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REVIEW

Recommendations for the use of cardiovascular tests in diagnosing diabetic autonomic neuropathy☆

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Abstract Despite its prevalence, clinical and prognostic impact, diabetic autonomic neuropathy, is widely under-diagnosed. The need for training and expertise to perform the cardiovascular tests (usually the task of diabetologists) is one possible reason. The availability of computer-assisted systems has allowed a wider diffusion of testing, but has also highlighted the need for an adequate knowledge of physiopathological backgrounds for their correct application and interpretation. The recommendations presented here were developed by the Neuropathy Study Group of the Italian Society of Diabetology and then endorsed by the Italian Association for the Study of Neurovegetative System, to promote the widespread adoption of good clinical practice in diabetic cardiovascular autonomic testing by outlining main evidence-based aspects, i.e. which tests, how to perform them, adequate interpretation of the results and their diagnostic use, confounding conditions that can impact on tests reliability. Therefore, these recommendations include the essential aspects of the physiopathological substrate

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of the tests, the controversial points in their analysis, their diagnostic characteristics, as well as safety. Detailed information is given on the physiological (age, weight, body position, resting heart rate and blood pressure, respiratory pattern, exercise, meals, acute blood glucose changes) and pathophysiological confounding factors, with emphasis on the effects of drugs. Instructions on how to perform the tests and interpret their results are also considered together with indications of candidate patients and periodicity of testing. A patient instruction sheet on why and how to perform the tests is included. Finally, the specific requirements for computerized systems to perform and evaluate cardiovascular tests are provided.

Preface to recommendations

Diabetic autonomic neuropathy (DAN) is a widespread disorder of the cholinergic, adrenergic and peptidergic autonomic fibres in the context of diabetes without other causes. It is characterized by a subclinical form that is detectable only by tests, and a clinical form, with the presence of signs and symptoms [1].

Cardiovascular diabetic autonomic neuropathy (CAN), the most studied form of DAN, is a frequent and early complication of diabetes, with a prevalence of around 20% in unselected patients [2,3]. Clinical correlates of CAN are age, diabetes duration, glycaemic control, diabetic sensorimotor polyneuropathy and the microangiopathic diabetic complications; emerging ones are cardiovascular risk factors - such as blood pressure (BP) - and cardiovascular diseases [3,4].

DAN causes significant morbidity due to the disabling impact of its clinical features (Table 1). Moreover, CAN is associated with a definite increased risk of mortality, with a relative risk of 6.2 and 3.65 in two distinct meta-analyses [3,5,6]. A predictive value of CAN with regard to cardiovascular morbidity has also been described [7]. The association of CAN with silent myocardial ischemia and/or coronary heart disease is well documented [5,6]. In the Detection of Ischaemia in Asymptomatic Diabetics (DIAD) study, heart rate response to Valsalva manoeuvre was the strongest determinant of silent myocardial ischemia [8]. CAN has also been associated with increased perioperative morbidity, with stroke, and with progression of diabetic nephropathy [9]. A number of potential pathogenetic mechanisms have been proposed to explain the excess mortality and morbidity associated with CAN (Table 1) [5,6,9].

Despite its prevalence and clinical impact, DAN is still widely under-diagnosed. The need for training and expertise to perform the cardiovascular tests (usually the task of diabetologists) is one possible reason. The availability of computer-assisted systems has indeed allowed a wider diffusion of CAN assessment, but at the same time has also highlighted the need for an adequate knowledge of physiopathological backgrounds and the confounding factors of cardiovascular tests. Despite the guidelines for the diagnosis of DAN [1,10,11] and documents on orthostatic hypotension and clinical autonomic testing [12,13] by the American Diabetes Association, the American Academy of Neurology and the American Autonomic Society, there is no widespread standardized approach to CAN testing [14].

These recommendations have been developed by the Neuropathy Study Group of the Italian Society of Diabetology (SID) [15] and have since been endorsed by the Italian Association for the Study of Neurovegetative System (AINV). They are intended to promote the widespread adoption of good practice in cardiovascular autonomic testing in diabetic patients, by readdressing the physiopathological substrate, the confounding factors, and providing instructions on how to perform and then interpret cardiovascular tests.

The full version of these recommendations is published online (Supplementary material) and contains a comprehensive background of this printed summarized version.

Preface

DAN is a common complication of diabetes, burdened by disabling clinical outcomes in its symptomatic forms. CAN is associated with an increased risk of overall mortality, cardiovascular morbidity, and anaesthetic complications during surgery. It can therefore be considered as a marker of risk as well as a potential promoter of morbidity and mortality.

Methods

The analysis of the available literature from PubMed (1966 to July 2007) used the following search terms: autonomic testing cross-referenced with diabetes, autonomic neuropathy cross-referenced with diabetes, diagnosis and cardiovascular tests. Additional terms were used when necessary. Literature search was updated to 2009 in the last revision. The methodology adopted for rating recommendations was that suggested for diagnostic studies by the European Federation of Neurological Societies [16]. For some aspects, the lack of clear evidence from the available literature led to the formulation of Good Practice Points (GPP) [16].

Recommendation on the role of symptoms in the diagnosis of DAN

The symptomatic forms of DAN are relatively uncommon, with the exceptions of erectile dysfunction and gastrointestinal symptoms, both multifactorial in origin [17]. The main autonomic symptoms to be investigated are those shown in Table 2.

Table 1 Spectrum of clinical manifestations of diabetic autonomic neuropathy.

Cardiovascular system
• Resting tachycardia with loss of reflex heart rate variations
• Exercise intolerance: reduced increase of heart rate and blood pressure
• Left ventricular dysfunction: reduced diastolic filling at rest
• Silent myocardial ischemia
• Orthostatic hypotension
• Postprandial hypotension
• Loss of circadian rhythm of blood pressure (nondipping, reverse dipping)
• Perioperative instability
• QT interval prolongation
• Sympatho-vagal imbalance
Peripheral vascular function
• Increased peripheral blood flow and warm skin
• Increased arteriovenous shunting and swollen veins
• Raised venous pressure and oedema
• Loss of cutaneous vasomotor reflexes in response to thermal stimuli and to injury
• Loss of venoarteriolar reflex with oedema and microvascular damage
• Increased incapillary permeability
• Mönckeberg sclerosis
Respiratory system
• Cardiorespiratory arrests
• Reduced bronchial reactivity
• Altered ventilatory responses
• Sleep apnoeic episodes (?)
Gastrointestinal system
• Altered esophageal motility
• Diabetic gastroparesis
• Diabetic diarrhoea
• Faecal incontinence
• Constipation
Urogenital system
• Diabetic cistopathy
• Erectile dysfunction
• Retrograde ejaculation
Hormonal secretion
• Decreased hypoglycaemia awareness
• Hypoglycaemia-associated autonomic failure
• Anaemia with erythropoietin deficiency
• Altered renin production
Pupillary function
• Reduced pupillary motor function
• Argyll-Robertson-type pupil
Sudomotor function
• Anhidrosis with dry skin at lower limbs
• Hyperhidrosis of the trunk
• Gustatory sweating

Although potentially disabling, autonomic symptoms are aspecific, poorly related to cardiovascular test abnormalities [18] and as such are not a reliable indicator of the presence of autonomic neuropathy [10].

Table 2 Autonomic symptoms.

Orthostatic hypotension
• Dizziness, blurred vision, fainting when standing up
Sweating abnormalities
• Sweating during meals, in particular with certain food
• Hyperhidrosis of the trunk or face especially nocturnal, with anhidrosis of legs or feet
Gastrointestinal symptoms
• Digestive difficulties, epigastric fullness and abdominal distension, nausea after meals, vomiting at awakening
• Watery nocturnal diarrhoea
• Faecal incontinence
• Constipation
Urinary abnormalities
• Lengthening of the interval between micturitions, lack of full bladder awareness
• Difficulty to start micturition (hesitancy)
• Prolonged dripping (terminal dribbling)
• Incapacity to hold urine (incontinence)
Erectile dysfunction
• Reduced capacity to reach and maintain erection
• Lack of spontaneous nocturnal or morning erections
• Reduced or absent ejaculation

Recommendation

Autonomic symptoms are aspecific and do not permit diagnosis of DAN. It is therefore necessary in any case to complete diagnostic assessment with the cardiovascular tests. Nonetheless, autonomic symptoms may be disabling and should be looked for in any diabetic patient so as to then perform the needed specific investigations - including cardiovascular tests - to obtain a differential diagnosis (level B).

Recommendation on which cardiovascular tests are indicated for CAN diagnosis and how to perform them

We recommend for CAN diagnosis the deep breathing test, the lying to standing test and the Valsalva manoeuvre among the heart rate tests, and the orthostatic hypotension test among the BP tests. The deep breathing test should be performed at six breaths per minute and all six respiratory cycles must be considered. The ratio between the 3 maximum and the 3 minimum RR intervals (the intervals between two consecutive ECG R waves), albeit belonging to different respiratory cycles, is recommended because it provides the highest values. In the lying to standing test the maximum/minimum 30:15 ratio is the preferable method to calculate the result as it detects the highest variability. In the Valsalva manoeuvre test the choice of the shortest RR interval during (and not after) the manoeuvre is proposed here as the most appropriate to calculate the Valsalva ratio (Fig. 1) [19—24].

In the orthostatic hypotension test, in measuring the standing BP fall we suggest considering only the standing values at 1 and 2 min. Despite the definition of orthostatic

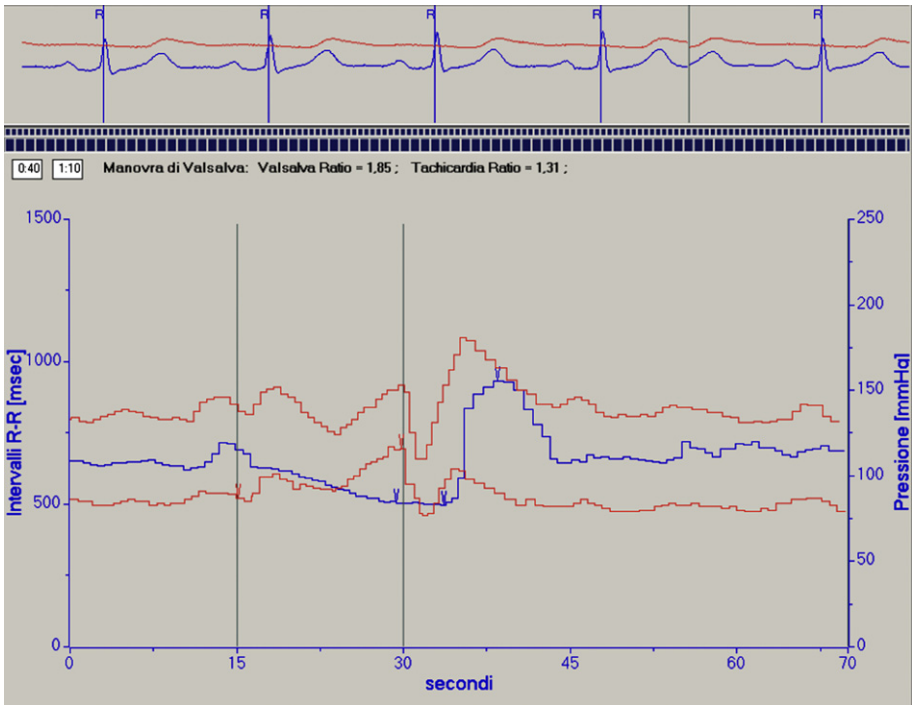

Figure 1 RR interval and systolic and diastolic blood pressure during Valsalva manoeuvre. In phase 1, in the first 2-3 seconds of the expiratory strain, a rapid rise in blood pressure and a prolongation of the RR interval (bradycardia) are observed. In phase 2, during strain, blood pressure initially decreases and then recovers and RR intervals gradually shorten (tachycardia). In phase 3, at release in the first few seconds, blood pressure rapidly decreases and RR intervals shorten further. In phase 4, there is a gradual increase in blood pressure over resting values (blood pressure overshoot) and a rapid prolongation of RR interval (bradycardia). The Valsalva ratio is obtained by dividing the longest RR interval in phase 4 by the shortest RR interval in phase 2. With the permission of K. Thomaseth and F. Bellavere.

hypotension by an ad hoc Consensus [12], we suggest as valid and appropriate the cut-off of 30 mmHg for the systolic BP fall on the basis of the values observed in the normal population [20,25,26]. An abnormal result of this test denotes an advanced form of CAN with prognostic implications but may also be due to other causes, such as reduced effective plasma volume or drug interference. The ease of the orthostatic hypotension test, the frequent presence in diabetic patients of multiple causes of orthostatic hypotension and its clinical and prognostic relevance, make advisable the use of this test on all patients at least on a yearly basis, even outside the complete programme of diagnosis and follow-up of CAN.

Other heart rate and BP tests, such as the cough test, the standing to lying test, the squatting test and the sustained handgrip test, are not recommended for CAN diagnosis [24,27].

Recommendation

Cardiovascular tests based on heart rate response to deep breathing, lying to standing and Valsalva manoeuvre, and on BP response to standing (orthostatic hypotension test) are an essential and irreplaceable part of CAN diagnosis (level B).

Regardless of the programme of diagnosis and follow-up of CAN, the measurement of BP in supine and standing positions should be performed in all diabetic patients at least at yearly intervals (level B).

Other tests of heart rate and BP, such as cough test, standing to lying test, squatting test, and sustained handgrip test, despite exploring some distinctive aspects of neural cardiovascular control, are not included among the tests recommended for CAN diagnosis (level C).

In performing and analysing the tests, adopt the modalities proposed here and verify that the software used complies with these modalities (GPP).

