The Squatting Test

A Useful Tool to Assess Both Parasympathetic and Sympathetic Involvement of the Cardiovascular Autonomic Neuropathy in Diabetes

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The heart rate responses observed after both squatting and standing are thought to be of reflex nature and may be useful to assess the functional integrity of parasympathetic and sympathetic nerves in diabetes. In the standard maneuver, each subject stood still for 3 min, then squatted down for 1 min, and at last stood up during an inspiratory phase. In 10 healthy subjects (25–31 years of age), lengthening of the R-R interval during squatting was abolished by atropine, whereas propranolol markedly attenuated shortening of the R-R interval at standing from squatting. Squatting test (SqT) ratios (SqT vagal [SqTv] = ratio between the R-R interval mean before squatting and the longest R-R interval after squatting; SqT sympathetic [SqTs] = ratio between the basal R-R interval and the shortest R-R interval at standing) were calculated in 558 healthy subjects and 346 diabetic patients (insulin-dependent diabetes mellitus/non-insulin-dependent diabetes mellitus: 103/243). Normal ranges (95 and 99% confidence intervals [CIs]) for subjects 20–74 years of age showed a statistically significant negative correlation with age. SqTv was outside the 99% CI in 145 (42%) diabetic patients and in 7 (1.3%) of the control subjects. The corresponding figures for SqTs were 40 and 0.8%, respectively. Age and duration of diabetes had a negative influence on SqT ratios. SqT ratios were compared with other reflex tests currently used for diagnosis of autonomic neuropathy: deep breathing (DB), lying-to-standing (LS), Valsalva manuever, and blood pressure change after standing (orthostatic hypotension [OH]). Autonomic involvement was arbitrarily defined as mild (one test pathological), definite (two tests pathological), or severe (three or more tests pathological). In patients with definite or severe involvement, SqT ratios and DB and LS tests showed the least overlap between healthy subjects and diabetic patients; however, for patients with mild or no autonomic involvement, SqT ratios were significantly better than DB, LS, or OH tests. In conclusion, *1*) SqT ratios can discriminate between healthy subjects and diabetic patients to an equal or greater extent than the other tests; *2*) SqT ratios give information on both parasympathetic and sympathetic activity; and *3*) SqT ratios are better than other single tests in identifying mild autonomic involvement. These results may be important for early intervention trials. *Diabetes* 43:607–12, 1994

William Hunter noted in 1784 that cyanotic patients obtained relief from faintness by assuming the posture we now call squatting (1). Sharpey-Schafer (2) observed that in normal subjects squatting caused an increase in systemic arterial pressure followed by bradycardia. His original hypothesis that squatting increased cardiac output and that the bradycardia was secondary to baroreflex activity was later confirmed (3). Thus, squatting is likely to produce its circulatory effects by improving venous return. It has been claimed that patients with orthostatic intolerance should be told about this blood pressure–raising maneuver (4). The circulatory modifications after the active change in posture from squatting to standing also have been investigated. This passage causes an almost immediate decrease in arterial pressure followed by an increased heart rate (HR) (5).

The HR responses observed after both squatting and standing are thought to be of a reflex nature and therefore may be useful to assess the functional integrity of cardiovascular autonomic nerves in pathological situations, as in diabetic neuropathy. The bradycardia during squatting may be under vagal control, and evidence suggests that tachycardia after standing from squatting is secondary to sympathetic stimulation (6). If so, combining the two maneuvers, we would have a single test that simultaneously evaluates parasympathetic and sympathetic activity based on HR responses. Although we currently have many valid reflex tests that evaluate parasympathetic activity (7), most sympathetic tests are based on blood pressure responses to various stimuli with low sensitivity.

In this study, we *1*) describe the HR responses to the squatting test (SqT) in normal subjects and analyze the autonomic mechanisms involved using pharmacological autonomic blockade; *2*) evaluate the effect of age in a large group of healthy people and establish confidence intervals (CIs) for the HR responses to SqT; *3*) show how responses in

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HR, heart rate; SqT, squatting test; SqTv ratio, squatting test vagal ratio; SqTs, squatting test sympathetic ratio; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; CI, confidence interval; DB, deep breathing; LS, lying-to-standing; VM, Valsalva maneuver; OH, orthostatic hypotension.

