) (Table 2). Prevalence of autonomic symptoms, which were not detected at T0 and T4, was also higher at T16 ($p < 0.05$). A postural pressure drop >20 mmHg consistently persisted at follow-up in 2 patients.

The presence of Abs to autonomic nervous structures at T0 carried over a 68% probability for development of one altered CV test at T16, while the absence of Abs at T0 carried a 91% probability of not having an abnormal DB test and an 89% probability of not having an altered Valsalva ratio at T16.

The prevalence of somatic symptoms, not detected at T0, increased from 14% (13/92) at T4, to 18% (12/66) at T16, and the prevalence of impaired reflexes from 6% (5/85) at T0, to 16%

(15/92) at T4 to 29% (19/66) at T16 ($p < 0.05$ vs T0). Four patients consistently showed absent or depressed ankle reflexes, and 4 were still complaining of somatic symptoms since T4.

Quality of life

The total DQOL score was lower (better QoL) in men (72.68 ± 12.2) than women (84.70 ± 20.1) ($p < 0.001$). Presence of autonomic symptoms was associated with a higher (worse) score (94.3 ± 25.8) than in the patients without symptoms (75.4 ± 15.4) ($p < 0.001$).

With reference to the 4 subscales explored, Satisfaction did not significantly correlate with any of the explored clinical variables. Impact was better in men (29.6 ± 5.9) than women (34.1 ± 6.7) (p=0.011), and associated with symptoms of autonomic neuropathy (36 ± 10.4 vs 30.9 ± 5.8) (p=0.008). Social/Vocational Worries was also better in men (9.0 ± 2.1 vs 11.4 ± 6.4) (p=0.03), and worse in patients with autonomic symptoms (14.8 ± 7.4 vs 9.3 ± 3.5) (p=0.01). The score for Diabetes-Related Worries was again better in men (6.3 ± 1.2 vs 8.5 ± 3.3), $p < 0.001$, and worse in presence of autonomic symptoms (9.3 ± 4.7 vs 6.9 ± 2.1) ($p = 0.023$).

A higher score for Diabetes-Related Worries was associated with past occurrence of severe hypoglycaemia (8.6 ± 4.3 vs 6.8 ± 1.5) ($p = 0.014$).

There were no correlations between the scores of the DQOL, or any of its subscales, and the presence and severity of somatic neuropathy or retinopathy.

Discussion

The pathogenic mechanisms underlying diabetic peripheral polyneuropathies are multiple, interrelated and, possibly, mutually perpetuating. Several lines of research indicate that immune determinants are likely to be involved in the events culminating in autonomic nerve damage, within the constellation of autoimmune stigmata that characterise type 1 diabetes (7-10, 21). The association, however, awaits confirmation. The present prospective study, conducted in young patients without established autonomic neuropathy at recruitment and followed for over 16 years until adulthood, strongly indicates that a cause-effect relationship may exist between autoantibodies to autonomic nervous tissues and development of diabetic autonomic neuropathy. Incipient or established CAN (22) reached a prevalence of 68% among the Ab-positive patients, significantly higher compared to the Ab-negative patients. The heart rate response to deep breathing, mainly mediated by the parasympathetic nervous system, was the most impaired CV test in the time course analysis, possibly reflecting early parasympathetic damage. Logistic regression analysis indicates that autoantibodies carry an almost 15-fold increased relative risk of developing an abnormal DB test over 16 years and an almost 6-fold increase of developing at least one abnormal CV test, independent of other variables.

Previous studies showed that, once present, autoantibodies to autonomic nervous structures persist (12, 13) and were consistently detected in our patients at recruitment and 4 years later. The proportion of autoantibody-positives was similar to that reported in adult patients with established diabetic autonomic neuropathy (7-10). While in our previous reports on this cohort we could not detect a clear association with CAN, this longer follow-up study highlights a significant association. This suggests that autoimmune mechanisms targeting sympathetic and parasympathetic structures may play a primary aetiological role in the development and progression of autonomic dysfunction in type 1 diabetes in the long term. Indeed, positivity for autoantibodies had a high positive predictive value for the later development of autonomic neuropathy.

Our study is in line with the only other prospective study detecting a relative risk of 7.5 to develop at least one abnormal autonomic test amongst Ab positive patients over a period of 13-14

years, unrelated to glycaemic control (13). In addition, in line with others (6, 23, 24), our study indicates that autonomic abnormalities also have an independent relationship with glycaemic control. In fact, multivariate regression analysis showed that previous long term glycaemic control was the only other determinant for increased relative risk of altered CV tests. In this series, diabetes duration appeared to be a minor predictor of CAN, in contrast to other reports (25).