Recommendation on standardization and patient's compliance

As cardiovascular tests measure heart rate and BP responses to the activation of a neural reflex arch from the stimulus to the effector organ, an abnormal result can derive from an anomaly in any step of the reflex arch. Then, it is necessary to be aware of the confounding factors that can impact on the standardization of stimuli, on autonomic or cardiovascular function.

The patient's cooperation, through instruction, acquaintance, and familiarization with the test is essential to achieve a standardized stimulus, in particular for the deep breathing test and the Valsalva manoeuvre. A patient instruction sheet with detailed information on the tests is provided in the full online version (Supplementary material).

Recommendation

The reliability of cardiovascular tests depends on the standardization of the procedure and on the exclusion of confounding factors (level C).

It is necessary to provide sufficient instructions and to allow the patients to familiarize themselves with the test in order to obtain adequate stimuli (GPP).

Recommendation on physiological confounding factors

Among the physiological factors affecting the test results, the most important are: 1. age, with a greater impact on the deep breathing test (normal age-related values of the cardiovascular tests are provided in the full online version (Supplementary material)) [19—22]; 2. the respiratory pattern, mainly for the deep breathing test [28]; 3. the body position and duration of supine rest [23]; 4. resting heart rate and BP [23]; 5. physical exercise [29]; 6. coffee, alcohol and cigarette consumption [1]; and 7. meals, mainly for the orthostatic hypotension test [22].

Recommendation

To interpret the results of cardiovascular tests based on heart rate it is advisable to use normal age-related values (level B).

Available data does not indicate the need to apply normal sex-related values for cardiovascular test results (level B).

It is essential to carefully prepare the patient to perform deep breaths at 6 breaths per minute during the deep breathing test, and to avoid deep or irregular breaths after the Valsalva manoeuvre and after standing. The correct performing of tests should be verified and the reliability of any single result notably different from the others should be questioned (level C).

The deep breathing test may be performed indifferently in the supine or sitting position; it is preferable to perform the Valsalva manoeuvre in the sitting position, and to allow at least a 2-min rest in the supine position before the orthostatic test (level C).

Although the degree of the responses to the deep breathing test, when expressed as the E/I ratio, and to the orthostatic test are slightly influenced by the resting heart rate, no correction is needed for the resting heart rate. Caution in interpreting the results should be exercised in the case of a resting heart rate $>$100 bpm. Alterations in cardiac rhythm preclude the performing of the heart rate tests (level C).

The confounding effect of supine systolic BP - for values $>$160 mmHg and $<$120 mmHg - should be taken into account when evaluating BP response to the orthostatic test (level C).

Patients should be requested to avoid strenuous physical exercise in the 24 h preceding the cardiovascular tests. Exercise training might positively influence the heart rate test results (GPP).

Although the acute effect of coffee on regular and non regular consumers is not well defined, it is recommended that caffeine beverages, as well as smoking and alcohol, be avoided at least 2 h prior to the tests (GPP).

It is advisable to perform the tests when fasting or at least 2 h after a light meal (GPP).

Recommendation on pathophysiological confounding factors

Among the pathophysiological factors that can affect the cardiovascular tests, worth mentioning are: 1. obesity, because despite the absence of conclusive evidence of the confounding effect of obesity in diabetic patients (113) at least morbid obesity might attenuate chest reflexes [29—32]; 2. hypoglycaemia and hyperglycaemia for their effects on sympathetic activity and baroreflex sensitivity [33]; 3. insulin for its hypotensive effect in patients with severe autonomic failure [22]; 4. hypoxia and hypercapnia for their ability to induce sympathetic activation and baroreflex modulation; 5. chronic renal disease for its association with both sympathetic overactivity and uremic neuropathy; 6. cardiovascular diseases, such as hypertension, coronary artery disease and congestive heart failure [9], although in the Hoorn study in the general population, the presence of cardiovascular disease did not act as a strong determinant of abnormalities in cardiovascular autonomic function [34]; 7. drugs, since several antihypertensive or psychoactive drugs may interfere with cardiovascular autonomic function with the effects not necessarily being class-dependent but possibly both drug- and a disease-specific (Table 3) [35—37]. In the Hoorn study, the use of antihypertensive drugs was an independent determinant of cardiovascular autonomic function with a stronger effect in normotolerant subjects than in diabetic patients [34]. Although the interference of a drug on cardiovascular tests is not easily predictable in any given patient, when a pharmacological wash-out is not feasible, diuretics, sympatholytic agents and tricyclic antidepressants should be considered the most interfering drugs, in that they may cause false positive responses mainly of the orthostatic tests (Table 3).

These data support the following recommendations.

Recommendation

The presence of morbid obesity might represent an interfering factor on the response to the deep breathing test (GPP).

Tests should be avoided during hypoglycaemia or marked hyperglycaemia (GPP).

It is recommended that cardiovascular tests be performed at least 2 h after short-acting insulin administration (level C).

Test results should be interpreted with caution in the case of chronic obstructive pulmonary disease or respiratory failure (GPP).

In the presence of kidney faillure the role of comorbid uremic neuropathy should be taken into account in interpreting the results (level C).

Tests results should be interpreted with caution in the presence of cardiovascular diseases, particularly heart failure (level C).

It is recommended that cardiovascular tests be performed after an appropriate wash-out of potentially interfering drugs, particularly diuretics, sympatholytic agents and psychoactive drugs. When this is not feasible, results of the tests should be interpreted with caution (level C).

Although in some cases it can be difficult to attribute abnormal results to cardiovascular diseases, drugs, or CAN itself, given the prognostic significance of CAN, it is still

Table 3 Drugs interfering with the heart rate and blood pressure tests.

Class	Drug	Effect on HR	Effect on BP
Diuretics	Thiazydic-furosemide		↑ orthostatic hypotension
	Spironolactone	↑ HRV in general population ↓ HRV in diabetic patients	
β-blockers	Atenolol	↓ LF-RR, ↑ HF-RR in general population	
	Bisoprolol	↑ HRV in general population	
	Metoprolol	↓ LF, ↑ HF in general population ↑ HRV in diabetic patients	
α-blockers			↑ orthostatic hypotension
Calcium antagonists	Diltiazem	↑ HRV in general population and diabetic patients	
	Verapamil	↑ HRV in general population, no effect in diabetic patients	
	Nifedipine	No effect	
ACE-inhibitors	Captopril	↑ HRV in general population	
	Lisinopril	↑ HRV in general population	
	Trandolapril	↑ HRV in general population, no effect on HR tests in diabetic patients	
	Enalapril	No effect	
	Quinapril	↑ HRV and ↑ HF in diabetic patients, ↑ HR tests in diabetic patients	
Angiotensin-II-receptor antagonists	Losartan	↑ HRV in general population, controversial ↑ HR tests in diabetic patients	
	Eprosartan	↓ HRV in general population	
Digitalis	Digoxin	↑ HRV in general population	
Psychodrugs	Benzodiazepines midazolam, diazepam	↓ HRV in general population	
	Lorazepam	Controversial ↓ HF-RR in general population	
	Alprazolam	↑ HF-RR in general population	↓ MF-BP in general population
	Tricyclic antidepressants (amitriptyline > imipramine, nortriptyline > desipramine, doxepin > fluvoxamine)	↓ HRV, ↓ HF-RR, ↓ HR tests in general population	↑ orthostatic hypotension
	Carbamazepine	↓ HRV in epilepsy	
Acetylsalicilic acid		↑ HF-RR, ↓ LF-RR in general population	

Legend: HR, heart rate; BP, blood pressure; HRV, heart rate variability; HF-RR, high frequency component of HRV; LF-RR, low frequency component of HRV; MF-BP, medium frequency component of BP.
References for this Table are in the text and in the full online version.

useful to evaluate the presence of autonomic dysfunction in such patients (GPP).

Recommendation on requirements for computerized systems to perform and evaluate cardiovascular tests

The fundamental technical requirements for computerized systems used to perform and evaluate cardiovascular tests are provided in detail in the full online version. A primary requirement for a computerized system is to supply all the information needed to control and check the correct performing of the test and the validity of the results for the physician who has to certify the diagnosis.

Recommendation

Check that the computerized systems used for the performing and analysis of cardiovascular tests correspond to the recommended technical requirements. Ensure that

technical requirements are clearly presented in the user manual of the system (GPP).

Recommendation on reasons for diagnosing CAN

The reasons for CAN diagnosis were reaffirmed by the American Diabetes Association in 2005 [11], and include the following: 1. Early detection of abnormalities and timely diagnostic and therapeutic intervention; 2. Differential diagnosis and allocation of symptoms suggesting autonomic dysfunction; 3. For its prognostic impact, the motivation of both physician and patient to pursue strict glycaemic control, to correct cardiovascular risk factors and to use any specific therapies for CAN; 4. for the planning of moderate-to high-intensity physical exercise.

Recommendation

It is appropriate to diagnose CAN in order to correctly identify clinical pictures, attribute possible symptoms to an autonomic dysfunction and to initiate symptomatic therapies (level B); to motivate intensive therapeutic strategies to correct hyperglycaemia and cardiovascular risk factors (level C); and to obtain information towards some aspect of therapeutic management, such as BP control and physical activity programs (level C).

Recommendation on indications for the diagnosis of CAN: choosing candidate patients and when to do the tests

Although the prevalence of CAN is clearly related to diabetes duration, there is also evidence of the presence of cardiovascular tests abnormalities at the time of diagnosis, in adolescents and young patients, and in older patients with a short diabetes duration. Moreover, a relationship between CAN and glycaemic control, cardiovascular risk factors and the other diabetic complications is well documented [3,4]. Candidate patients for the diagnosis of CAN have therefore been identified [11] as those indicated in the following recommendation.

Recommendation

Diagnosis of CAN should be performed:

- In type 2 diabetic patients at diagnosis,
- In type 1 diabetic patients 5 years after the diagnosis,
- Independently from diabetes duration, in the diabetic patients with symptoms suggesting autonomic dysfunction,
- In diabetic patients before planning a program of moderate-to-high-intensity physical exercise, especially in the presence of high cardiovascular risk,
- In diabetic patients with a history of poor glycaemic control, high cardiovascular risk and microangiopathic complications, especially when planning major surgical procedures (level C).

Recommendation on diagnostic characteristics of cardiovascular tests: sensitivity, specificity, reproducibility

The deep breathing, lying to standing and Valsalva manoeuvre tests are considered indexes mainly of parasympathetic function, that is "cardio-vagal", while the orthostatic hypotension test is considered a test of sympathetic function, that is "adrenergic" [13,27] Being non-invasive, safe, clinically relevant, easy to perform, sensitive, specific, reproducible and standardized, these tests are considered established measures of autonomic function with high quality (classes I and II) and high strength of evidence (B) [13]. While the orthostatic hypotension test has low sensitivity and high specificity [26,27], all heart rate tests seem to have high sensitivity and specificity (80—90%) for CAN (diagnosed using Ewing's tests) [5,19]. Reproducibility data available in literature provide coefficients of variation lower than 10% for E/I ratio and 30:15 ratio, and of 10—15% for Valsalva ratio in both healthy and diabetic subjects [38]. None of the three heart rate tests shows a clear superiority in diagnostic characteristics over the remaining two [38].

Recommendation

Based on their characteristics of sensitivity, specificity, reproducibility, ease of performing, and standardization, cardiovascular tests of deep breathing, orthostatism, Valsalva manoeuvre, and orthostatic hypotension test are established measures of autonomic function. There is no clear evidence of any diagnostic superiority between the heart rate tests (level B).

Recommendation on safety of cardiovascular tests

Cardiovascular tests have a high value to risk ratio. The Valsalva manoeuvre increases intrathoracic, intraocular (by 7.9 mmHg) [39] and intracranial pressure and may theoretically be associated with a small risk of intraocular haemorrhage or lens dislocation [13]. In clinical practice there is the habit of avoiding the Valsalva manoeuvre in the presence of either proliferative retinopathy with the risk of retinal haemorrhage, or severe uncontrolled hypertension, although there is no report in the literature of complications with sequelae for this test in diabetic patients [13].

Recommendation

Cardiovascular tests are a safe diagnostic tool that is free from complications (level C). Given the lack of definitive evidence of the safety of the Valsalva manoeuvre in the presence of proliferative retinopathy and the risk of retinal haemorrhage, this test is not advisable in these circumstances (GPP).

Recommendation on rationale of the battery of tests

It is simplistic to believe that a single test may evaluate global autonomic function [13]. Although the three heart

rate tests explore mainly parasympathetic function, the nervous pathways and reflex mechanisms involved are not identical, playing sympathetic activity and baroreflex a contributory role in the orthostatic test and the Valsalva manoeuvre [40]. The diagnostic definition of CAN based on several tests reduces the probability of false positives. The use of a battery of tests allows indication of severity or progression of CAN or just a more robust definition of complication. The need to use several tests of both vagal and sympathetic function is restated in the available guidelines [1,11,19].

Recommendation

For the diagnosis and monitoring of CAN, a battery of independent tests of parasympathetic and sympathetic function is required (level B).

Recommendation on requirements for the diagnosis of CAN

No agreement exists on the number of abnormal cardiovascular tests required to reach the diagnosis of CAN. Previous guidelines underlined that to establish the presence of CAN it is advisable to detect abnormalities in more than one test on several occasions [10]. In a meta-analysis evaluating the association between CAN and mortality, the predictive value for mortality changed accordingly to the definition of CAN on the basis of one or at least 2 abnormal tests, with the relative risks being 1.2 and 3.45, respectively [36]. This supports the view that a true negative prognostic value can be attributed only to a definite CAN and that more than one abnormal test is required to diagnose a definite CAN.

