Diabetologia (2008) 51:1046–1050
DOI 10.1007/s00125-008-0987-y

ARTICLE

The Neuropad test: a visual indicator test for human diabetic neuropathy

C. Quattrini · M. Jeziorska · M. Tavakoli · P. Begum · A. J. M. Boulton · R. A. Malik

Received: 3 October 2007 / Accepted: 22 February 2008 / Published online: 27 March 2008

Abstract
Aims/hypothesis The commercially available Neuropad test was developed as a simple visual indicator test to evaluate diabetic neuropathy. It uses a colour change to define the integrity of skin sympathetic cholinergic innervation. We compared the results of Neuropad assessment in the foot with established measures of somatic and autonomic neuropathy.
Methods Fifty-seven diabetic patients underwent Neuropad assessment, quantitative sensory and autonomic function testing, and evaluation of intra-epidermal nerve fibre density in foot skin biopsies.
Results Neuropad responses correlated with the neuropathy disability score (r_s=0.450, p<0.001), neuropathic symptom score (r_s=0.288, p=0.03), cold detection threshold (r_s=0.394, p=0.003), heat-as-pain perception threshold visual analogue score 0.5 (r_s=0.279, p=0.043) and deep-breathing heart rate variability (r_s=−0.525, p<0.001). Intra-epidermal nerve fibre density (fibres/mm) compared with age- and sex-matched control subjects (11.06±0.82) was non-significantly reduced (7.37±0.93) in diabetic patients with a normal Neuropad response and significantly reduced in patients with a patchy (5.01±0.93) or absent (5.02±0.77) response (p=0.02). The sensitivity of an abnormal Neuropad response in detecting clinical neuropathy (neuropathy disability score ≥5) was 85% (negative predictive value 71%) and the specificity was 45% (positive predictive value 69%).
Conclusions/interpretation The Neuropad test may be a simple indicator for screening patients with diabetic neuropathy.

Keywords Autonomic neuropathy · Diabetic neuropathy · Skin innervation · Sudomotor dysfunction

Abbreviations

CASE	computer-aided sensory evaluator
CDT	cold detection threshold
DB-HRV	deep-breathing heart rate variability
DNS	diabetic neuropathy symptom score
HP-VAS	heat-as-pain perception threshold visual analogue score
IENFD	intra-epidermal nerve fibre density
NDS	neuropathy disability score
Q-SART	quantitative sudomotor axon reflex testing

Introduction

The evaluation of nerve damage in human diabetic neuropathy is important to define those at risk of developing sensory loss, pain and foot ulceration. Simple clinical tests include assessment of neurological deficits using the neuropathy disability score (NDS), vibration perception threshold or the 10 g monofilament to define severe neuropathy and hence risk

C. Quattrini · M. Tavakoli · A. J. M. Boulton · R. A. Malik (✉)
Division of Cardiovascular Medicine, Core Technology Facility, University of Manchester, 46 Grafton Street, Manchester M13 9NT, UK
e-mail: rayaz.a.malik@man.ac.uk

M. Jeziorska
Division of Regenerative Medicine, University of Manchester, Manchester, UK

P. Begum
Department of Gastrointestinal Sciences, Clinical Sciences Building, Salford Royal Hospitals, Salford, UK

C. Quattrini · M. Tavakoli · A. J. M. Boulton · R. A. Malik
Manchester Diabetes Centre, Manchester Royal Infirmary, Manchester, UK

of ulceration [1]. Tests which evaluate earlier stages of neuropathy include neurophysiology, quantitative sensory testing [2] and nerve [3] or skin biopsy [4]. However, all of these tests are expensive, require specialist assessment and, in the case of biopsy, are invasive.