A multi-step process can be envisaged in which nerve damage is first initiated by vascular, metabolic and/or autoimmune mechanisms, and perpetuated/amplified by the same autoimmune mechanisms, as neuronal autoantigens are released. Autoimmunity is evoked to explain the pathogenesis of idiopathic and paraneoplastic dysautonomic syndromes, with levels of autoantibodies targeting ganglionic acetylcholine receptors (AchR) correlating with the severity of autonomic dysfunction (14, 26, 27). Those studies indicate a potential therapeutic role for acetylcholinesterase inhibitors in the enhancement of autonomic function (15). Circulating autoantibodies to autonomic nervous structures and subclinical autonomic neuropathy are present in other autoimmune disorders, such as rheumatoid arthritis and lupus (28). In type 1 diabetes, the target autoantigens within the vagus nerve and cervical ganglia have not been identified. They might include the ganglionic neurotransmission and the smooth muscle calcium channels (29). Intriguingly, borderline levels of ganglionic AChR antibodies were detected in a patient with both celiac disease and type 1 diabetes and one patient with celiac disease and subclinical neuropathy (30). Furthermore, in the context of autoimmune diabetes, there is evidence for predominant active B-cell response *in situ* against pancreatic nervous system elements (31, 32), suggesting a shared propensity for β cell and neuronal tissue autoimmunity.

Diabetic autonomic neuropathy, possibly the least recognised and most overlooked of diabetic complications, has increasingly gained attention as an independent predictor of silent myocardial ischemia and mortality, as consistently indicated by several cross sectional studies (2, 3, 33). The pooled prevalence rate risk for silent ischemia is estimated at 1.96 by meta-analysis studies (5). In this report, established CAN (22) was detected in near 20% of young adult patients with acceptable metabolic control, after over approximately 23 years of diabetes duration, against 12%

of patients of the same cohort with subtle asymptomatic autonomic dysfunction (one abnormal CV test) a decade earlier, in line with other studies in type 1 diabetes (2, 24). Approximately 30% of the patients, developed signs of peripheral somatic neuropathy, not associated with autonomic dysfunction. This discrepancy suggests the participation of pathogenic mechanisms different from metabolic control and a distinct clinical course, as indicated by the DCCT study, where hyperglycaemia had a less robust relationship with autonomic than somatic neuropathy (6). Furthermore, symptoms at different levels of the autonomic system, which were previously absent, appeared in 10% of patients at T16. In line with the analysis of the risk determinants, autonomic symptoms were associated both with the presence of autoantibodies and a worse glycaemic control. Orthostatic hypotension was the earliest documented symptom in the course of this longitudinal survey (12). However, it was only detected at follow up in the two patients with altered CV tests and impaired quality of life. The data suggest that early detection of autonomic derangement might be susceptible to correction by appropriate intervention, at least in its functional if not organic component (34). Similarly to what happened with the slowly evolving history of islet cell autommunity, identification of target antigens within nervous tissue will probably allow the setting of specific immunometric assays, overcoming the limits of immunofluorescence techniques. However, the assay employed in this paper is both feasible and reproducible (11-13) and it is hoped that demonstration of its predictive value will stimulate both the development of more specific techniques and more systematic search for autonomic neuropathy in the patients.

Furthermore, this study shows that autonomic neuropathy, together with female sex and the occurrence of severe hypoglycaemias, is a major determinant for poor quality of life in patients with type 1 diabetes. This is in agreement with previous reports (35), and linked to such invalidating symptoms as orthostatic hypotension and chronic diarrhoea. In fact, the subscales involved were Impact of diabetes, Social/Vocational Worries and Diabetes-Related Worries. In contrast, somatic neuropathy, not associated with impaired QoL in this cohort, was mostly present as non-painful paraesthesias, whereas previous reports of impaired QoL were in people with painful neuropathy or detected by the more specific NeuroQoL (36), which we did not use because a validated Italian

version is not available for this questionnaire. Previous reports had shown better quality of life among men than women, both in general and diabetic populations (37, 38) and a relationship between quality of life and hypoglycaemia was also reported by others (35, 38). In this cohort, the impact of severe hypoglycaemia was specifically related to the Diabetes-Related Worries subscale.

In conclusion, the present study provides persuasive evidence for a primary pathogenic role of autoimmunity in the development of autonomic diabetic neuropathy. However, the mechanisms through which autoantibodies impair their target organ function, whether through classical complement action, pro-apoptotic effects of complement, enhanced antigen presentation or channelopathy (26, 39, 40), remain to be elucidated.

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Table 1. Clinical characteristics of the diabetic patients at T16 screening.