Recommendation

In the light of the available evidence and on the basis of the expert opinion, at least two abnormal tests of heart rate are required for the diagnosis of CAN. The presence of one abnormal test identifies the condition of early or uncertain CAN, to be confirmed over time. The presence of orthostatic hypotension identifies a condition of severe or advanced CAN (level C).

Recommendation on staging of CAN

Various methods for staging CAN have been proposed until now, considering the number and/or kind of abnormal tests: 1. the early, intermediate and severe stage, defined according to the presence of abnormalities in the deep breathing, Valsalva manoeuvre and orthostatic hypotension test, respectively [10]; 2. the early, definite and severe involvement according to the presence of one abnormal or two borderline heart rate tests, two or more abnormal heart rate tests, and presence of orthostatic hypotension, respectively [19].

Due to the limited sensitivity of the orthostatic hypotension test (the only test of sympathetic function currently in use) an approach to staging of CAN that considers a progression from an early to an advanced involvement is considered more appropriate than a progression from a parasympathetic to a sympathetic neuropathy [19,41]. Moreover, no chronological hierarchy among the heart rate tests has been clearly proved so far.

Recommendation

A staging of CAN based on the battery of cardiovascular tests allows the identification of the transition from early to advanced involvement (level C).

Recommendation on periodicity of the cardiovascular test performing

The early performing of cardiovascular tests enables a baseline evaluation that is useful for the comparison with follow-up tests [11]. Indication on periodicity of test performing is suggested in the following recommendation.

Recommendation

In the considered opinion of the experts, in the case of altered cardiovascular tests in the baseline evaluation, it is advisable to repeat the tests annually in order to confirm the diagnosis of CAN and evaluate its progression. Moreover, even in the absence of alterations of cardiovascular tests, it is advisable to repeat the tests annually in diabetic patients with poor glycaemic control, high cardiovascular risk and microangiopathic complications, whereas in the others patients a longer interval is recommended (GPP).

Disclosure

Nothing to disclose.

Authors' contributions

Study concept and design: VS (general organisation), FB, LS, RQ, GB, PM, GV, KE.

Literature research and drafting of various sections of the manuscript: VS (preface, comments to the analysis, confounding factors, interpretation of cardiovascular tests), FB (history, requirements for computerized systems), LS (description of heart rate tests), RQ (orthostatic hypotension test), GB (other cardiovascular tests), PM (symptoms), GV (other cardiovascular tests), KE (other cardiovascular tests).

Participation in 2 two-day meetings to discuss results, to revise drafts, and to acquire a shared position: VS, FB, LS, RQ, GB, PM, RM.

Analysis of patients' cardiovascular test database: VS, FB, LS, GB, RM.

Critical revision of the manuscript: VS, FB, LS, SM, PC.

Preparation of the final manuscript: VS, FB, SM.

All authors have read and approved the final manuscript.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.numecd.2010.07.005.

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Recommendations for the use of cardiovascular tests in diagnosing diabetic autonomic neuropathy

Short title: Cardiovascular autonomic tests in diabetes

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Abstract

Despite its prevalence, clinical and prognostic impact, diabetic autonomic neuropathy, is widely under-diagnosed. The need for training and expertise to perform the cardiovascular tests (usually the task of diabetologists) is one possible reason. The availability of computer-assisted systems has allowed a wider diffusion of testing, but has also highlighted the need for an adequate knowledge of physiopathological backgrounds for their correct application and interpretation. The recommendations presented here were developed by the Neuropathy Study Group of the Italian Society of Diabetology and then endorsed by the Italian Association for the Study of Neurovegetative System, to promote the widespread adoption of good clinical practice in diabetic cardiovascular autonomic testing by outlining main evidence-based aspects, i.e. which tests, how to perform them, adequate interpretation of the results and their diagnostic use, confounding conditions that can impact on tests reliability. Therefore, these recommendations include the essential aspects of the physiopathological substrate of the tests, the controversial points in their analysis, their diagnostic characteristics, as well as safety. Detailed information is given on the physiological (age, weight, body position, resting heart rate and blood pressure, respiratory pattern, exercise, meals, acute blood glucose changes) and pathophysiological confounding factors, with emphasis on the effects of drugs. Instructions on how to perform the tests and interpret their results are also considered together with indications of candidate patients and periodicity of testing. A patient instruction sheet on why and how to perform the tests is included. Finally, the specific requirements for computerized systems to perform and evaluate cardiovascular tests are provided.

Preface to Recommendations: definition, epidemiology, clinical and prognostic impact of diabetic autonomic neuropathy

Diabetic autonomic neuropathy (DAN) is a widespread disorder of the cholinergic, adrenergic and peptidergic autonomic fibres in the context of diabetes without other causes. It is characterized by a subclinical form that is detectable only by tests, and a clinical form, with the presence of signs and symptoms (1). DAN manifests itself in a varied manner involving - to different degrees and at different times - the various functions controlled by the autonomic nervous system (Table 1).

Cardiovascular diabetic autonomic neuropathy (CAN), the most studied form of DAN, is a frequent and early complication of diabetes. Considering the population study Oxford Community Diabetes Study (2) and two wide multicentric European studies in unselected patients, the EURODIAB IDDM Complications Study in 3250 type 1 patients (3) and the Diacan Multicenter Study Group in 1171 type 1 and type 2 diabetic patients (4), the prevalence of CAN according to cardiovascular tests abnormalities is estimated to be between 16.8 and 20.9% in type 1 and between 15.8 and 22.1% in type 2 diabetic patients. Although abnormalities in cardiovascular tests or other autonomic function indexes are already present at the time of diagnosis both in type 1 (7.7%) and type 2 diabetes (5-7.3%) (5-8), prevalence of CAN increases mainly with age and diabetes duration. In the population-based Rochester Diabetic Neuropathy Study the prevalence of cardiovagal impairment was 27% in type 1 (mean age 51 years and mean duration 24 years) and 44% in type 2 diabetic patients (mean age 64 years and mean duration 15 years) (9). In a community-based study in Denmark the prevalence of CAN in 40-70 years old type 1 diabetic patients was 38% (10). In 506 unselected insulin-dependent diabetic patients aged 12 to 85 years the frequency of CAN (at least 2 abnormal tests out of 4) increased from 16.6% in the whole cohort to 24.5% in diabetics aged 40 to 49 years and 30.7% in those with diabetes of 20 or more years duration (11). Longitudinal clinic-based studies have given insights into the progression rate of CAN suggesting an absolute increase in prevalence of CAN of about 2 and 6% per year in type 1 and type 2 diabetic patients, respectively (5, 12-14).

There are several more obvious clinical correlates of CAN in addition to age and diabetes duration, i.e. glycaemic control, diabetic sensorimotor polyneuropathy, and the microangiopathic diabetic complications; emerging ones are cardiovascular risk factors, such as blood pressure (BP), cardiovascular diseases, and insulin levels (in type 2 diabetes) (5, 12, 15-17). Although CAN often coexists with diabetic sensorimotor polyneuropathy (17), there is no complete parallel behaviour between the two neuropathies and their development may diverge in type 2 diabetes (5). Therefore, diagnostic tests for diabetic sensorimotor polyneuropathy do not adequately substitute those for CAN, and CAN should not be considered as either a small or obscure component of diabetic sensorimotor polyneuropathy (18).

DAN is a cause of significant morbidity because of the disabling impact of its clinical features (Table 1). Moreover, CAN is associated with a definite increased risk of mortality. In a meta-analysis of 12 studies, regarding 2615 diabetic patients followed for 1-11.5 years, the median of mortality at 5 years was 25% in diabetic patients with CAN compared to 4% in those without CAN, with a relative risk of 6.2 (15). A further meta-analysis of 15 longitudinal studies regarding 2900 patients followed for 1-16 years showed that the diagnosis of CAN based on at least two abnormal cardiovascular tests determines a relative risk of mortality of 3.65 (95% C.I. 2.66-4.47) (19). In a subsequent 10-year prospective study in 391 type 1 diabetic patients, combined abnormality in heart rate variability and QTc was a strong predictor of mortality independently of conventional risk factors with an adjusted hazard ratio of 6.7 (20). Prolonged QTc was an independent predictor of mortality also in the nondiabetic and diabetic elderly population of the Monica/Kora Augsburg Cohort Study (21).

CAN is also associated with perioperative morbidity, i.e. cardiorespiratory arrests during anaesthesia, increased instability during surgery and abnormal cardiovascular reactions even during minor surgery

(22, 23), more severe intraoperative hypothermia (24).
An association of CAN with silent myocardial ischemia and/or coronary artery disease is well documented although not unanimously and at a smaller degree as previously suggested (18, 25, 26). In a meta-analysis of 12 studies regarding 1468 diabetic patients silent myocardial ischemia was present in 10% of those without CAN and in 20% of those with CAN with a prevalence rate ratio of 1.96 (18). At baseline, DIAD study showed that in 1123 type 2 diabetic patients heart rate response to Valsalva manoeuvre was the strongest determinant of silent myocardial ischemia (odds ratio 5.6) (27). Some longitudinal studies have documented the predictive value for stroke of cardiovascular tests abnormalities or QT interval prolongation (28-30). There is also evidence of an association between CAN and nondipping or reverse dipping, defined as a day-night variation in BP of less than 10% or of less than 0%, respectively (31). The demonstration of a significant correlation between the day-night change in LF/HF ratio (index of sympathovagal balance) and the day-night difference in BP supported a possible pathogenetic link between a relative nocturnal sympathetic predominance and nondipping in diabetic patients (31, 32) and also in elderly hypertensive patients (33). Nondipping or reverse dipping is a recognized correlate of end-organ damage of hypertension in cross sectional studies (34) and an independent predictor for cardiovascular events and for the progression of diabetic nephropathy in some longitudinal studies (31, 35-38). The prognostic value of nondipping seems to be more evident for the reverse dipping or the so-called rising pattern, condition more strongly related to CAN (39). CAN itself and autonomic pupillary abnormalities have been shown to predict independently the progression of diabetic nephropathy (40, 41), although not unanimously (42). A number of potential pathogenetic mechanisms have been proposed to explain the excess mortality and morbidity associated with CAN: reduced heart rate variability, imbalance of sympathovagal activity leading to unfavourable sympathetic predominance, QT interval prolongation, impaired baroreflex sensitivity, cardiac sympathetic dysinnervation, reduction in sympathetically mediated vasodilatation of coronary vessels, silent myocardial ischemia, dysregulation of cerebral circulation, postural hypotension, and nondipping are both conditions associated with CAN and predictors or promoters of cardiovascular morbidity and mortality (19, 31). Progression of diabetic nephropathy (40, 41) or contributory role to diabetic foot complications (43) are other suggested potential and meaningful links between CAN and diabetes burden.
Despite the definite evidence of a predictive value of CAN on mortality and morbidity, the diagnosis of CAN is not widely performed (18, 44). The possible reasons for this phenomenon are the low level of interest in an unfamiliar complication, scepticism about available therapeutic options and therefore about the utility of diagnosis, the only recent availability of standardized diagnostic methods, and finally the need for training and expertise to perform the cardiovascular tests, usually the task of diabetologists. The availability of computer-assisted systems for cardiovascular tests has allowed a wider diffusion of their use, but at the same time has also outlined a need for an adequate physiopathological background knowledge for their correct application and interpretation. Unlike peripheral nervous function measurements, cardiovascular tests are not a direct measure of the autonomic nervous system and may be influenced by several interfering factors. Moreover, there are still problems in the standardization of the tests, which is reflected in the wide variability in methods and structures in European laboratories for the study of the autonomic nervous system (45).
Guidelines for the diagnosis of CAN by the American Diabetes Association and/or the American Academy of Neurology were published in 1988, 1992 and 2005 (1, 44, 46); in 1996 two more documents on orthostatic hypotension and clinical autonomic testing by the American Academy of Neurology and the American Autonomic Society were added (47, 48).
These recommendations have been developed by the Neuropathy Study Group of the Italian Society of Diabetology (SID) (49) and have since been endorsed by the Italian Association for the Study of Neurovegetative System (AINV). They are intended to promote the widespread adoption of good

practice in cardiovascular autonomic testing, by readdressing the essential aspects of the physiopathological substrate, the confounding factors, and providing instructions on how to perform and then interpret cardiovascular tests.
This is the full version of recommendations the summarized version of which is published in the printed format of the journal.

Preface
Diabetic autonomic neuropathy is a common and early complication of diabetes, burdened by disabling clinical outcomes in its symptomatic forms. Cardiovascular diabetic autonomic neuropathy is associated with an increased risk of overall mortality, cardiovascular morbidity, and anaesthetic complications during surgery. It can therefore be considered as a marker of risk as well as a potential promoter of morbidity and mortality.