Autonomic dysfunction has been principally evaluated centrally by assessing heart rate variability [5] and peripherally by assessing sweating using complex and expensive equipment including the sympathetic skin response and quantitative sudomotor axon reflex testing (Q-SART) [6]. The commercially available Neuropad has recently been proposed as a simple test to diagnose sudomotor dysfunction and hence peripheral neuropathy. An adhesive pad containing cobalt salts is attached to the plantar aspect of the foot and changes colour from blue to pink within 10 min and defines if sudomotor and hence cholinergic sympathetic function is normal [7]. An abnormal Neuropad response is associated with sympathetic dysfunction and clinical neuropathy [7] and the reproducibility of the test has been shown to be excellent [8].

In this study we assessed the Neuropad indicator test in a cohort of diabetic patients who underwent a comprehensive assessment of somatic and autonomic neuropathy as well as assessment of intra-epidermal nerve fibre density (IENFD) in skin biopsies from the dorsum of the foot.

Methods

This study was approved by the Local Research Ethics committee and all patients gave informed consent to take part in the study.

Neuropathy assessment All 57 diabetic patients underwent assessment of the NDS as previously described [1]. We set the cut-off for neuropathy/no neuropathy at NDS=5/10. Patients underwent computer-aided sensory evaluator (CASE) IV quantitative sensory assessment [9] including: heat-as-pain perception threshold visual analogue score (HP-VAS), cold detection threshold (CDT) and deep-breathing heart rate variability (DB-HRV). Orthostatic hypotension was also assessed as a measure of sympathetic dysfunction [10] and was defined by a postural drop in BP of at least 20 mmHg [11]. Symptoms were assessed using the diabetic neuropathy symptom score (DNS) [12] and the short form of McGill's Pain Questionnaire [13, 14].

Sudomotor dysfunction Testing was carried out in a warm and quiet environment (24±1°C). The Neuropad test (miro Verbandstoffe, Wiehl-Drabenderhöhe, Germany) was applied on the plantar aspect of the great toe and removed after 10 min to evaluate the colour change as normal (blue colour turned completely pink, score=0), patchy (patches of blue and pink, score=0.5), abnormal (remained blue, score=1.0).

Skin biopsy A 3 mm punch skin biopsy was taken from the dorsum of the foot, approximately 2 cm above the second metatarsal head, under 1% lidocaine local anaesthesia. Skin samples were immediately fixed in 4% (wt/vol.) paraformaldehyde for 18–24 h and then cryoprotected in 30% (wt/vol.) sucrose for 4 h and cut into 50 μm thick sections.

Immunohistochemistry Melanin bleaching with 0.25% (wt/vol.) potassium permanganate and 3% (wt/vol.) oxalate solution was used prior to staining. For protein block, a mixture of Tris-buffered saline with 0.5% (wt/vol.) powdered milk, 1% Triton X-100 and 5% (vol./vol.) normal swine serum was applied for 4 h. The sections were incubated overnight at room temperature with 1:1,200 Biogenesis polyclonal rabbit anti-human protein gene product (PGP9.5) antibody (Serotec, Oxford, UK). Swine anti-rabbit secondary antibody 1:300 (45 min) was then applied; sections were quenched with 1% (vol./vol.) H_2O_2 in 30% (vol./vol.) MeOH-PBS (30 min) prior to a 45 min incubation with 1:500 horseradish peroxidase–streptavidin (Vector Laboratories, Peterborough, UK) and 3,3′-diaminobenzidine chromogen was used to demonstrate the reaction. Sections were transferred onto gelatin-subbed slides, coverslipped and observed by light microscopy.

Image analysis An image analysis camera (Sony 2CCD, CCD-IRIS; Weybridge, Surrey, UK) and suitable computer program (Leica QWin Standard V2.4; Leica Microsystem Imaging, Cambridge, UK) were used to quantify IENFD (number of fibres per mm of basement membrane) as previously described [15].