TABLE 1
Demographic characteristics of the populations studied

	Healthy subjects	Diabetic patients
n	558	346
Age (years)	46 ± 16	43 ± 18
Sex (M/F)	289/269	180/164
Body mass index (kg/m^2)	26.1 ± 2.8	26.5 ± 4.5
Diabetes duration (years)	—	9.8 ± 4.5
IDDM/NIDDM	—	103/243

Data are means ± SD.

diabetic subjects differ from those found in normal subjects; and *4*) also show that the SqT presents favorable features for the assessment of both vagal and sympathetic activity by comparing responses among cardiovascular reflex tests.

RESEARCH DESIGN AND METHODS

A total of 558 healthy normal subjects were studied. Subjects were divided for age-intervals of 5 years from 20 to 74 years: each interval included a minimum of 40 subjects, ~50% men and 50% women. Most of those <65 years of age were hospital staff, otherwise healthy people attending outpatient departments of our institution, or healthy relatives of inpatients. Those 65–74 years of age were volunteers from day centers for the elderly in Naples. A total of 346 diabetic patients attending the outpatient diabetic department also were studied within a period of 30 months. The actual numbers of diabetic patients in each of the 5-year age-bands were 8 at 20–24 years of age; 13 at 25–29 years of age; 20 at 30–34 years of age; 25 at 35–39 years of age; 51 at 40–44 years of age; 45 at 45–49 years of age; 47 at 50–54 years of age; 35 at 5–59 years of age; 49 at 60–64 years of age; 41 at 65–69 years of age; and 12 at 70–74 years of age. According to the criteria of the National Diabetes Data Group (1979), 103 had insulin-dependent diabetes mellitus and 243 had non-insulin-dependent diabetes mellitus. The demographic characteristics of both control subjects and diabetic patients are given in Table 1. In both groups, subjects with cardiac failure, angina, cerebrovascular disease, and major cardiac arrhythmias were excluded. No diabetic patient was in overt metabolic decompensation as judged by HbA_{1c} value and average daily plasma glucose level (<12.5 mM). No one was involved in a physical exercise program.

Protocol of the SqT. A standard squatting maneuver was performed in the morning after an overnight fast, while a continuous electrocardiogram was recorded. All subjects refrained from smoking and drinking coffee in the morning of the test day and avoided prescription medicines for at least 8 h. Each subject stood still for 3 min (phase I) and then squatted (taking about 2 s), remaining in this position for 1 min (phase II). Lastly, the subject stood up during an inspiratory phase and remained in the standing position for 1 min (phase III). The SqT-induced HR response was expressed by SqT vagal ratio (SqTv), which is the ratio between the baseline R-R interval (mean of 10 beats just before squatting) during phase I and the longest R-R interval in the first 15 s of phase II; and SqT sympathetic ratio (SqTs), which is the ratio between the baseline R-R interval as described before and the shortest R-R interval in the first 10–20 s of phase III. Each subject performed the test twice after a 5-min resting period. All subjects were asked to perform the test in complete relaxation without speaking and in the absence of any noise. Room temperature was kept between 20 and 24°C.

Pharmacological studies. Ten young normal subjects (25–31 years of age) underwent pharmacological studies on 2 different days. On the first day, two control SqT tests were performed and repeated 15 min after intravenous atropine sulfate (3 mg total dose). On the second day, the SqT test was performed twice before and after intravenous propranolol (10 mg total dose). According to previous studies, the doses used give adequate vagal and cardiac sympathetic blockade (8).

Other cardiovascular tests. On the same morning of the standard SqT, all subjects underwent a battery of four cardiovascular reflex tests (9): HR response to six forced respirations in 1 min (deep breathing [DB], maximum/minimum HR expressed by the expiration:inspiration ratio between the mean of the three longest R-R intervals in expiration and the mean of the three shortest R-R intervals in inspiration), HR response to standing (lying-to-standing [LS], modified 30:15 ratio; ratio of the 30th to the 15th R-R interval after changing from supine to upright position); HR response to a standardized Valsalva maneuver (VM) (ratio of the longest R-R interval after the VM to the shortest interval during the VM), and blood pressure change after standing (orthostatic hypotension [OH], change in systolic blood pressure beginning 30 s after the subject assumes the upright posture). HR was recorded continuously with a Lifepak 6 (PhysioSystem, Milan, Italy) recorder. Each value was calculated as the mean of at least two consecutive tests that were done in the same sequence with a few minutes rest between each test.