Brief history of cardiovascular tests
Although autonomic symptoms in diabetes have been known for over a century, the systematic diagnosis of CAN only goes back fifty years or so (50-53). In the early 1970s interest in finding an objective test to assess autonomic function developed. Since the fundamental principles of cardiac neural control were well known, it was decided to adopt manoeuvres capable of investigating the activity - and the integrity - of the autonomic nervous system of the heart. It was already accepted that heart rhythm depended on the 'decelerating' or 'accelerating' effects on heart rate of nerve fibres with opposing roles: respectively, parasympathetic and sympathetic. No one however had attempted to measure the efficiency and the interactive control on heart beat of these autonomic components using simple physiological stimuli, until Wheeler and Watkins in London in 1973 (54) and Baldwa and Ewing in Edinburgh in 1977 did so (55), using the deep breathing test and the standardized Valsalva manoeuvre.
These were the first two objective and quantitative cardiovascular tests to be introduced into clinical practice. The two tests were enormously successful. To obtain better physiopathological definition, Ewing later introduced further tests (56): heart rate variation during a change in posture (lying to standing), BP change on standing (postural hypotension), and BP rise with prolonged isometric contraction (sustained handgrip); suggesting what is now the ‘classic’ battery of five tests bearing his name. The first three tests based on heart rate variation, and the other two based on BP variation were considered adequate to explore the efficiency of the two main efferent nervous pathways, respectively. Thus, using only a simple electrocardiogram and measuring BP, it was possible to: a) obtain a certain diagnosis of a suspected CAN, b) quantify the degree of CAN, c) detect the presence of a sub-clinical CAN, and d) measure the relationship between the two main autonomic efferent components, sympathetic and parasympathetic.
The use of Ewing’s classic battery of tests underwent a simplified evaluation through a scoring system, introduced in 1983 (57), with the intention of making the tests easier to interpret for any clinician. A further potential application of the tests is becoming increasingly important, namely e) the attribution of a prognostic value to cardiovascular tests with regard to mortality (58), sudden death and (possibly) silent coronary artery disease (27) that are associated with both CAN and diabetes. The cardiovascular tests are now being used all over the world in the diagnosis of diabetic and non diabetic autonomic dysfunction, also in cardiological and neurological fields (59).

Methods
This report is derived from the analysis of the available literature from PubMed (1966 to July 2007) using the following search terms: autonomic testing cross-referenced with the term diabetes, autonomic neuropathy cross-referenced with the terms diabetes, diagnosis, and cardiovascular tests. The

bibliographies of the selected articles were reviewed for relevant articles. For some aspects, such as confounding factors on cardiovascular tests additional research was conducted using other specific terms: drug classes (ACE inhibitors, sartans, benzodiazepines, antidepressants, sympathetic blockers, diuretics), caffeine, smoking, cardiovascular disease, pulmonary disease, blood glucose, physical activity. Moreover, major textbooks on autonomic neuropathy and diabetic neuropathy, reviews and consensus on diabetic neuropathy and on autonomic testing were used. Finally, unpublished Authors' data was also considered and some conclusions were based on the Authors' expertise in CAN. Literature search was updated to 2009 in the last revision by the Authors.
The methodology adopted for rating the quality of evidence and strength of recommendations was that suggested for diagnostic studies by the European Federation of Neurological Societies (60). For some aspects, the lack of clear evidence from the available literature led to the formulation of Good Practice Points (GPP) (60).

The role of symptoms in the diagnosis of DAN

Despite the frequency and spread of functional autonomic alterations measurable at various levels (Table 1), the symptomatic forms of DAN are relatively uncommon (61, 62), with the exceptions of erectile dysfunction (multifactorial in origin) and of gastrointestinal symptoms, which are otherwise common in the general population and have no strong link in diabetic patients with either the motor dysfunctions detected at gastrointestinal level or the cardiovascular tests (63).
The main autonomic symptoms to be investigated are those of orthostatic intolerance, gastric alterations, diarrhoea and constipation, bladder dysfunction, erectile dysfunction, and sweating alterations (Table 2). In general, the symptoms of autonomic neuropathy tend to be intermittent, may worsen, and rarely disappear (64).
Simple and validated questionnaires for the evaluation of autonomic symptoms are not available. The Autonomic Symptom Profile is the only validated questionnaire but contains some 169 questions related to 11 domains of different autonomic functions (65). The Diabetes Bowel Symptom Questionnaire has been proposed as a measure of gastrointestinal symptoms and glycaemic control in diabetes suitable for epidemiological and clinical studies (66). In the Rochester Diabetic Neuropathy Study a correlation between the autonomic symptoms - evaluated using the Autonomic Symptom Profile questionnaire - and the autonomic deficits scored according to sudomotor function and cardiovascular tests assessment, was present but weak in type 1 diabetic patients, and completely absent in type 2 diabetic patients (9). In another study orthostatic symptoms were poorly related to postural systolic BP fall in both type 1 and type 2 diabetes (67).
Although potentially disabling, autonomic symptoms are aspecific and as such are not a reliable indicator of the presence of autonomic neuropathy (46). It is therefore necessary to complete diagnostic assessment with the cardiovascular tests and to exclude other non autonomic causes of symptoms.

Recommendation (level B)
Autonomic symptoms are aspecific and do not permit diagnosis of DAN. It is therefore necessary in any case to complete diagnostic assessment with the cardiovascular tests.
Nonetheless, autonomic symptoms may be disabling and should be looked for in any diabetic patient so as to then perform the needed specific investigations - including cardiovascular tests - to obtain a differential diagnosis.

Cardiovascular tests: description of tests based on heart rate

The tests based on heart rate variations are the deep breathing test, the lying to standing test and the Valsalva manoeuvre. These tests require a continuous recording of heart rate by either a simple electrocardiograph (ECG) or by an analogue signal acquisition, subsequently elaborated via a specialist software. It is essential to inspect the ECG trace (on paper or monitor) in order to exclude artifacts or any type of arrhythmias from the calculations.

Deep breathing test

Physiology: in normal subjects heart rate varies with respiratory cycles, showing an increase during inspiration and a decrease during expiration. This phenomenon, called sinus arrhythmia, is under parasympathetic control, being abolished with atropine and vagus nerve section in animals. The depth of respiration increases sinus arrhythmia; the test has been standardized by asking the subject to breathe deeply at six breaths per minute (5 seconds in and 5 seconds out) for one minute (68).

Performing: the subject is kept in supine or sitting position for at least 1 minute and then invited to start the test with a deep inspiration to the maximum total lung capacity for 5 seconds, followed by a forced expiration down to the residual volume. Such a respiratory cycle is repeated 6 times in 1 minute. The time to alternate the respiratory cycle is signalled directly to the patient by the operator or, preferably, by a time-keeping instrument. It is important that the subject does not change the respiratory phase before the 5 seconds have elapsed.

Analysis: the result can be expressed as the expiration-inspiration ratio (E/I ratio) or as the difference between maximum and minimum heart rate. The E/I ratio is obtained by calculating the ratio between the average of the 3 longest RR intervals during expiration and the average of the 3 shortest RR intervals during inspiration. The difference in heart rate is calculated by measuring the difference between the average of the 3 highest heart rates and the average of the 3 lowest heart rates over the 6 breathing cycles.

Lying to standing test

Physiology: in normal subjects heart rate increases after standing to maintain an appropriate stroke volume, and then decreases. The maximum increase in heart rate generally occurs between the 10th and the 20th beat after standing, whereas heart rate generally returns to lower values between the 25th and the 35th beat. Initial cardiac acceleration upon standing is an exercise reflex with vagal withdrawal, while subsequent acceleration is baroreflex-mediated with both vagal inhibition and sympathetic stimulation. The cardiac deceleration that follows is an expression of baroreflex-mediated vagal enhancement. Pharmacologic testing suggests most of the heart rate changes are parasympathetic. Atropine influences the heart rate response to standing in that it abolishes the increase, while propranolol has no effect (17). The test is therefore considered a measure of the parasympathetic function. **Performing**: after lying in the supine position for at least 5 minutes, the subject is invited to stand up quickly but remain relaxed, with their arms at rest alongside the body, without speaking or moving until the end of the test (generally 30-45 seconds after standing up). The test might also be performed with a simultaneous recording of ECG and BP, to measure both heart rate and BP response to standing; in this case the supine lying period should last for at least 5-10 minutes before starting BP measurements.

Analysis: the result of the test is expressed by the 30:15 ratio, obtained as the ratio of the longest RR interval measured between the 25th and the 35th beat after standing up to the shortest RR interval measured between the 10th and the 20th beat after standing up.

Valsalva manoeuvre

Physiology: the Valsalva manoeuvre is a forced expiration with an open glottis against resistance. This manoeuvre causes changes in both BP and heart rate (Figure 1). During the expiratory straining, the intrathoracic pressure increases and causes a reduction of the venous return to the heart, with a subsequent BP decrease, reflex tachycardia and peripheral vasoconstriction. At the release of the strain, intrathoracic pressure decreases to normal values, the venous return increases abruptly with subsequent BP overshoot, baroreceptor activation and bradycardia. During strain, tachycardia is initially determined by vagal withdrawal and afterwards by sympathetic activation. The peripheral reflex vasoconstriction is due to the sympathetic nervous system activation.
Performing: the subject in the sitting position is invited to blow with an open glottis into a mouthpiece connected to a manometer, without taking a deep breath beforehand, and to maintain a constant expiratory effort equivalent to an intraoral pressure of 40 mmHg for 15 seconds. After this period, the expiratory straining is suddenly released, and the subject should breathe regularly and remain silent and motionless until the end of the test. To perform the manoeuvre correctly, it is essential that the expiration occurs with an open glottis and not with the use of the cheeks. In the latter case, the reduction of the venous return, which is the initial event precipitating the chain of BP and heart rate modifications, does not occur. The occurring of facial flushing and plethora, and neck vein engorgement testify to the correctness of the manoeuvre (69).
Analysis: the heart rate variation induced by BP changes during the Valsalva manoeuvre is obtained by measuring the Valsalva ratio, calculated as the ratio between the longest RR interval after the expiratory straining and the shortest RR interval during the expiratory straining.

Cardiovascular tests: description of test based on blood pressure

The BP test is based on the variations induced by the change from supine to standing position (orthostatic hypotension test).

Orthostatic hypotension test
Physiology: the test is considered an index of sympathetic function, as the maintaining of BP during standing is mediated by an activation of the sympathetic nervous system that increases cardiac output and above all vascular peripheral resistance.
Performing: after at least five minutes of supine rest, BP is measured three or more times, 1 minute apart, until it stabilizes, then the subject is invited to stand up quickly, with help if needed, and BP is measured twice, after 60 and 120 seconds in the standing position. The arm in which BP is measured should be placed horizontally at the level of the right atrium and supported to avoid any isometric physical exercise that might increase BP. Standing with the arm hanging alongside the body results in an overestimation of BP values (up to 10 mmHg) and may therefore reduce the sensitivity of the manoeuvre in detecting orthostatic hypotension (70).
Analysis: to interpret the test, the difference between the last systolic BP value measured in the supine position and the lowest value during standing is considered.
An abnormal result does not necessarily reflect the presence of CAN but may be the consequence of other autonomic nervous system dysfunctions, or conditions characterized by reduced effective plasma volume, or drugs. When the abnormal test is due to CAN, it denotes a particularly severe form with serious prognostic implications.
The ease of the manoeuvre, the common presence of multiple causes of orthostatic hypotension in diabetic patients and its clinical and prognostic relevance, make advisable the use of the orthostatic hypotension test on all patients at least on a yearly basis, even outside the complete program of diagnosis and follow-up of CAN.

Recommendation (level B)
Cardiovascular tests based on heart rate and BP variations are an essential and irreplaceable part of CAN diagnosis.
Regardless of the programme of diagnosis and follow-up of CAN, the measurement of BP in supine and standing positions should be performed in all diabetic patients at least at yearly intervals.

Other cardiovascular tests

Other tests of heart rate and BP have been proposed. These tests, although they explore some various aspects of nervous cardiovascular control, are not commonly used in the diagnosis of CAN.

Cough test
The test evaluates the cardioacceleratory response to coughing and explores cardiac parasympathetic integrity. In supine position the subject is requested to give three vigorous maximal coughs evenly spaced over 3 s. Intermittent coughing determines intrathoracic and arterial pressure fluctuations that activate a heart rate response characterised by an immediate RR shortening after the last cough followed by a slower RR lengthening to resting values in about 12-14 s. The cough-induced cardioacceleration is essentially reflex in nature and under cholinergic control in that a vagal withdrawal occurs due to both the contraction of abdominal and expiratory muscles and baroreceptor activation. The cough-induced heart rate response is expressed by the CT ratio, i.e. the ratio between the baseline RR interval and the shortest RR interval in the first 12 s after coughing. A limitation of the test might be the difficulty in correctly performing three vigorous coughs (71).

Standing to lying test
The test evaluates the immediate heart rate response when changing from standing to supine position. The easy-to-perform test is aimed at evaluating both parasympathetic and sympathetic function. The analysis is in two phases, an early phase when the S-L_1 is calculated as the ratio between the mean RR interval before lying and the shortest RR interval over the first 5 beats after lying, and a late phase when the S-L_2 is obtained as the ratio between the longest RR interval between the 20th and 25th beat and the shortest RR interval over the first 5 beats after lying. S-L_1 and S-L_2 are considered a pure parasympathetic test and a predominantly sympathetic test, respectively (72).

Squatting test
The test evaluates the heart rate response on an active change from standing to squatting and vice versa. Squatting-induced bradycardia appears to be mediated by the vagus nerve, as it is completely abolished by atropine and unaffected by propranolol. Conversely, cardioacceleration from squatting to standing is thought to represent a sympathetic activation - abolished by propranolol - triggered by the orthostatic fall in BP (73). The SqT vagal ratio is measured as the ratio between the RR interval mean before squatting and the longest RR interval after squatting. The SqT sympathetic ratio is obtained as the ratio between the baseline RR interval and the shortest RR interval at standing. A limitation of the test might be the difficulty in squatting especially for elderly or obese patients.

Sustained handgrip test
The test is a measure of sympathetic function because the increase in diastolic BP during sustained isometric muscle contraction is mediated by the sympathetic nervous system, which in turn causes an increase in cardiac output and peripheral vascular resistance.
BP is measured three times in the sitting position, then the subject is invited to press a handgrip dynamometer with the contralateral hand with the greatest possible strength. Then, he/she is asked to

grasp the dynamometer up to 5 minutes, maintaining one-third of the previously-evaluated maximum effort. BP values are measured every minute during the effort.
The difference between the maximum diastolic BP during the effort and the mean resting diastolic BP is considered. The test result is normal when the increase in diastolic BP is higher than 15 mmHg, borderline between 15 and 10 mmHg, and abnormal when lower than 10 mmHg (74). The sustained handgrip test is no longer considered a necessary component of the cardiovascular test battery for the diagnosis of CAN for a number of reasons: its difficulty to perform, its limited sensitivity, specificity and reproducibility (75), and potential harm due to hemodynamic stress (although there are no reports of adverse events due to the test).