Statistical analysis Statistical analysis was performed using SPSS 15.0 for Windows. Results are presented as means± SEM. Spearman analysis was used to test for correlation of Neuropad ranks with all other measures of neuropathy. The Mann–Whitney test was used for comparison between two groups and the Kruskal–Wallis test was used to compare more than two groups. Post hoc multi-group comparison analysis was performed by a Tukey test (in the case of equal variances as assessed by Levene's test) or a Dunnet T3 test (in the case of unequal variances) tests. A χ^2 test was used to study associations between two dichotomous variables.

Results

Fifty-seven diabetic patients (20 type 1 and 37 type 2) aged 56±1.4 years were classified in accordance with the

Table 1 General clinical characteristics of the patients distributed according to the three categories of Neuropad responses

	Age (years)	Type 1/type 2	Duration (years)	HbA_{1c} (%)	BMI (kg/m^2)	PH (mmHg)	NDS
p value	NS	NS	0.01	NS	NS	NS	0.004
Neuropad normal	54±3	6/10	14±3	7.5±0.4	28.5±1.2	−13±3	3.3±0.6
Neuropad patchy	56±3	6/11	24±3*	8.4±0.2	29.7±1.3	−19±2	5.5±0.8
Neuropad abnormal	59±2	8/16	25±2*	8.0±0.3	30.4±1.0	−20±3	6.5±0.7*

PH, Postural hypotension
*$p<0.05$ compared with the Neuropad-normal group. Patients with a patchy and abnormal Neuropad associated significantly with PH (>20 mmHg systolic reduction) ($p=0.001$, χ^2 test)

Neuropad response as: normal ($n=16$), patchy ($n=16$) and abnormal ($n=21$). Age, BMI and HbA_{1c} did not differ between patient groups with different Neuropad responses, whereas duration of diabetes ($p<0.01$) and severity of neuropathy assessed via NDS ($p<0.05$) and degree of postural hypotension did (Table 1). According to the NDS, 12 patients had no neuropathy (NDS<3); 18 mild neuropathy (NDS 3–5), 15 moderate neuropathy (NDS 6–8) and 12 severe neuropathy (NDS 9–10). The NDS was significantly higher in patients with an abnormal Neuropad response (6.5±0.7) compared with patients with a normal response (3.3±0.6), $p<0.05$, and the Neuropad responses correlated with the severity of neuropathy defined by the NDS ($r_s=0.450$, $p<0.001$). Patients were further grouped into those with NDS<5 (40%) and NDS≥5 (60%). The sensitivity of an abnormal Neuropad response (either blue or patchy) in detecting neuropathy was 85% (negative predictive value 71%), while the specificity was 45% (positive predictive value 69%).

Symptoms Seventeen patients had no symptoms of neuropathy; 70% had a DNS>0, 55% DNS>1, 39% DNS>2 and 25% DNS>3. Thirty-two patients had neuropathic pain (HP-VAS score ≥1). The Neuropad response correlated with the neuropathic symptoms as assessed by the DNS Questionnaire ($r_s=0.288$, $p=0.03$) and pain subtypes 'unsteady' ($r_s=0.379$, $p=0.004$), 'numb' ($r_s=0.363$, $p=0.006$) and 'throbbing' ($r_s=0.336$, $p=0.012$) as characterised by the McGill Pain Questionnaire.

Small fibre function The results of CASE IV assessment are summarised in Table 2. The CDT response was significantly greater in diabetic patients with a patchy (91±3) and an abnormal (92±4) response compared with patients with a normal response (69±7), $p=0.007$, and the Neuropad ranking correlated with CDT ($r_s=0.394$, $p=0.003$; Table 3). While there was no significant difference in HP-VAS 0.5 between a normal and abnormal Neuropad response there was a correlation between it and Neuropad ranking overall ($r_s=0.279$, $p=0.043$; Table 3). The DB-HRV response was significantly lower in diabetic patients with a patchy (29±9), $p<0.05$, and abnormal (19±7), $p<0.05$, response compared with diabetic patients with a normal response (64±8) and correlated with the overall Neuropad ranking ($r_s=-0.525$, $p<0.001$; Table 3). No relationship was found with HP-VAS 5.0, HP-VAS 0.5–5.0 or postural hypotension (drop in BP >20 mmHg), which occurred in 36% of patients. However, the χ^2 test showed a significant association between postural hypotension and an abnormal Neuropad test ($p=0.001$).