Statistical analysis. The influence of age on HR variation in healthy subjects was examined by regression analysis. Multiple correlation analysis was used to study the relation of age and resting HR on HR variation. The association between indexes was assessed with linear regression. When appropriate, paired Student's *t* test was applied to examine differences in HR responses during pharmacological maneuvers. The difference in HR variability between men and women was compared by unpaired Student's *t* tests. Multiple regressions were used to analyze the interrelationship among SqTv and SqTs, age, and diabetes duration. All data are presented as means ± SD, 95 and 99% CIs are provided. Because of the skewed distribution of results, the original data were transformed to normality by the method of Box and Cox (10), in which a general power transformation is used to convert the original data to normality. CIs were then calculated, and the data were transformed back.

RESULTS

SqT ratios in healthy subjects. SqT ratios were independent of resting HR. The correlation coefficient of age on SqT ratios ranged from 0.62 ($P < 0.001$) for SqTv to 0.57 ($P < 0.001$) for SqTs. The SqT ratios plotted as a function of age in 558 control subjects are shown in Figs. 1 and 2. HR variability was generally lower in women than in men, but the difference was not significant ($P = 0.6$). Therefore, results for both sexes were analyzed together. The values of 95 and 99% CIs for SqT ratios are given in Table 2. Results for individual subjects were rarely outside these limits. SqTv and SqTs ratios were outside the 95% CI in 20 (3.6%) and in 17 (3%) subjects, respectively, and outside the 99% CI in 7 (1.3%) and 5 (0.8%) subjects, respectively. The reproducibility of the SqT ratios was assessed in a separate study of 10 healthy subjects 20–35 years of age, who were studied on three separate occasions over 3 days. The variation coefficients were 6.6% (range, 3–9%) for SqTv and 6.7% (range, 3.5–9.3%) for SqTs. Learning was not involved in the response because the mean difference of SqT ratios was not different (0.1) from zero ($P = 0.2$).

Pharmacological studies. Fig. 3 shows the R-R intervals obtained during the three phases of the SqT test in the 10 normal subjects before and after pharmacological studies. The normal response (basal SqT before intervention studies) consisted of an immediate lengthening of the R-R interval with a maximum between 10–15 s after squatting, followed by a return to the resting level and a shortening of R-R interval with a minimum over the 10–20 s after standing from squatting, followed by a relative stabilization. The similar pattern of response during the 2 control days in young healthy adults confirms the reproducibility of the test. After atropine, the resting R-R intervals shortened, but no appreciable changes in the R-R interval were observed over the 60 s after squatting (phase II). After propranolol alone, the pattern of the R-R response after squatting was not significantly different from that of control periods; however, the R-R shortening after standing from squatting was practically undone (Table 3).

SqT ratios in diabetic patients. The responses to the cardiovascular reflex tests obtained in diabetic patients are

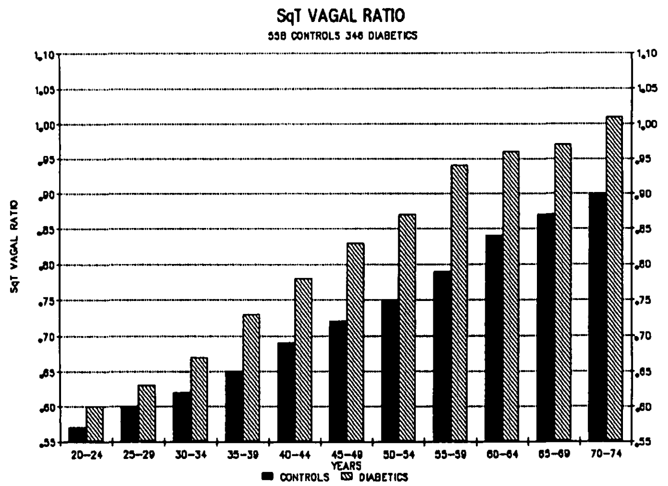

FIG. 1. Distribution of the SqTv ratio in healthy subjects and diabetic patients divided by 5-year intervals from 20 to 74 years. Mean values are shown.