Recommendation (level C)
Other tests of heart rate and BP variations, despite exploring some distinctive aspects of neural cardiovascular control, are not included among the tests recommended for CAN diagnosis.

Comments to the analysis of cardiovascular tests

Deep breathing test
There is almost unanimous agreement that deep breathing must be performed at six breaths a minute with a test duration of one minute and that all six respiratory cycles must be considered (76). This breathing rate guarantees the maximum heart rate variation (17, 75). No agreement exists with regard to the best method to describe respiratory sinus arrhythmia during the test, i.e. based on heart rate or on RR interval and calculated as difference (in bpm or seconds) or as a ratio (77-79). Each measure has its specific properties and likely there is no clear evidence to prefer heart rate difference to E/I ratio or viceversa (77) and many software provide both measures. Some debate exists, moreover, as to the choice of the minimum and maximum RR intervals during the breathing cycles to be considered for the E/I ratio: the ratio between the mean of all 6 maximum and 6 minimum RR intervals (80), the selection of the highest ratio among those of the 6 respiratory cycles (73), the average of the ratios of three consecutive cycles (81), and the ratio between the 3 maximum and the 3 minimum RR intervals albeit belonging to different respiratory cycles (82). The latter modality provides the highest values and represents the one put forward in these recommendations.

Lying to standing test
The index originally proposed to evaluate this test was the 30:15 ratio, that is the ratio between the 30th and the 15th RR interval after standing. However, it was noticed that the greatest tachycardia and bradycardia are not always observed at the 15th and 30th beat respectively after standing up. Accordingly, the index was subsequently calculated as the ratio between the longest RR interval around the 30th beat and the shortest RR interval around the 15th beat, the ratio between the longest RR interval between the 25th and the 35th beat and the shortest RR interval between the 10th and the 20th beat (82, 83), and the ratio between the longest RR interval between the 20th and 40th beat and the shortest between the 5th and the 25th beat after standing (75). The latter two modalities - the maximum/minimum 30:15 ratio - are preferable as they detect the highest variability.

Valsalva manoeuvre
When evaluating the response, some Authors suggest calculating the ratio between the longest RR interval after the Valsalva manoeuvre and the shortest RR interval both during and after the manoeuvre, since the greatest tachycardia usually occurs immediately after the manoeuvre (48, 52, 80). The choice of the shortest RR interval during the manoeuvre is, however, proposed here as the most appropriate.

Orthostatic hypotension test

In the evaluation of BP fall during standing, a length of 2 minutes of orthostatism is put forward here as an adequate period of observation, although in patients with orthostatic hypotension and autonomic failure a further fall in BP may occur after 2 minutes (84). Similarly, in measuring BP fall we suggest considering only the standing values at 1 and 2 minutes and not at 30 seconds, in this way choosing the late BP variations (1-2 minutes) rather than the more marked changes of the early adaptation phase (30 seconds) (80).

Although an ad hoc Consensus in 1996 (47) established for the definition of orthostatic hypotension a cut-off value of BP fall of 20 mmHg for systolic or 10 mmHg for diastolic BP, some considerations point to a reappraisal of these diagnostic criteria in diabetes. Firstly, the few studies that have assessed the distribution of BP fall on standing in the healthy people found values of systolic BP fall ≥30 mmHg as those close to ≥97.5 percentile of control subjects (9, 75). Secondly, in a recent study the 30 mmHg cut-off value demonstrated no inferiority in diagnostic characteristics for CAN when compared to the 20 mmHg criterion and instead a better likelihood ratio for a positive result, i.e. a greater ability to estimate the probability of CAN (67). Thirdly, orthostatic hypotension is burdened with an adverse prognostic impact in both the general and diabetic population with an additional increase in risk over that driven by abnormalities of heart rate tests (5, 64, 85, 167). The label of orthostatic hypotension given to a diabetic patient implies an additional negative impact beyond the already detrimental meaning of CAN. Thus, for these reasons we believe that the more conservative cut-off value originally proposed of 30 mmHg for the systolic BP fall (81) is still valid and even preferable. Furthermore, although some Authors argue that the diastolic rather than the systolic fall is more characteristic of autonomic failure (80), some considerations make this proposal unacceptable. In diabetic patients, in particular, the values of systolic BP fall are more frequently abnormal than diastolic ones (75), and the absolute value of orthostatic change in diastolic BP is less evident and therefore more likely to produce measurement errors, which are in any case possible with the use of the common sphygmomanometer (86).

Recommendation (GPP)

In performing and analysing the tests, adopt the modalities proposed here and verify that the software used complies with these modalities.

Cardiovascular tests: confounding and interfering factors

As cardiovascular tests measure heart rate and BP responses to the activation of a neural reflex arch that includes stimulus, receptor, afferent nerve, central network, efferent nerve, and effector organ, an abnormal result can derive from an anomaly in any step of the reflex arc. It is therefore necessary to be aware of the confounding factors that can impact on the standardization of stimuli, on autonomic function or cardiovascular function. Information should be obtained on the patient's clinical state and on the presence of conditions potentially affecting the autonomic nervous system or cardiovascular system. Moreover, tests should be avoided and postponed in the presence of intercurrent diseases associated with fever, infection, or dehydration. Fever induces vasodilatation in the skin, a reduction in venous return to the heart, a drop in BP, an increase in heart rate and blood flow, in addition to changes in the respiratory rate and regularity (168). Dehydration is often associated with orthostatic intolerance. It determines a sympathetic activation as a protection against excessive hypotension, which is probably triggered by hyperosmolality and a decrease in central venous pressure (169).

Recommendation (level C)

The reliability of cardiovascular tests depends on the standardization of the procedure and on the exclusion of confounding factors.

Patient's compliance. The patient's cooperation, through instruction, acquaintance, and familiarization with the test is essential to achieve a standardized stimulus, in particular for the deep breathing test and Valsalva manoeuvre. In particular for the Valsalva manoeuvre, only tests correctly performed should be considered for calculation (59). Table 3 shows a patient instruction sheet with detailed information on the tests.

Recommendation (GPP)
It is necessary to provide sufficient instructions and to allow the patients to familiarize themselves with the test in order to obtain adequate stimuli.

Age. Heart rate variability, both spontaneous and induced by provocative stimuli, decreases with age (87). The inverse relationship between age and reflex response of heart rate is either linear or logarithmic and tends to level off after 60 years of age (83); it is more evident for the deep breathing test but present also in the orthostatic test and to a lesser extent in the Valsalva manoeuvre (75, 80, 82, 83, 88-92). It is therefore necessary to use age-related normal values to interpret the test results, as normal values proposed by Ewing (81) can produce false negative results in young subjects and false positive results in the elderly. In subjects older than 65, it may be more difficult to differentiate between normal and abnormal results (89, 92), although it is still possible to accurately diagnose autonomic neuropathy (83). In literature, age-related normal values are available (Table 4) but care must be taken with regard to the methods used to perform and interpret the tests; the choice of reference values from the literature should be based on a substantial agreement with the methods adopted by the Authors. It is advisable to employ the normal values foreseen by the software being used, provided that both the characteristics of the examined population and the literature references of the published works containing those findings are reported. In the case of a manual reading of the ECG trace, the published age-related normal values can be used as a reference (Table 4), even though the reliability of such an approach is limited by the non-homogeneous performing and interpretation of the tests among Authors.

Recommendation (level B)
To interpret the results of cardiovascular tests based on heart rate it is advisable to use normal age-related values.

Gender. In most available studies in healthy people a significant effect of gender on cardiovascular tests has not been demonstrated (75, 80, 82, 83, 89, 92, 170).

Recommendation (level B)
Available data does not indicate the need to apply normal sex-related values for cardiovascular test results.

Respiratory pattern. The greatest source of poor inter-subject reproducibility in the tests based on heart rate is the variation in the respiratory pattern. There is a positive correlation between the variability in heart rate changes and variability in air volume breathed during the deep breathing test in both healthy and diabetic subjects (93). Moreover, both heart rate response and respiratory volume during deep breathing increase when the test is repeated over time, due to the patient's familiarization with the test, a lower level of anxiety and consequent greater breathing depth (94, 95).

Recommendation (level C)
It is essential to carefully prepare the patient to perform deep breaths at 6 breaths per minute during the deep breathing test, and to avoid deep or irregular breaths after the Valsalva manoeuvre and after standing. The correct performing of tests should be verified and the reliability of any single result notably different from the others should be questioned.

Body position. Body position and duration of supine rest before the tests influence the results of the tests. Some Authors suggest that in the supine position the vagal control during deep breathing is more evident (80) and a modest acute volume depletion does not influence RR interval (96). It has also been noted - however - that heart rate responses do not vary significantly between supine and sitting position (97), or are slightly reduced (98), and that the reproducibility is better in the sitting position, probably because breathing in the sitting position involves only the thoracic muscles, whereas in the supine position it also involves the abdominal ones resulting in greater variability of the response (94).
It is preferable to perform the Valsalva manoeuvre in the sitting position because cardiovascular responses are higher than in the supine position; the standing position, albeit associated with even more marked responses, is obviously less applicable (97, 98).
It is preferable to perform the lying to standing test after at least 2-3 minutes of rest in the supine position, because the duration of supine rest amplifies BP fall and subsequent BP recovery. A supine rest of 20 minutes increases by 30% the heart rate response obtained after 1 minute of pre-test supine rest (97).

Recommendation (level C)
The deep breathing test may be performed indifferently in the supine or sitting position; it is preferable to perform the Valsalva manoeuvre in the sitting position, and to allow at least a 2-minute rest in the supine position before the orthostatic test.

Resting heart rate. An inverse relationship has been described between resting heart rate and the amount of its response to deep breathing - when measured as E/I ratio, not when measured as heart rate difference - (77, 78, 88,) and to the orthostatic test (30:15 ratio) (74, 75), but not to the Valsalva manoeuvre (75). The influence of resting heart rate is lower than that of age and duration of supine rest, and does not require a correction of the results according to the resting heart rate. Nonetheless, the deep breathing test, exploring vagal function and requiring a significant vagal tone, becomes more difficult to interpret in the presence of resting tachycardia (>100 bpm) (80) and some Authors suggest to exclude causes of tachycardia other than those due to vagal neuropathy (77) and to provide information on resting heart rate or RR interval together with the test result (78).
Abnormalities of cardiac rhythm, such as atrial fibrillation, sinus-atrial dysfunction (atrial rhythm), or a high number of ectopic beats, and obviously the presence of a pacemaker rhythm, preclude or invalidate the performing of cardiovascular tests (77).

Recommendation (level C)
Although the degree of the responses to the deep breathing test, when expressed as the E/I ratio, and to the orthostatic test are slightly influenced by the resting heart rate, no correction is needed for the resting heart rate. Caution in interpreting the results should be exercised in the case of a resting heart rate >100 bpm. Alterations in cardiac rhythm preclude the performing of the heart rate tests.

Blood pressure. The orthostatic BP values are correlated to those in the supine position, and 25% of the variance of the systolic BP fall is due to the supine BP (86). Subjects with elevated supine BP tend

to have a higher BP fall during the orthostatic test; therefore, if BP is >160 mmHg or <120 mmHg, the confounding effect of resting BP should be taken into account (false positive results with high BP and false negative results with low BP) (86).

Recommendation (level C)
The confounding effect of supine systolic BP - for values >160 mmHg and <120 mmHg - should be taken into account when evaluating BP response to the orthostatic test.

Physical exercise. Physical exercise acutely determines a reduction in vagal activity and an increase in sympathetic one. The effect on vagal activity persists for several hours, up to 12, after vigorous and prolonged exercise, the recovery time being dependent on the cardiorespiratory fitness of the subject (99).
In healthy subjects and in patients with diabetes or heart diseases, there is controversial evidence that regular exercise is associated with an increase in heart rate variability, in time- and frequency-domain vagal indexes of heart rate variability, and in baroreflex sensitivity, and with a reduction in frequency-domain sympathetic indexes of heart rate variability (100-108).

Recommendation (GPP)
Patients should be requested to avoid strenuous physical exercise in the 24 hours preceding the cardiovascular tests. Exercise training might positively influence the heart rate test results.

Coffee and alcohol consumption and smoking. Caffeine has a sympathomimetic effect and is an established pressor agent also used to treat dysautonomic postprandial hypotension (109, 110). An enhancement in the sympathoadrenal response to acute hypoglycaemia is one of the mechanisms underlying the protective effect against hypoglycaemia unawareness of even modest amounts of caffeine (111). Chronic use of caffeine seems to increase vagal indexes of heart rate variability in healthy subjects and type 1 diabetic patients (112). However, the acute effect on the tests in regular and not regular consumers remains unsettled.
Alcohol has been associated with an increase in heart rate and a small transient early rise in BP followed by a BP fall due to the induced vasodilatation. Alcohol seems to have different acute effects on muscle and skin blood flow, with a predominant direct vasoconstrictor effect on muscle blood flow and an indirect vasodilator effect on skin blood flow via its metabolites (110).
In the general population cigarette smoking acutely increases sympathetic activity and catecholamine levels, and produces an acute and transient decrease in vagal activity (113).

Recommendation (GPP)
Although the acute effect of coffee on regular and not regular consumers is not well defined, it is recommended that caffeine beverages, as well as smoking and alcohol, be avoided at least 2 hours prior to the tests.