IENFD The IENFD was significantly reduced in diabetic patients (5.69±0.51) compared with 15 age and sex matched non-diabetic control individuals (11.06±0.82, $p<0.001$, Fig. 1). Diabetic patients with a normal Neuropad result had a non-significant reduction in IENFD (7.37±0.93). This was significantly reduced in patients with either a patchy (5.01±0.93) or absent (5.02±0.77) result ($p=0.02$). IENFD correlated with the Neuropad response ($r_s=-0.271$, $p=0.04$).

Table 2 CASE IV assessment in the patients distributed according to the three categories of Neuropad response

	CDT (pc)	HP-VAS 0.5 (pc)	HP-VAS 5.0 (pc)	HP-VAS 0.5–5.0 (pc)	DB-HRV (pc)
p value	0.007	NS	0.020	0.015	0.002
Neuropad normal	69±7	31±7	26±8	33±8	64±8
Neuropad patchy	91±3*	54±11	62±9*	69±9*	29±9*
Neuropad abnormal	92±4*	58±7	51±7	36±7	19±7*

pc, Percentile
*$p<0.05$ compared with the Neuropad-normal group

Table 3 Correlation of the Neuropad ranking (normal, abnormal and patchy) with the three quantitative sensory function tests of small fibre denervation

Test	Correlation
CDT	
r_s	0.394
p value	0.003
HP-VAS 0.5	
r_s	0.279
p value	0.043
DB-HRV	
r_s	−0.525
p value	0.0003

Discussion

Sweat function has an important protective role and the presence of peripheral autonomic neuropathy characterised by anhidrosis is integral to the pathogenesis of diabetic foot ulceration [16]. When sweat gland function is lost because of peripheral autonomic denervation, foot ulceration is facilitated [17].

Our data show that the Neuropad test is able to detect the presence of clinically relevant neuropathy with good sensitivity, confirming the data from Papanas et al. [7]. A lower specificity in comparison with sensitivity is also confirmed [18] but, notably, with limited reduction in positive predictive value. The Neuropad correlations with CASE IV measurements, as well as with neuropathic symptoms, substantiate its validity as an effective screening instrument for small fibre neuropathy. Therefore the test appears to have the potential to be used as a first-line screening tool, and once a patient is found to have a positive Neuropad test, additional assessment is recommended.

Interestingly, tests of autonomic dysfunction, including altered heart rate variability and postural hypotension were also associated with an abnormal Neuropad response.

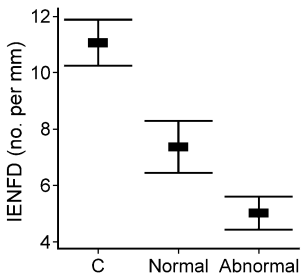

Fig. 1 IENFD in diabetic patients with normal and abnormal Neuropad responses. There were significantly lower IENFDs in patients with a pathological Neuropad test (abnormal) compared with those with a normal Neuropad test (normal), p=0.02. IENFD in non-diabetic control individuals is included for reference (C). Horizontal bars, mean; error bars, SEM

Although somatic and cardiac autonomic neuropathies are considered to be separate clinical entities [19], a common link is that of involvement of small fibres [20, 21]. Although the association with somatic autonomic neuropathy demands a more specific validation with quantitative sudomotor testing using the gold standard for sudomotor testing, Q-SART [22, 23], this was not available in our laboratory.