TABLE 2
Normal ranges for SqT ratios in normal subjects 20–74 years of age

Age (years)	n	SqTs ratio Lower 95% CI	SqTs ratio Lower 99% CI	SqTv ratio Higher 95% CI	SqTv ratio Higher 99% CI
20–24	56	1.55	1.54	0.59	0.61
25–29	53	1.47	1.46	0.61	0.62
30–34	67	1.43	1.42	0.63	0.64
35–39	42	1.36	1.34	0.66	0.67
40–44	54	1.32	1.31	0.71	0.72
45–49	40	1.29	1.28	0.73	0.74
50–54	48	1.26	1.24	0.77	0.78
55–59	60	1.20	1.19	0.82	0.83
60–64	53	1.15	1.14	0.86	0.87
65–69	45	1.10	1.09	0.89	0.90
70–74	40	1.07	1.06	0.92	0.93

Abnormal values are less than the lower 99% CIs for SqTs ratios and greater than the higher 99% CIs for SqTv ratios.

shown in Table 4. SqTv and SqTs ratios were outside the 95% CI in 160 (46%) and 156 (45%) patients, respectively, and outside the 99% CI in 145 (42%) and 141 (41%) patients, respectively. Age ($P < 0.001$) and duration of diabetes ($P < 0.01$) exerted a significant influence on SqT ratios in diabetic subjects. Also shown in Table 4 are the responses to the other tests in both control and diabetic populations. Although no significant differences among the frequency distribution of the abnormal tests were observed in normal subjects, the highest number of tests outside the 95% CI was seen with LS, VM, and DB, followed by the SqT ratios, and lastly by OH with no test outside the 99% CI in the whole population of normal subjects. The situation was quite different in diabetic patients, for whom the greatest number with responses outside the 95 and 99% CIs was seen with the SqT ratios. The difference was significant for both ratios: the χ^2 with Yates's continuity correction was significant for LS ($\chi^2 = 9.02$, $P < 0.001$), but not for DB ($\chi^2 = 2.81$, $P > 0.05$).

Diabetic patients were divided, based on their autonomic involvement, into four categories, and the frequency distribution of abnormal (outside the 99% CI) responses for each test was calculated (Table 5). Those diabetic patients for whom no test (DB, LS, VM, or HO) was abnormal were arbitrarily considered to be free of cardiovascular autonomic neuropathy. The degree of autonomic involvement was arbitrarily defined as mild, definite, or severe if a single test, two tests, or three tests or more were abnormal. In the healthy population, 14 subjects (2.5%) had one test outside the 99% CI and only 4 subjects (0.7%) had two tests outside the 99% CI. The corresponding figures for the SqT ratios in the healthy population were 12 subjects (2.1%) with one ratio outside the 99% CI and one subject (0.2%) with the two ratios outside the 99% CI. In the diabetic population, 180 patients were considered to be unaffected by cardiovascular autonomic neuropathy; the remaining subjects had neuropathic involvement, which was mild in 51 patients, definite in 76, and severe in 39. As a whole, the number of diabetic patients with abnormal SqT ratios was larger than that of diabetic patients with other responses abnormal. For patients with definite or severe autonomic involvement (two or more tests abnormal), SqT ratios were not significantly better than results for DB or LS. However, for patients with mild (or no) autonomic involvement, the SqTv ratio was significantly