Meals. Postprandial hypotension is a relevant phenomenon in elderly subjects and in patients with autonomic dysfunction. The pathogenesis is complex and not completely known, involving alterations of both the neural control of splanchnic blood flow and the release of gastrointestinal hormones. In a small group of recently diagnosed type 2 diabetic patients and in healthy subjects, oral glucose load was found to cause postprandial hypotension (systolic BP fall of at least 20 mmHg) in 44 and 33% of cases, respectively; the amount of postprandial hypotension was related to the gastric emptying (171). Oral glucose load is also associated with sympathetic activation in healthy subjects (114).

Recommendation (GPP)
It is advisable to perform the tests when fasting or at least 2 hours after a light meal.

Pathophysiological confounding factors

Obesity. There is enough agreement that in the obese patients autonomic function is disturbed, in particular with depressed parasympathetic activity (172) and a sympathetic overactivity, this latter occurring with some regional heterogeneity (105, 173). In obesity and the metabolic syndrome impaired heart rate cardiovascular tests (172), lower heart rate variability, a higher ratio of low-to-high frequency spectral components of heart rate variability, and delayed heart rate recovery after exercise have been described. These are consistent with lower cardiac vagal activity and a relative sympathetic predominance (174). These autonomic abnormalities may represent both a cause and consequence of obesity, and can contribute significantly to the adverse clinical prognosis of the obese population (174). Weight loss is accompanied by an improvement in cardiovascular autonomic function, in particular by an attenuation in the resting whole-body norepinephrine spillover rate and muscle sympathetic nerve activity, an improvement in cardiac autonomic modulation, and a reversal of blunted sympathetic responsiveness at both peripheral and central nervous system levels (105, 174). The nature of the association between obesity and autonomic dysfunction is complex and incompletely explored and can involve metabolic, hormonal, and mechanical aspects (105, 174). However, there is no conclusive evidence of a confounding effect of obesity on cardiovascular tests in diabetic patients (115, 116). The presence of morbid obesity is potentially able to reduce lung expansion and to attenuate chest reflexes due to the influence of mechanical factors such as intrathoracic fat deposits (117).

Recommendation (GPP)
The presence of morbid obesity might represent an interfering factor on the response to the deep breathing test.

Hypoglycaemia and hyperglycaemia. Hypoglycaemia causes sympathetic activation and QT interval prolongation, although this is less evident in diabetic patients with CAN (118). Antecedent hypoglycaemia in healthy subjects has been found to reduce baroreflex sensitivity, decrease muscle sympathetic nerve activity response to transient nitroprusside-induced hypotension and reduce plasma norepinephrine response to lower body negative pressure (119**).** In healthy subjects acute hyperglycaemia increases the QT interval and reduces the response to the squatting test, possibly due to reduced baroreflex activity (120, 121). There are anecdotal reports of acute autonomic dysfunction during ketoacidosis (122).

Recommendation (GPP)
Tests should be avoided during hypoglycaemia or marked hyperglycaemia.

Insulin. In normal subjects insulin acutely causes dose-dependent and endothelial-dependent vasodilatation of peripheral vessels through the release of nitric oxide; at the same time, it causes vagal withdrawal and sympathetic activation through both a main central mechanism and a secondary vasodilatation-mediated baroreflex activity. In healthy people this leads to a slight reduction in diastolic BP, an increase in heart rate and stroke volume, and a reduction in peripheral resistance (123, 124). In severe autonomic failure, by contrast, insulin has a hypotensive effect, due to the absence of significant sympathetic activation (59). Therefore, insulin has a potentially disrupting effect on the orthostatic hypotension test.

Recommendation (level C)
It is recommended that cardiovascular tests be performed at least 2 hours after short-acting insulin administration.

Respiratory diseases. Hypoxia and hypercapnia can cause to various degrees sympathetic activation and baroreflex modulation. A subclinical autonomic neuropathy with abnormal heart rate tests has been described in chronic obstructive pulmonary disease, especially in severe forms and when hypoxia coexists (125-127).
In patients with obstructive sleep apnea, a sympathetic activation has been documented mainly by measuring muscle sympathetic neural activity, even when awake without apnea (175). This sympathoexcitation has been attributed to chemoreflex activation by hypoxia and hypercapnia, as well as to altered baroreflex responsiveness, and can be reversed by long-term continuous positive air-way pressure (176).

Recommendation (GPP)
Test results should be interpreted with caution in the case of chronic obstructive pulmonary disease or respiratory failure.

Kidney disease and anaemia. Autonomic neuropathy diagnosed using the cardiovascular reflex tests has been reported in about 60% of predialysis and haemodialysis non diabetic patients (177, 178). Putative mechanisms for uremic neuropathy are the presence of uremic neurotoxins including parathyroid hormone and β2-microglobulin, the inhibition of the axonal Na-K pump and hyperkalemia leading to alterations in axonal membrane excitability, and the dysfunction of Na/Ca^{2+} exchanger with consequent increase in intracellular calcium and axonal loss (179, 180). The scenario is complicated by the demonstration that a state of sympathetic overactivity may occur in patients with chronic renal disease mainly as a consequence of afferent signals arising in the failing kidneys (173). Little information however is available on the course of this latter autonomic dysfunction or on its relationship with late uremic autonomic neuropathy. Thus, if it is relatively accepted that the combination of diabetes with kidney failure markedly increases the likelihood of CAN (181), no data exists on the potential confounding effect on cardiovascular tests of the sympathetic overactivity associated with kidney failure. Finally, in patients with nephrotic syndrome, water and sodium retention, extracellular volume expansion, hypovolemia, and fluid mobilization from extra to intravascular compartments might influence, to a varying degree, BP and heart rate responses to standing.
Apart from the evidence of a bidirectional link between autonomic dysfunction and anemia, i.e. the association between severe autonomic dysfunction with EPO-deficient anemia and the association between anemia of different etiology with impaired heart rate variability (182-185), anemia is accompanied by a hyperdynamic circulatory state due to an increase in cardiac output that is mediated by a lower afterload, an increased preload, and positive inotropic and chronotropic effects, while increased heart rate is due to hypoxia-stimulated chemoreceptors and increased sympathetic activity (183, 186). These anemia-induced compensatory cardiovascular adjustments might potentially affect reflex cardiovascular responses.

Recommendation (level C)
In the presence of kidney faillure the role of comorbid uremic neuropathy should be taken into account in interpreting the results.

Cardiovascular diseases. Essential hypertension has been described as a condition of overactivity of the sympathetic nervous system (187). In diabetic hypertensive patients a higher prevalence of abnormal heart rate tests has been described, whereas the increased sympathetic tone commonly observed in essential hypertension seems not to be preserved (128).
The presence of coronary artery disease was shown to impair cardiovascular test results in type 2 diabetic patients under multiple cardiovascular drug treatments by increasing the prevalence of abnormal deep breathing tests from 19 to 48% (129). In another study, in younger (≤45 years) and older (>45 years) non-diabetic and diabetic subjects, the presence of coronary artery disease, congestive heart failure or treated hypertension was associated with an impairment of heart rate test but only in younger and not older subjects, suggesting a predominance in older subjects of the age effect over the influences of cardiovascular diseases or drugs (130). In the Hoorn study in the general population, the presence of cardiovascular disease did not act as a strong determinant of abnormalities in cardiovascular autonomic function (131).

Recommendation (level C)
Tests results should be interpreted with caution in the presence of cardiovascular diseases, particularly heart failure.

Interfering drugs. Several antihypertensive or psychoactive drugs frequently used in diabetic patients may interfere with cardiovascular autonomic function and potentially with the cardiovascular tests, either inhibiting or exciting the responses. The effects are not necessarily class-dependent but can be specific both for a drug and a disease. The demonstration for many drugs of an effect on spontaneous heart rate variability (87) does not necessarily imply a significant interference with the cardiovascular tests. Data both on the effects of drugs on cardiovascular tests and in diabetic patients is rather scanty. In the Hoorn study use of antihypertensive drugs was an independent determinant of cardiovascular autonomic function explored by standard cardiovascular tests and heart rate variability measures, being - however - stronger in normotolerant subjects than in diabetic patients (131).
The potential systemic absorption of drugs administered via inhalation or eye drops should also be taken into consideration.
β-blockers appear to increase heart rate variability in healthy subjects and in patients with myocardial infarction and heart failure (132); and this is considered to be an expression of sympathetic inhibition and vagal activation. However, they do not show any substantial effect on the deep breathing test (17). Metoprolol has been shown to improve in 20 type 1 diabetic subjects the vagal time- and frequency-domain indexes of heart rate variability (133). Therefore, β-blockers in addition to the reduction in resting heart rate, modify sympatho-vagal balance in favour of vagal activity. Peripheral or central sympatholytic agents mainly interfere with BP. Class 1C antiarrhythmic agents depress heart rate variability (132). On the basis of these considerations, we should not expect an inhibitory interference of β-blockers on heart rate tests, whilst α-blockers might enhance orthostatic BP fall.
Non-dihydropyridine calcium-antagonists, diltiazem and verapamil, reduce cardiac sympathetic activity in patients with hypertension or stable angina and in diabetic patients after myocardial infarction (134), while dihydropyridine calcium-antagonists, such as nifedipine, do not seem to have any effect (135).
There is evidence that ACE-inhibitors - captopril, lisinopril, quinapril and trandolapril, but not enalapril - exert a possible positive modulation on heart rate variability (136-138). Data on the therapeutic effect of ACE-inhibitors on CAN is controversial (136, 139, 140). Even more preliminary and controversial is the data on the influence of angiotensin-II-receptor antagonists on cardiovascular nervous control on both general and diabetic populations (135, 140). For these drugs, an interference action on the tests - even present - should not be inhibitory.

Diuretics favour a volume depletion and are powerful interfering factors on the orthostatic hypotension test. Spironolactone, despite its increasing effect on heart rate variability and baroreflex sensitivity in normal subjects and patients with heart failure (135), seemed to slightly reduce the vagal indexes of heart rate variability in 42 type 2 diabetic patients (141).
Digoxin would increase time-domain vagal indexes of heart rate variability without modifying heart rate or the heart rate response to tilt test (138).
There are some indications that acetylsalicylic acid would enhance the sympathoadrenergic response to hypoglycaemia in healthy subjects (142), and at the same time would exert an inhibitory effect on sympathetic activity but a favourable one on vagal activity (143).
Tricyclic antidepressants, chlorpromazine and thioridazine have anticholinergic and antiadrenergic effects; venlafaxine, trazodone and mirtazapine have noradrenergic effects (144). Several observations have documented that tricyclic antidepressants reduce heart rate variability, time- and frequency-domain vagal indexes of heart rate, and also responses to cardiovascular tests (145, 146). Mild or no interference has been reported with serotoninergic antidepressants, such as paroxetine and citalopram. There is a single report that carbamazepine reduces heart rate variability (147), with a nocturnal rise in sympathetic activity after sudden withdrawal (148). Other anticonvulsants and tramadol do not have significant effects (144). Although with some controversy, benzodiazepines, such as midazolam, diazepam and lorazepam, have been found to produce a dose-dependent attenuation in vagal tone through a central mechanism interacting with GABA (γ-amino-butyric acid) receptors (149, 150), whilst alprazolam inhibits sympathetic tone (151). Therefore, with many psychoactive drugs, particularly neuroleptics and tricyclic antidepressants, a reduction in heart rate response is possible, besides the well-known effect on orthostatic BP due - in the case of tricyclic antidepressants - to antagonism with α1-adrenergic receptors.
Although the interference of a drug on cardiovascular tests is not easily predictable in any given patient and therefore the reliability of the tests performed under treatment can be difficult to ascertain, when a pharmacological wash-out is not feasible, diuretics, sympatholytic agents and tricyclic antidepressants should be considered the most interfering drugs, in that they may cause false positive responses mainly of the orthostatic tests (Table 5).
In some cases it can be difficult to discriminate whether underlying cardiovascular disease, drugs taken, or CAN per se are responsible for abnormal cardiovascular responses. Nevertheless, given the prognostic significance of CAN, it is still useful to assess autonomic dysfunction in these patients.

Recommendation (level C)
It is recommended that cardiovascular tests be performed after an appropriate wash-out of potentially interfering drugs, particularly diuretics, sympatholytic agents and psychoactive drugs. When this is not feasible, results of the tests should be interpreted with caution.
Although in some cases it can be difficult to attribute abnormal results to cardiovascular diseases, drugs, or CAN itself, given the prognostic significance of CAN, it is still useful to evaluate the presence of autonomic dysfunction in such patients (GPP).

Requirements for computerized systems to perform and evaluate cardiovascular tests

In the nineteen-nineties, the diffusion of cardiovascular tests in the routine diagnosis of autonomic dysfunction raised the need to move from a manual reading of the electrocardiographic tracing on paper, which was laborious and prone to errors, to the use of computerized systems.
After initial difficulties, due to the substantial modifications of the electrocardiographic parameters during the tests (amplitude, axis, isoelectric line) and the potential interferences that required visual and manual controls of the computer behaviour, sophisticated and validated software systems have been

developed, able to obtain and mathematically elaborate the electrocardiographic signal with good reliability and a low margin of error.
However, there is a high heterogeneity, equally in terms of reliability, among the available software systems, and it is difficult to give an analytical opinion without having tested them directly.
It therefore seems preferable, following the useful suggestions of K. Thomaseth (Research National Council, CNR, Padua), to detail the fundamental requirements that serve as reference to verify the reliability of the chosen system.