With regard to the site of testing using Neuropad we chose the pulp of the great toe, which has a dense cholinergic sympathetic innervation and avoided the metatarsal head area as the latter is a common site of ulceration. Furthermore, the biopsy was not taken from the pulp of the toe as this would have imposed a considerable risk in terms of non-healing. Nevertheless the biopsied area was from a nearby area, the dorsum of the foot, which was clearly more distal than the normal site for skin biopsy, which is the thigh or ankle [24]. Studying the nerve fibres entering the sweat glands in the dermis would have been more anatomically relevant in demonstrating a sympathetic deficit [25]; however, the skin biopsy was neither large enough nor deep enough to allow quantification of sweat gland innervation adequately. The correlation between the Neuropad response and IENFD was poorer than with the other measures of neuropathy. We can only speculate that assessing IENFD, which provides a structural measure of the number of fibres, may not reflect nerve fibre and specifically sudomotor nerve function [25], as recent studies certainly suggest that IENFD relates well to other measures of somatic neuropathy [26, 27]. Nevertheless, as IENFD is widely accepted as a gold standard measure of skin denervation and neuropathy [26–28], we believe these findings add strength to the assertion that Neuropad reflects the severity of distal somatic neuropathy.

A potential clinical limitation of Neuropad may be the 10 min needed for screening in a busy diabetic clinic. A recent study has suggested that the maximum threshold time to define an abnormal Neuropad test may be lowered to 530 s [29]. However, the distinct practical clinical advantage of the Neuropad test is that patients can self-administer the test at home. Furthermore, the visual reinforcement of an abnormal test will hopefully make the patient more aware of their risk of neuropathy and ulceration [30].

In conclusion, this study has shown that an abnormal Neuropad response indicates both functional and structural denervation in the feet of diabetic patients. This has considerable clinical relevance in screening for diabetic neuropathy.

Acknowledgements The study was supported by Diabetes UK (RD03/0002624) and National Institutes of Health (R01 NS46259-01) and Neuropad tests were supplied by miro Verbandstoffe.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