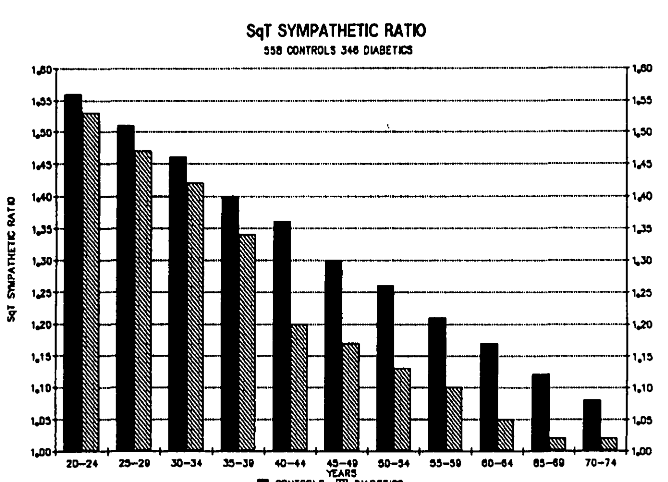

FIG. 2. Distribution of the SqTs in healthy subjects and diabetic patients divided by 5-year intervals from 20 to 74 years. Mean values are shown.

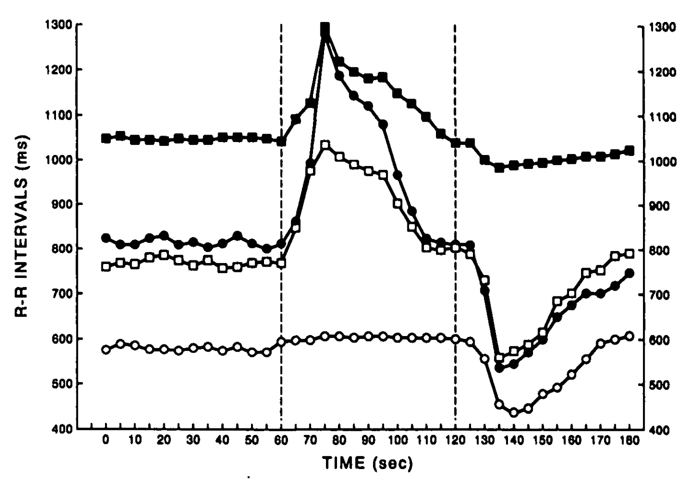

FIG. 3. HR responses, expressed as R-R intervals (ms), during the SqT. Phase I (standing) is from 0 to 60 s; phase II (squatting) is from 61 to 120 s; phase III (standing) is from 121 to 180 s. Control 1, 10 young normal subjects who underwent pharmacological studies; control 2, the entire population of healthy subjects.

TABLE 3
HR responses to SqT before and after cardiac autonomic blockade in 10 young normal subjects

	Standing (phase I)		Squatting (phase II)		Standing (phase III)	
	R-R (ms)	HR (beats/min)	R-R (ms)	HR (beats/min)	R-R (ms)	HR (beats/min)
Control	817 ± 75	73 ± 4	1289 ± 108	47 ± 3	536 ± 33	112 ± 9
Atropine	580 ± 49	103 ± 6	510 ± 68	100 ± 6	437 ± 57	137 ± 10
Propranolol	1049 ± 74	57 ± 5	1298 ± 113	46 ± 3	997 ± 72	60 ± 7

Data are means ± SD. Data for phase II and phase III represent peak responses.

better than results for DB ($\chi^2 = 4.07$, $P < 0.05$) or LS ($\chi^2 = 19.87$, $P < 0.001$), and the SqTs ratio was significantly better than results for OH ($\chi^2 = 47.6$, $P < 0.0001$). SqT ratios showed good correlations with other indexes both in normal subjects and diabetic patients, which ranged from 0.4 to 0.42 for VM and 0.6 to 0.63 for DB.

DISCUSSION

There are few quantitative studies on the initial circulatory events that follow active changes in posture to and from squatting. In normal subjects, the immediate cardiovascular responses on an active change from standing to squatting consist of R-R interval shortening, apparently associated with increased blood pressure. The reflex nature of squatting-induced bradycardia is confirmed by the results of pharmacological studies with autonomic blocking drugs; atropine completely abolished the response, suggesting that the R-R variation after squatting is mediated by the vagus nerve. Propranolol has no effect on the pattern of response, confirming that the sympathetic nerves are not involved.