General requirements of the software

First of all, the software should adhere precisely to the established protocols of performing of the tests, also in relation to the timing of the various phases of a test, and allow the immediate verification of the correct performing of the test by elaborating, in real time, the parameters required for the calculation of the indexes. A primary requirement for the system is therefore that of supplying all the information needed to control the correct performing and the validity of the results of the test for the doctor who has to certify the diagnosis.

Components of the system

In general, an ideal computerized system should include the following components:

1. a biomedical instrumentation (ideally telemetry) for the continuous acquisition, visible in real time on monitor, of the electrophysiological parameters, i.e. electrocardiogram (ECG), optionally instantaneous BP, breathing, and other useful signals such as blood flow of peripheral vessels and microcirculation;
2. an adequate interface device between instruments and computer to obtain the simultaneous acquisition of the physiological signals in digital form;
3. a computer with a dedicated software to automate the performing, evaluation and storage of the tests;
4. the devices needed to perform the tests, such as a dedicated manometer for the Valsalva manoeuvre and, optionally, a tilt table and a dynamometer for the handgrip test, which may eventually be interfaced with the computer to verify automatically the required parameters.

Requirements of the system components

1. Electrocardiographic acquisition should ideally consist of two ECG leads to ensure more reliable automatic recognition of QRS complexes compared to one single trace, thus minimizing possible artifacts, due to alterations of the electrical zero line and high frequency disturbances, often due to movement during the tests. These low- or high-frequency artifacts should be eliminated by using appropriate band-pass filters in both the electrocardiograph and, especially, in the software. Sophisticated 12-lead diagnostic electrocardiographs are unsuitable because of their wide bandwidth, which is too easily influenced by interference that can overload signal amplifiers. As regards the instruments measuring other physiological parameters, these should also be robustly equipped, e.g. with an impedance transducer for breathing or a reliable representation of the sphygmic wave for BP measurement.
2. Devices used to acquire the signals, the so-called analog-digital converters (A/D), should satisfy the following technical requirements:

- for the analog component and for each independent channel: differential input, variable gain amplifier and anti-aliasing analog filter;
- for the digital component: A/D converter, at least 12 bit, and a sampling rate higher than 200 Hz per channel, i.e. for the acquisition of 4 signals (in round robin) the sampling rate should be at least 800 Hz.

3. The computer and the software should enable the following:

- correct acquisition of the original physiological signals (ECG, BP, blood flow, etc.) to be verified by inspecting the temporal flow of data, with real time visualization on the monitor and possibly afterwards on printed paper;
- correct identification of the acquired signals (R wave and peak of QRS complex, T wave peak and end, systolic and diastolic BP for each beat, etc.) to be verified with markers directly on graphs;
- the series of RR intervals acquired and, of any other signals, to be examined in real time during the tests, in order to be able to interrupt and repeat the test should there be temporary problems, for example, with artifacts or bad performing of the manoeuvre;
- the timing of the tests given by the computer to be synchronized with the acquisition and storage of the direct and acquired signals; the RR intervals used to calculate the indexes of the test, for example, the minimum/maximum RR interval during/after the effort of Valsalva manoeuvre, should be shown on the tachogram and their relative values indicated at the end of the test;
- the possibility to manually modify the choice of RR intervals used for the calculation of the indexes in case the software does not correctly recognise a QRS complex, therefore altering the result of the test
-

Recommendation (GPP)

Check that the computerized systems used for the performing and analysis of cardiovascular tests correspond to the recommended technical requirements. Ensure that technical requirements are clearly presented in the user manual of the system.

Performing and interpretation of cardiovascular tests

Reasons for diagnosing cardiovascular diabetic autonomic neuropathy

The reasons for CAN assessment were reaffirmed by American Diabetes Association in 2005 (44), and include the following: 1. early detection of abnormalities and timely diagnostic and therapeutic interventions; 2. differential diagnosis and allocation of symptoms suggesting autonomic dysfunction, such as erectile dysfunction, orthostatic, gastrointestinal and urinary symptoms; 3. for its prognostic impact, the motivation of both physician and patient to pursue strict glycaemic control, to undertake pharmacological and non-pharmacological measures to correct cardiovascular risk factors and to use any specific therapies for CAN; 4. for the planning of moderate-to high-intensity physical exercise (152).

Recommendation

It is appropriate to diagnose CAN in order to correctly identify clinical pictures, attribute possible symptoms to an autonomic dysfunction and to initiate symptomatic therapies (level B); to motivate intensive therapeutic strategies to improve glycemic control and correct cardiovascular risk factors (level C); and to obtain information towards some aspect of therapeutic management, such as BP control and physical activity programs (level C).

Indications for the diagnosis of cardiovascular diabetic autonomic neuropathy: choosing candidate patients and when to do the tests

Although the prevalence of CAN is clearly related to the diabetes duration (5, 9-14), there is also evidence of the presence of cardiovascular tests abnormalities at the time of diagnosis in both type 1

and type 2 diabetic patients (6-8), in adolescents and young patients, in older patients with a short diabetes duration. Moreover, a subclinical CAN may be detected early by autonomic function tests (15).
On the other hand, a relationship between CAN and glycaemic control, cardiovascular risk factors and the other diabetic complications, such as diabetic polyneuropathy, retinopathy and nephropathy is well documented (5, 12, 15-17, 31). Then, candidate patients for the diagnosis of CAN have therefore been identified (44) as those indicated in the following recommendation.

Recommendation (level C)
Diagnosis of CAN should be performed

- *in type 2 diabetic patients at diagnosis,*
- *in type 1 diabetic patients 5 years after the diagnosis,*
- *independently from diabetes duration, in the diabetic patients with symptoms suggesting autonomic dysfunction,*
- *in diabetic patients before planning a program of moderate-to-high-intensity physical exercise, especially in the presence of high cardiovascular risk,*
- *in diabetic patients with a history of poor glycaemic control, high cardiovascular risk and microangiopathic complications, especially when planning major surgical procedures.*

Diagnostic characteristics of cardiovascular tests: sensitivity, specificity, reproducibility
The cardiovascular tests originally introduced for the diagnosis and monitoring of CAN (81) are the tests of heart rate variation during deep breathing, Valsalva manoeuvre and orthostatism, and the tests of BP variation during orthostatism (orthostatic hypotension) and sustained isometric muscular strain. The first three test are considered indexes mainly of parasympathetic function, that is "cardio-vagal"; the latter, of sympathetic function, that is, "adrenergic" (46, 48).
Being non-invasive, safe, clinically relevant (they correlate with tests of somatic nervous function and have a prognostic value), easy to perform, sensitive and specific (with regard to CAN itself, to other autonomic neuropathies and to diabetic polyneuropathy, diagnosed using cardiovascular tests, frequency domain indices of heart rate variability, sudomotor function tests, electrodiagnosis and the clinical exam, respectively) (95, 188-191), reproducible and standardized, these tests are considered established measures of autonomic function with high quality (classes I and II) and strength of evidence (B) according to American Academy of Neurology system for rating the quality of evidence and strength of recommendations (48). The isometric handgrip test has, instead, limited sensitivity, specificity (75) and reproducibility, and is therefore regarded as an investigational test only (48). The orthostatic hypotension test, the only adrenergic test left, is considered to have low sensitivity and high specificity (153, 67), and is subject to several interferences from drugs and disease conditions.
All heart rate tests seem to have high sensitivity and specificity (80-90%) for CAN (diagnosed using Ewing's tests). None of the three tests of heart rate shows a clear superiority of sensitivity, specificity and reproducibility over the others (154). From surveys of diabetic patients of the Authors of the present recommendations, among heart rate tests the orthostatic test and the Valsalva manoeuvre show a slightly higher sensitivity than the deep breathing test (values from 80 to 91%, for the first two tests, versus values from 79 to 83% for deep breathing tests), whereas data on specificity suggests, although not unanimously, a slight superiority for the Valsalva manoeuvre (unpublished results). Moreover, the low sensitivity and high specificity of the orthostatic hypotension test are confirmed (30.8 and 98.2%, respectively) (67).
Reproducibility data available in literature indicate in normal subjects coefficients of variation of 6-8.9% for E/I ratio, 8-15.4% for Valsalva ratio, and 5.3-9% for 30:15 ratio (88, 93); whereas in diabetic

patients coefficients of variation are found to be 5.3, 6.8, 22.8% for E/I ratio (92, 154, 192), 10.3, 10.5, 25.1% for the Valsalva ratio (93, 154, 174), and 11.4% for 30:15 ratio (192).

Recommendation (level B)
Based on their characteristics of sensitivity, specificity, reproducibility, ease of performing, and standardization, cardiovascular tests of deep breathing, orthostatism, Valsalva manoeuvre, and orthostatic hypotension test are established measures of autonomic function. There is no clear evidence of any diagnostic superiority between the heart rate tests.

Safety of cardiovascular tests
Valsalva haemorrhagic retinopathy has been described as a rare and generally self-limited condition that develops in otherwise healthy eyes as a consequence of prolonged Valsalva manoeuvre during a number of activities, including strenuous exercise, vomiting, weight-lifting, severe coughing, colonoscopy, and maternal labour (193). The Valsalva manoeuvre increases intrathoracic, intraocular and intracranial pressure and may theoretically be associated with a small risk of intraocular haemorrhage or lens dislocation (48). In particular, during the Valsalva manoeuvre with an expiratory airway pressure of 35-40 mmHg for 15 second, intraocular pressure has been reported to increase by 7.9 mmHg - from a mean baseline of 14 mmHg to a peak of 22 mmHg - in both diabetic patients and control subjects (155). For this reason in clinical practice there is the habitual avoidance of the Valsalva manoeuvre in patients with either proliferative retinopathy/the risk of retinal haemorrhage or severe uncontrolled hypertension, although in daily life conditions similar to the Valsalva manoeuvre can commonly occur. Apart from an anecdotal report of vitreous hemorrhage in a patient with proliferative diabetic retinopathy after an extended episode of vomiting (194), no data exists in literature on the occurrence of Valsalva retinopathy induced by cardiovascular tests. Moreover, in around 100 published studies of about 4000 cases, there is no report of complications with sequelae. The 1441 patients in the Diabetes Control and Complications Trial Study (156) and the 880 patients in the Rochester Diabetic Study underwent cardiovascular tests periodically for 6.5 and 8 years (respectively) without complications. In over 20,000 cardiovascular tests performed in one US laboratory there has been no report of complications (48). To sum up what we know, although cardiovascular tests seem to have a high value-to-risk ratio, avoiding the Valsalva manoeuvre in patients with proliferative retinopathy might still be a sensible tactic.

Recommendation (level C)
Cardiovascular tests are a safe diagnostic tool that is free from complications.
Given the lack of definitive evidence of the safety of the Valsalva manoeuvre in the presence of proliferative retinopathy and the risk of retinal haemorrhage, this test is not advisable in these circumstances (GPP).

Rationale of the battery of tests
It is simplistic to believe that a single test may evaluate global autonomic function (48). Moreover, although the three tests based on heart rate explore mainly parasympathetic function, the nervous pathways and reflex mechanisms involved are not identical, playing sympathetic activity and baroreflex a contributory role in the orthostatic test and the Valsalva manoeuvre (157). There is no clear evidence that one individual heart rate test may substitute the other two, or have such an evident diagnostic superiority as to be used on its own (191). The diagnostic definition of CAN based on several tests reduces the probability of false positives, considering that the lower normal limits of the tests are placed around a prefixed percentile (1st, 2.5th, 5th) of the distribution in the normal control

population, thus 'abnormal' values may be found also in normal subjects. The use of a battery of tests allows indication of severity or progression of CAN or just a more robust definition of complication. The need to use several tests of both vagal and sympathetic function is restated in the available guidelines (1, 44, 195).

Recommendation (level B)
For the diagnosis and monitoring of CAN, a battery of independent tests of parasympathetic and sympathetic function is required.

Requirements for the diagnosis of cardiovascular diabetic autonomic neuropathy
For some Authors, the abnormality of one heart rate test among the 2 or 3 actually performed, is a sufficient requirement to document the presence of early CAN (18, 44, 46). The San Antonio Consensus - however - underlined that, in order to establish the presence of CAN, it is advisable to detect abnormalities in more than one test on several occasions (46). Ziegler at al. (75, 158) proposed as a requisite for the diagnosis of definite and borderline CAN the presence of at least 3 and 2 abnormal results, respectively, among 7 autonomic cardiovascular indexes (including in addition to the traditional 5 cardiovascular tests other time and frequency domain indexes of RR interval variability). To evaluate the overall cardiovascular test abnormality it has also been used the autonomic score, obtained by the sum of the score given to each single test (0 for a normal, 1 for a borderline, 2 for an abnormal result): the diagnostic cut-off value has been considered 5/10 (57). In the meta-analysis by Maser et al (159) on the association between CAN and mortality, the predictive value for mortality changed accordingly to the definition of CAN on the basis of one or at least two abnormal tests, being the relative risks 1.2 and 3.45 respectively. In a more recent study the Hazard ratio for stroke increased from 1.7 to 2.7 in diabetic patients with one to three abnormal cardiovascular tests (30). This suggests that only a definite CAN has a true negative prognostic value and that more than one abnormal test is required to diagnose a definite CAN.

Recommendation (level C)
In the light of the available evidence and on the basis of the expert opinion, at least two abnormal tests of heart rate are required for the diagnosis of CAN. The presence of one abnormal test identifies the condition of early or uncertain CAN, to be confirmed over time. The presence of orthostatic hypotension identifies a condition of severe or advanced CAN.