1. Abbott CA, Carrington AL, Ashe H et al (2002) The North–West Diabetes Foot Care Study: incidence of, and risk factors for, new diabetic foot ulceration in a community-based patient cohort. Diabet Med 19:377–384
2. Dyck PJ, Davies JL, Litchy WJ, O'Brien PC (1997) Longitudinal assessment of diabetic polyneuropathy using a composite score in the Rochester Diabetic Neuropathy Study cohort. Neurology 49:229–239
3. Malik RA, Veves A, Walker D et al (2001) Sural nerve fibre pathology in diabetic patients with mild neuropathy: relationship to pain, quantitative sensory testing and peripheral nerve electrophysiology. Acta Neuropathol (Berl) 101:367–374
4. Lauria G, Cornblath DR, Johansson O et al (2005) EFNS guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathy. Eur J Neurol 12:747–758
5. O'Brien IA, O'Hare P, Corrall RJ (1986) Heart rate variability in healthy subjects: effect of age and the derivation of normal ranges for tests of autonomic function. Br Heart J 55:348–354
6. Maselli RA, Jaspan JB, Soliven BC, Green AJ, Spire JP, Arnason BG (1989) Comparison of sympathetic skin response with quantitative sudomotor axon reflex test in diabetic neuropathy. Muscle Nerve 12:420–423
7. Papanas N, Papatheodorou K, Christakidis D et al (2005) Evaluation of a new indicator test for sudomotor function (Neuropad) in the diagnosis of peripheral neuropathy in type 2 diabetic patients. Exp Clin Endocrinol Diabetes 113:195–198
8. Papanas N, Papatheodorou K, Papazoglou D, Christakidis D, Monastiriotis C, Maltezos E (2005) Reproducibility of the new indicator test for sudomotor function (Neuropad) in patients with type 2 diabetes mellitus: short communication. Exp Clin Endocrinol Diabetes 113:577–581
9. Arezzo JC (1999) New developments in the diagnosis of diabetic neuropathy. Am J Med 107:9S–16S
10. Itoh H, Uebori S, Asai M, Kashiwaya T, Atoh K, Makino I (2003) Early detection of orthostatic hypotension by quantitative sudomotor axon reflex test (QSART) in type 2 diabetic patients. Intern Med 42:560–564
11. Kempler P, Tesfaye S, Chaturvedi N et al (2001) Blood pressure response to standing in the diagnosis of autonomic neuropathy: the EURODIAB IDDM Complications Study. Arch Physiol Biochem 109:215–222
12. Meijer JW, Bosma E, Lefrandt JD et al (2003) Clinical diagnosis of diabetic polyneuropathy with the diabetic neuropathy symptom and diabetic neuropathy examination scores. Diabetes Care 26:697–701
13. Melzack R (1987) The short-form McGill Pain Questionnaire. Pain 30:191–197
14. Masson EA, Hunt L, Gem JM, Boulton AJ (1989) A novel approach to the diagnosis and assessment of symptomatic diabetic neuropathy. Pain 38:25–28
15. Polydefkis M, Yiannoutsos CT, Cohen BA et al (2002) Reduced intraepidermal nerve fiber density in HIV-associated sensory neuropathy. Neurology 58:115–119
16. Vinik AI (2003) Management of neuropathy and foot problems in diabetic patients. Clin Cornerstone 5:38–55
17. Boulton AJ (1998) Lowering the risk of neuropathy, foot ulcers and amputations. Diabet Med 15(Suppl 4):S57–S59
18. Papanas N, Papatheodorou K, Papazoglou D, Christakidis D, Monastiriotis C, Maltezos E (2007) The new indicator test (Neuropad): a valuable diagnostic tool for small-fiber impairment in patients with type 2 diabetes. Diabetes Educ 33:257–266
19. Thomas PK (1973) Metabolic neuropathy. J R Coll Physicians Lond 7:154–160
20. Hanson P, Schumacker P, Debugne T, Clerin M (1992) Evaluation of somatic and autonomic small fibers neuropathy in diabetes. Am J Phys Med Rehabil 71:44–47
21. Dutsch M, Marthol H, Michelson G, Neundorfer B, Hilz MJ (2004) Pupillography refines the diagnosis of diabetic autonomic neuropathy. J Neurol Sci 222:75–81
22. Novak V, Freimer ML, Kissel JT et al (2001) Autonomic impairment in painful neuropathy. Neurology 56:861–868
23. Low PA, Benrud-Larson LM, Sletten DM et al (2004) Autonomic symptoms and diabetic neuropathy: a population-based study. Diabetes Care 27:2942–2947
24. McArthur JC, Stocks EA, Hauer P, Cornblath DR, Griffin JW (1998) Epidermal nerve fiber density: normative reference range and diagnostic efficiency. Arch Neurol 55:1513–1520
25. Levy DM, Terenghi G, Gu XH, Abraham RR, Springall DR, Polak JM (1992) Immunohistochemical measurements of nerves and neuropeptides in diabetic skin: relationship to tests of neurological function. Diabetologia 35:889–897
26. Quattrini C, Tavakoli M, Jeziorska M et al (2007) Surrogate markers of small fiber damage in human diabetic neuropathy. Diabetes 56:2148–2154
27. Quattrini C, Jeziorska M, Boulton A, Malik RA (2008) Reduced VEGF expression and intra-epidermal nerve fiber loss in human diabetic neuropathy. Diabetes Care 31:140–145
28. Polydefkis M, Hauer P, Griffin JW, McArthur JC (2001) Skin biopsy as a tool to assess distal small fiber innervation in diabetic neuropathy. Diabetes Technol Ther 3:23–28
29. Papanas N, Giassakis G, Papatheodorou K et al (2007) Use of the new indicator test (Neuropad) for the assessment of the staged severity of neuropathy in type 2 diabetic patients. Exp Clin Endocrinol Diabetes 115:58–61
30. Boulton AJ (1995) Why bother educating the multi-disciplinary team and the patient–the example of prevention of lower extremity amputation in diabetes. Patient Educ Couns 26:183–188