The active change from squatting to standing causes cardioacceleratory responses mediated by sympathetic hyperactivity. The immediate decrease in the arterial pressure at the start of standing is an interesting finding and is strictly related to the hemodynamic changes that have occurred in the previous squatting phase. Thus, the increase in the stroke output and pulse pressure obtained during squatting causes a reflex vasodilatory response that is still present when the hydrostatic fall in stroke output takes place on standing. Together with a decompression of arterial vessels in the legs (5), this will explain the sustained fall in arterial blood pressure (mean decrease: systolic = 36.4 ± 16; diastolic = 21 ± 4; mean = 27 ± 10 mmHg), which triggers a reflex HR response. These changes contrast with those obtained in the active change from the lying to the standing position. This change of posture, performed after 1 or 20 min of supine rest, was accompanied by an immediate increase in systolic pressure, probably explained by a compression of arteries by contracting postural muscles during this kind of orthostatic test (11). Further evidence for the sympathetic mediation of the R-R interval shortening during the orthostatic load (from squatting to standing) comes from the results of pharmacological intervention studies: propranolol abolished the HR increase after standing, suggesting that cardiac sympathetic nerves are normally involved in this response.

Age is an important determinant of HR variability in normal subjects (12,13). SqT ratios do not escape the rule. After ensuring an even distribution of age and sex in each decade from 20 to 70 years, we calculated age-related normal ranges. From the analysis of the results, SqT ratios appear to be superior to any other single test in discriminating between responses that are in or outside the 99% CI in diabetic subjects with autonomic neuropathy. Moreover, the ratios have higher sensitivity compared with other tests used because results for 12 diabetic patients with no neuropathy on the classic reflex test battery fell outside the 99% CI for SqT ratios.

The search for the ideal battery of cardiovascular autonomic function tests looks never ending (14–16). However, some consideration of the feasible application of the SqT test seems justified. Because the difference between tests so far proposed may be technical, with some limitations concerning age-related normal ranges, quicker and less cumbersome tests should be sought. A direct sympathetic cardiac activity cannot yet be investigated by any other test based on R-R interval variation, so we have evaluated the usefulness of the SqT ratio alone or in combination with only another test (e.g., DB) for the diagnosis of cardiac autonomic neuropathy. If we look at the prevalence of mild autonomic involvement (arbitrarily defined) in our group of diabetic patients, we find a prevalence rate of 14.7%, that is, in 51 subjects one test was abnormal. Although the SqTv ratio showed the least overlap between control and diabetic populations and it was able to identify ~75% of diabetic patients falling in the category (SqTs identified 69%), the crude prevalence of a single abnormal SqTv or SqTs in the entire diabetic population was ~13% (38 abnormal ratios in patients with mild neuropathy plus 8 abnormal ratios in the patient category called none [Table 5]), a value not statistically different from the prevalence rate of 14.7% obtained with the classic battery of tests. It could be argued that a higher rate of responses outside the 99% CI may be related to higher frequencies of false-positive responses. However, the prevalence of a single response outside the 99% CI in the healthy population was 2.5% for classic tests (DB, LS, VM, and HO) and 2.1% for SqT ratios (NS). Moreover, these data also indicate that both parasympathetic and sympathetic involvement is present simulta-

TABLE 4
Distribution in control subjects and diabetic patients of abnormal responses of each test

	Healthy subjects (n = 558)		Diabetic patients (n = 346)	
Test	95% CI n (%)	99% CI n (%)	95% CI n (%)	99% CI n (%)
SqTv	20 (3.6)	7 (1.3)	160 (46)	145 (42)
SqTs	17 (3.0)	5 (0.8)	156 (45)	141 (40)
DB	21 (3.7)	6 (1.0)	148 (43)	125 (36)
LS	31 (5.6)	8 (1.4)	134 (40)	106 (31)*
VM	23 (4.3)	0 (0)	89 (26)	75 (21)*
OH	21 (3.8)	0 (0)	39 (11.3)	29 (8.4)*

*Significantly different vs. SqT ratios.