Staging of cardiovascular diabetic autonomic neuropathy
Heart rate tests are compromised more early than the BP ones, and clinical symptoms of sympathetic dysfunction are belated. However, given that the test of orthostatic hypotension (the only test of sympathetic function currently in use) has limited sensitivity, an approach to staging of CAN that considers a progression of CAN from an early to an advanced involvement is believed more correct than a progression from a parasympathetic to a sympathetic neuropathy (18, 160, 161).
If it has been established that the orthostatic hypotension test is far from sensitive and very specific and as such an abnormal test result is less common with a prevalence of 5.9-13% according to 30 mmHg criterion (4, 9, 67, 81, 162) and occurs after any abnormality seen in heart rate tests, no chronological hierarchy among the heart rate tests has been proved. The San Antonio Consensus proposed a staging of CAN based on precocious abnormality in the deep breathing test (early stage), abnormality in the Valsalva manoeuvre (intermediate stage), and finally the presence of orthostatic hypotension (severe stage) (46). In the Technical Review on DAN of the American Diabetes Association (18), compared to the San Antonio Consensus, the Valsalva manoeuvre was put forward

as the best test to monitor the progression of CAN. It should be noted that Ewing originally proposed a classification based on early involvement (one abnormal heart rate test or two borderline), definite involvement (two or more abnormal heart rate tests) and severe involvement (presence of orthostatic hypotension) (81). The score system (57) has the dual advantage of both quantifying the progression of CAN and providing an overall quantitative result of the battery of tests. It should also be noted that, reviewing the pools of diabetic patients studied by the Authors of these recommendations, there is no clear chronology in the abnormalities in heart rate test results, whilst orthostatic hypotension test is confirmed as occurring late. In particular, there is no evidence of a precocious alteration of deep breathing test compared to orthostatic test and Valsalva manoeuvre. In support of such consideration there are the observations of a higher prevalence of abnormality in the Valsalva ratio and 30:15 ratio compared to E/I ratio (34.8 and 44.5% vs. 27.8%, respectively) (4), or of a substantial equality in the prevalence of abnormality between 30:15 ratio and E/I ratio (29.4 and 23.8%, respectively) (163), confirmed by the surveys of the present Authors (prevalence of abnormalities for E/I ratio 21-27%, for 30:15 ratio 20-35%, and for Valsalva ratio 20-38%) (unpublished results).

Recommendation (level C)
A staging of CAN based on the battery of cardiovascular tests allows the identification of the transition from early to advanced involvement.

Periodicity of the cardiovascular test performing
The early performing of cardiovascular tests enables a baseline evaluation that is useful for the comparison with follow-up tests (44). If no abnormalities are detected, it is advisable to repeat annually the tests (44), in particular in the diabetic patients at higher risk to develop CAN. It could be useful to monitor with the cardiovascular tests the progression of CAN, even if a definite CAN has been already diagnosed, particularly to identify the onset of orthostatic hypotension.

Recommendation (GPP)
In the considered opinion of the experts, in the case of altered cardiovascular tests in the baseline evaluation, it is advisable to repeat the tests annually in order to confirm the diagnosis of CAN and evaluate its progression. Moreover, even in the absence of alterations of cardiovascular tests, it is advisable to repeat the tests annually in diabetic patients with poor glycaemic control, high cardiovascular risk and microangiopathic complications, whereas in the others patients a longer interval is recommended.

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Authors' contributions

Study concept and design: VS (general organisation), FB, LS, RQ, GB, PM, GV, KE.

Literature research and drafting of various sections of the manuscript: VS (preface, comments to the analysis, confounding factors, interpretation of cardiovascular tests), FB (history, requirements for computerized systems), LS (description of heart rate tests), RQ (orthostatic hypotension test), GB (other cardiovascular tests), PM (symptoms), GV (other cardiovascular tests), KE (other cardiovascular tests).

Participation in 2 two-day meetings to discuss results, to revise drafts, and to acquire a shared position: VS, FB, LS, RQ, GB, PM, RM.

Analysis of patients' cardiovascular test database: VS, FB, LS, GB, RM.

Critical revision of the manuscript: VS, FB, LS, SM, PC.

Preparation of the final manuscript: VS, FB, SM.

All authors have read and approved the final manuscript.

Legends of figures

Figure 1. RR interval and systolic and diastolic blood pressure during Valsalva manoeuvre.
In phase 1, in the first 2-3 seconds of the expiratory strain, a rapid rise in blood pressure and a prolongation of the RR interval (bradycardia) are observed. In phase 2, during strain, blood pressure initially decreases and then recovers and RR intervals gradually shorten (tachycardia). In phase 3, at release in the first few seconds, blood pressure rapidly decreases and RR intervals shorten further. In phase 4, there is a gradual increase in blood pressure over resting values (blood pressure overshoot) and a rapid prolongation of RR interval (bradycardia). The Valsalva ratio is obtained by dividing the longest RR interval in phase 4 by the shortest RR interval in phase 2. With the permission of K. Thomaseth and F. Bellavere.

Table 1. **Spectrum of clinical manifestations of diabetic autonomic neuropathy**

Cardiovascular system

- Resting tachycardia with loss of reflex heart rate variations
- Exercise intolerance: reduced increase of heart rate and blood pressure
- Left ventricular dysfunction: reduced diastolic filling at rest
- Silent myocardial ischemia
- Orthostatic hypotension
- Postprandial hypotension
- Loss of circadian rhythm of blood pressure
- Perioperative instability
- QT interval prolongation
- Sympatho-vagal imbalance

Peripheral vascular function

- Increased peripheral blood flow and warm skin
- Increased arteriovenous shunting and swollen veins
- Raised venous pressure and oedema
- Loss of cutaneous vasomotor reflexes in response to thermal stimuli and to injury
- Loss of venoarteriolar reflex with oedema and microvascular damage
- Increased incapillary permeability
- Mönckeberg sclerosis

Respiratory system

- Cardiorespiratory arrests
- Reduced bronchial reactivity
- Altered ventilatory responses
- Sleep apnoeic episodes (?)

Gastrointestinal system

- Altered esophageal motility
- Diabetic gastroparesis
- Diabetic diarrhoea
- Faecal incontinence
- Constipation

Urogenital system

- Diabetic cistopathy
- Erectile dysfunction
- Retrograde ejaculation

Hormonal secretion

- Decreased hypoglycaemia awareness
- Hypoglycaemia-associated autonomic failure
- Anaemia with erythropoietin deficiency
- Altered renin production

Pupillary function

- Reduced pupillary motor function
- Argyll-Robertson-type pupil

Sudomotor function

- Anhidrosis with dry skin at lower limbs
- Hyperhidrosis of the trunk
- Gustatory sweating

Table 2. **Autonomic symptoms**	
Orthostatic hypotension • Dizziness, blurred vision, fainting when standing up **Sweating abnormalities** • Sweating during meals, in particular with certain food • Hyperhidrosis of the trunk or face especially nocturnal, with anhidrosis of legs or feet **Gastrointestinal symptoms** • Digestive difficulties, epigastric fullness and abdominal distension, nausea after meals, vomiting at awakening • Watery nocturnal diarrhoea • Faecal incontinence • Constipation	**Urinary abnormalities** • Lengthening of the interval between micturitions, lack of full bladder awareness • Difficulty to start micturition (hesitancy) • Prolonged dripping (terminal dribbling) • Incapacity to hold urine (incontinence) **Erectile dysfunction** • Reduced capacity to reach and maintain erection • Lack of spontaneous nocturnal or morning erections • Reduced or absent ejaculation

Table 3. **Instructions for the diabetic patient when performing cardiovascular tests.**

Several important involuntary bodily functions, such as heart rate, blood pressure, and intestinal and bladder function, are regulated by the autonomic component of the nervous system, known as the autonomic nervous system.

Diabetes can compromise the integrity of such a system and consequently alter the functions of several organs, even without perceived symptoms. For these reasons it is important to study the autonomic nervous system and identify possible alterations well in advance.

The tests you are undergoing will evaluate your heart rate and BP response to some simple stimuli, such as deep breathing or standing. In fact, your responses depend on the nervous control of the heart and blood vessels.

To study the autonomic function we use four tests performed in an outpatient clinic. The validity of the results of the autonomic neuropathy tests depend on the correct performing of the manoeuvres outlined below.

The first three tests are performed with an electrocardiographic recorder and evaluate heart rate variation in response to:

1. deep breathing: you will be asked to breathe deeply 6 times in one minute, inhaling for 5 seconds and exhaling for 5 seconds, following specific instructions from the operator;
2. standing up from lying down: after a few minutes in a horizontal position, you will be asked to stand up quickly and stand still until the end of the test (at most a few minutes); usually the test is performed twice;
3. Valsalva manoeuvre: during this manoeuvre you will be asked to breathe out strongly against resistance by blowing into a mouthpiece connected to a manometer (that measures pressure); when instructed, you should blow into the mouthpiece until the needle of the manometer reaches 40 mmHg and try to maintain that level for 15 seconds; before starting, do not take a deep breath, just breathe normally, and during the test try to maintain constant strength without blowing out your cheeks; at the end of this, you should breathe normally - avoid breathing deeply or speaking until the end of the test; the test is repeated three times;

The fourth test is the orthostatic hypotension test that measures the variation of blood pressure from a lying down to a standing up position by measuring blood pressure several times, first in a horizontal position, and then 1 and 2 minutes after standing.

It is not necessary to avoid food on the day of the tests. You should take your usual medication, including antidiabetic medicines. Remember to inform the operator (it is a good idea to write a list beforehand) of your usual daily medication because these may influence the test results.

In the event of irregular heart beat (atrial fibrillation) or if you have a pacemaker, the tests cannot be performed, nor it is advisable to perform the tests in the event of an ongoing illness. In the hours leading up to the tests, you should avoid stress, intense physical exercise, smoking, and intake of coffee or alcohol; it is also advisable to urinate before the tests.

Table 4. **Age-related normal limits of cardiovascular tests reported by Ziegler et al. (75), Cardone et al. (82), Balzani et al. (90) and Bax et al. (91), who adopt the modality of performing and analysis of heart rate tests most similar to those reported in the present recommendations.** Attention must be paid to verify that the methods used to perform and interpret the tests in the cited studies are homogenous to one's own.

Test	**Author**	**Age (years)**						
		<20	**20-29**	**30-39**	**40-49**	**50-60**	**61-70**	**71-80**
Deep breathing								
E/I ratio	Ziegler	1.21	1.17	1.15	1.13	1.11	1.10*	
	Cardone		1.24	1.19	1.15	1.11	1.08	1.05
	Bax		1.29	1.26	1.21	1.12	1.10	1.06
HR difference	Balzani	21.6	18.3	15.3	12.9	10.8	9.1	
Lying to standing								
30:15 ratio	Ziegler	1.15	1.12	1.10	1.08	1.07	1.06*	
	Cardone		1.15	1.11	1.07	1.05	1.02	1.00
	Bax		1.19	1.16	1.09	1.05	1.04	1.02
	Balzani		1.21	1.13	1.05	0.99	0.91	0.85
Valsalva manoeuvre								
Valsalva ratio	Ziegler	1.22	1.21	1.19	1.18	1.17	1.16*	
	Cardone		1.42	1.35	1.29	1.24	1.19	1.15
	Bax		1.59	1.49	1.46	1.34	1.14	1.11
	Balzani		1.48	1.39	1.32	1.25	1.17	1.12

Orthostatic hypotension: non age-dependent normal limits			
Systolic blood pressure fall (mmHg)	**Normal**	**Borderline**	**Abnormal**
	<20	20-29	≥30

* age range 61-65 years

Table 5. **Drugs interfering with the heart rate and blood pressure tests**			
Class	**Drug**	**Effect on HR**	**Effect on BP**
Diuretics	Thiazydic-furosemide		↑ orthostatic hypotension
	Spironolactone	↑ HRV in general population (135) ↓ HRV in diabetic patients (141)	
β-blockers	Atenolol	↓ LF-RR, ↑ HF-RR in general population (164)	
	Bisoprolol	↑ HRV in general population (87)	
	Metoprolol	↓ LF, ↑ HF in general population (136) ↑ HRV in diabetic patients (133)	
α-blockers			↑ orthostatic hypotension
Calcium antagonists	Diltiazem	↑ HRV in general population and diabetic patients (135)	
	Verapamil	↑ HRV in general population (135), no effect in diabetic patients (135)	
	Nifedipine	No effect	
ACE-inhibitors	Captopril	↑ HRV in general population (165)	
	Lisinopril	↑ HRV in general population (165)	
	Trandolapril	↑ HRV in general population, no effect on HR tests in diabetic patients (139)	
	Enalapril	No effect	
	Quinapril	↑ HRV and ↑ HF in diabetic patients (165, 166), ↑ HR tests in diabetic patients (140)	
Angiotensin-II-receptor antagonists	Losartan	↑ HRV in general population (135), controversial ↑ HR tests in diabetic patients (135, 140)	
	Eprosartan	↓ HRV in general population (135)	
Digitalis	Digoxin	↑ HRV in general population (138)	
Psychodrugs	Benzodiazepines midazolam, diazepam	↓ HRV in general population (149)	
	Lorazepam	Controversial ↓ HF-RR in general population (149, 151)	
	Alprazolam	↑ HF-RR in general population (151)	↓ MF-BP in general population (151)
	Tricyclic antidepressants (amitriptyline> imipramine, nortriptyline > desipramine, doxepin > fluvoxamine)	↓ HRV, ↓ HF-RR, ↓ HR tests in general population (145, 146)	↑ orthostatic hypotension
	Carbamazepine	↓ HRV in epilepsy (147)	
Acetylsalicilic acid		↑ HF-RR, ↓ LF-RR in general population (143)	

Legend: HR, heart rate; BP, blood pressure; HRV, heart rate variability; HF-RR, high frequency component of HRV; LF-RR, low frequency component of HRV; MF-BP, medium frequency component of BP.