	Diabetic patients at T16 n = 66	Nervous tissue Ab positive patients n = 19	Nervous tissue Ab negative patients n = 47
Age (years ± SD) (range)	31.4 ± 2 (28-36)	31.3 ± 2 (28-36)	31.5 ± 2 (28-35)
M : F	33 : 33	9 : 10	24 : 23
Duration of diabetes (years ± SD) (range)	23.4 ± 3.7 (16-31)	22.6 ± 3.2 (16-28)	23.6 ± 3.8 (16-31)
Follow-up duration (years ± SD)	16.5 ± 1.2	16.2 ± 1.4	16.6 ± 1.1
HbA1c % (mmol/mol)* Previous year	7.81 ± 1.14 (61 ± 12)	8 ± 0.8 (64 ± 9)	7.7 ± 1.3 (61 ± 14)
Previous 5 years	7.8 ± 1 (62 ± 11)	7.9 ± 0.7 (63 ± 8)	7.8 ± 1.2 (61 ± 13)
Insulin (U/Kg)	0.73 ± 0.17	0.70 ± 0.15	0.74 ± 0.18
Microalbuminuria	7 (11%)	3 (16%)	4 (9%)
Background retinopathy	37 (54%)	12 (63%)	25 (53%)
Laser-treatment	7 (11%)	2 (11%)	5 (11%)
Smoke habit	21 (32%)	4 (21%)	17 (36%)
Ex-smokers	5 (7%)	1 (5%)	4 (9%)
Hypo/Hyperthyroidism	8 / 1	5 / 0	3 / 1

Coeliac disease	4	3	1
Hypertension	4 (6%)	0	4 (9%)
Anti-vagus nerve Ab positive	8 (12%)	8	-
Anti-cervical ganglia Ab positive	10 (15%)	10	-
Both Ab positive	1	1	-

Data are *n* (%) or means ± SD

*HbA1c levels refer to values of the previous year (mean of 3 values) and the previous 5 years (mean of available data)

Table 2. Abnormal cardiovascular tests, reflex examination and somatic or autonomic symptoms, according to the status of autoantibodies to autonomic nervous tissues.

	Patients at T16 n=66	Nervous tissue Ab positive patients n=19	Nervous tissue Ab negative patients n=47
Resting heart rate (bpm)	77 ± 12	79 ± 11	76 ± 13
Normal CV test	38 (%)	6 (%)	32 (%)
Abnormal/borderline CV tests	28 (42%)	13 (68%)*	15 (32%)
One CV test	16	5	11
Two CV tests	10	7	3
Three CV tests	1	1	0
Four CV tests	1	0	1
Autonomic symptoms	7 (11%)	5 (26%)†	2 (4%)
Postural pressure drop >20 mmHg	2	1	1§
Gastrointestinal symptoms	2	1	1
Genitourinary symptoms	2	1	1
Sudomotor symptoms	3	2	1
Absent or depressed reflexes	19 (29%)	5 (26%)	14 (30%)
Persistently depressed since T4	4	1	3
Somatic symptoms	12 (18%)	4 (21%)	8 (17/%)
Symptoms persistent since T4	4	1	3

Data are *n* (%) or means ± SD

* $p = 0.05$ vs Ab negative patients, both for one and more then one abnormal test (DB test abnormal in 8/13, 30:15 ratio abnormal in 6/13, Valsalva ratio abnormal in 7/13)

† $p = 0.05$ vs Ab negative patients

§ The same patient reported postural hypotension, gastrointestinal and genitourinary symptoms

Table 3. Multivariate logistic regression analysis testing the independent effect of the Ab presence on altered CV tests, adjusted for gender, disease duration and HbA1c level.

	β coefficient	RR (95% CI)	*p* value
AT LEAST ONE ALTERED CV TEST AT T16			
Sex	-0.239	0.788 (0.227-2.729)	0.707
Disease duration	0.132	1.141 (0.964-1.351)	0.125
5 years HbA1c	0.062	1.064 (1.002-1.13)	**0.04**
Ab presence	1.752	5.77 (1.56-21.33)	**0.009**
ALTERED DB TEST AT T16			
Sex	-0.960	0.383 (0.072-2.033)	0.26
Disease duration	0.199	1.22 (0.955-1.56)	0.112
5 years HbA1c	0.097	1.102 (1.021-1.19)	**0.013**
Ab presence	2.685	14.65 (2.48-86.46)	**0.003**
DB at T0	-.098	0.907 (0.808-1.018)	0.097

Figure legends.

Figure 1. Results of cardiovascular tests. Mean ± SD value of DB test (A), 30:15 ratio (B) and Valsalva ratio (C), in Ab negative (open circle) and Ab positive (close circle) patients. T0 recruitment, T4 at 4 year follow-up, and T16 at 16 year follow-up.

* $p < 0.05$ compared to values at T0 for both study groups.

§ $p < 0.05$ compared to values in Ab negative patients.