TABLE 5
Abnormal (outside the 99% CI) responses for autonomic tests in the diabetic population arbitrarily classified as having progressive degrees of autonomic involvement

Involvement	n	SqTv	SqTs	DB	LS	VM	OH
None (0 of 4)	180	8	0	0	0	0	0
Mild (1 of 4)	51	38	35	29	13	9	0
Definite (2 of 4)	76	63	61	60	58	34	0
Severe (3 of 4 or 4 of 4)	39	36	35	36	35	32	29

neously early in the course of autonomic neuropathy. This view is supported by the results of recent studies in which *meta*-[^{123}I]iodobenzylguanidine, a norepinephrine analogue, was used to assess adrenergic cardiac innervation; derangements of adrenergic cardiac innervation were found in 20 of 26 diabetic patients, whereas a reflex test indicated cardiac autonomic neuropathy only in 4 of 26 patients (17). Moreover, a study in which cardiac autonomic neuropathy was evaluated by power spectral analysis claims that sympathetic and parasympathetic impairment occurs simultaneously in all degrees of diabetic autonomic involvement (18).

The prevalence of definite cardiac autonomic involvement (two or more tests abnormal) was 33%. Even in this case, SqTv showed the least overlap between control and diabetic populations, but the difference among SqT ratios, DB, and LS was not significant (abnormal ratios were 99, 96, 96, and 93 for SqTv, SqTs, DB, and LS, respectively, in this population of 115 diabetic patients with definite autonomic neuropathy). It seems that in the late stages of autonomic neuropathy, the sensitivity of each single test (except VM and HO) ranges from 86 to 81%.

We have calculated the frequency distribution of responses outside the 99% CI to a mini-battery of tests consisting of SqT plus DB. A single diabetic patient was arbitrarily considered to be affected by a definite autonomic involvement if he or she had at least two tests outside the 99% CI (SqTv + SqTs or SqTv + DB or SqTs + DB). We have calculated sensitivity and specificity of this mini-battery with the values obtained by applying the classic battery of tests. Although the results obtained with whichever battery so far proposed cannot be considered as a gold standard for the diagnosis of cardiac autonomic neuropathy, the use of these reflex tests is consolidated and is becoming the norm. The last consensus statement on diabetic neuropathy claims that although invasive tests for cardiovascular function are not suitable for routine screening or for monitoring progress, noninvasive tests "have been validated and shown to be reliable and reproducible, to correlate with each other and with tests of peripheral somatic nerve function and to have prognostic value" (19). With the limitations given above about the absence of a true independent gold standard, we found a relative sensitivity value of 96% with a relative specificity of 97%; the positive predicted value was 95% and the negative predicted value was 97%. Note that only 3 subjects of the entire control population of 558 had positive results for the mini-battery (0.5%). Thus, of 115 diabetic patients with a diagnosis of definite autonomic neuropathy, the application of the mini-battery proposed is able to identify 110; moreover, the prevalence rates and the degree of cardiac autonomic neuropathy were different (Table 6). The overall prevalence of definite cardiovascular autonomic neuropathy with the mini-battery was ~42%, similar to the 46%

TABLE 6
Comparison between crude prevalence and degree of involvement of cardiac autonomic neuropathy diagnosed by applying both the classic battery of the four tests (DB, LS, VM, and OH) and the mini-battery we propose (DB plus SqT ratios)

Neuropathy	Classic battery n (%)	Mini-battery n (%)
None	180 (52)	168 (48.5)
Mild	51 (14.7)	29 (8.4)
Definite	76 (22)	110 (31.8)
Severe	39 (11.3)	39 (11.3)
Total (mild + definite + severe)	166 (48)	178 (51.4)

found in 103 diabetic patients with more sophisticated tests of autonomic function (spectral analysis and vector analysis [20]). The mini-battery proposed (DB followed by SqT with a 1-min rest between the two tests) is simple, quick (it takes 7 min to be performed), and less cumbersome for the patients. Because it is based on HR variation only, it can therefore be connected to a computer-assisted system. Moreover, with age-related normal ranges, it may be more sensitive.

The SqT test, as herein described and used, can discriminate between normal subjects and diabetic patients to a greater or equal extent than the other tests used, giving information on both sympathetic and parasympathetic activity. It is better than other single tests in identifying mild and sympathetic autonomic involvement, which may be important for early intervention trials (21).

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