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High Diagnostic Performance of the Indicator Plaster Neuropad for the Detection of Established Diabetic Autonomic Neuropathy

Ioanna Zografou [1,*], Panagiotis Doukelis [1], Theocharis Koufakis [1], Evangelia Kotzakioulafi [2], Polykarpos Evripidou [2], Zisis Kontoninas [2], Christos Savopoulos [2], Michael Doumas [1], Triantafyllos Didangelos [2] and Konstantinos Kantartzis [3,4,5]

1 Second Propaedeutic Department of Internal Medicine, Medical School, Hippokration Hospital, Aristotle University of Thessaloniki, 54642 Thessaloniki, Greece; pitdukel@yahoo.gr (P.D.); thkoyfak@auth.gr (T.K.); doumasm@auth.gr (M.D.)
2 First Propaedeutic Department of Internal Medicine, Medical School, University General Hospital of Thessaloniki "AHEPA", Aristotle University of Thessaloniki, 54642 Thessaloniki, Greece; ekotzaki@auth.gr (E.K.); polysevripidou@gmail.com (P.E.); drziko2401@gmail.com (Z.K.); chrisavopoulos@gmail.com (C.S.); didang@auth.gr (T.D.)
3 Department of Internal Medicine IV, Division of Endocrinology, Diabetology and Nephrology, University of Tübingen, 72076 Tübingen, Germany; konstantinos.kantartzis@med.uni-tuebingen.de
4 Institute for Diabetes Research and Metabolic Diseases (IDM), Helmholtz Centre Munich, University of Tübingen, 72076 Tübingen, Germany
5 German Center for Diabetes Research (DZD), 85764 München-Neuherberg, Germany
* Correspondence: ioannazo@yahoo.gr

Abstract: Aim: The aim of this study was to evaluate the specificity, sensitivity and accuracy of the Indicator Plaster Neuropad in detecting established Diabetic Autonomic Neuropathy (DAN). Methods: We studied 180 patients with Diabetes Mellitus (DM, mean age 49.5 ± 16 years, 82 with DM type 1). All patients underwent the following Cardiovascular Reflex Tests (CARTs): R-R variation during deep breathing (Mean Circular Resultant (MCR) and standard deviation (SD)), Valsalva maneuver, R-R variability after a rapid change from lying to standing position and postural hypotension. The presence of DAN was established if ≥2 CARTs were abnormal. According to the result the patients were divided into two groups, one with DAN and one without DAN. Assessment with Neuropad was performed also in all patients. Results: Abnormal perspiration with Neuropad (uncompleted or no change in color) was detected in 94 patients. Established DAN was detected in 85 patients. The sensitivity, specificity and accuracy of Neuropad for the diagnosis of established DAN were 87.1%, 78.9% and 82.8%, respectively and area under the curve was 0.846 and 95% CI (0.787, 0.905). Conclusions: Neuropad has high sensitivity, specificity, and accuracy in detecting established DAN, as defined by ≥2 abnormal CARTs.

Keywords: Neuropad; diabetic autonomic neuropathy; sensitivity; accuracy; diagnostic tests

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1. Introduction

Diabetic autonomic neuropathy (DAN) is a common and serious complication of Diabetes Mellitus (DM) [1,2]. It is a form of peripheral neuropathy affecting parasympathetic, sympathetic, or both types of nerves in people with DM. It can cause a variety of symptoms, as it can affect all organs of the body, including the autonomic nerve fibers that innervate the heart and blood vessels [3]. Impairment of autonomic innervation of the cardiovascular system is called cardiovascular autonomic neuropathy (CAN) and is the most important form of DAN. Symptoms of CAN include resting tachycardia, exercise intolerance, and

orthostatic hypotension, while in more severe cases CAN is associated with syncope, silent myocardial infarction, and an increased risk of cardiovascular mortality [4,5]. Therefore, early diagnosis of CAN is extremely important, as it might detect a number of patients at high risk of cardiovascular complications and increased mortality [5].

The prevalence of CAN varies in selected studies of asymptomatic people with DM from 10% to 65%, reflecting differences in the diagnostic tests and criteria used for the diagnosis [6,7]. The Screen-Detected Diabetes in Primary Care (ADDITION) study showed an annual incidence of CAN of 1.8% in newly diagnosed patients with type 2 DM during a median period of 6.5 years [7].

Despite its high frequency and importance, CAN is often neglected and underestimated. That happens because subclinical autonomic neuropathy is usually completely asymptomatic and can only be detected by specific cardiovascular reflex tests (CARTs). These tests were proposed by Ewing in 1985 and are recommended by the Toronto panel [8–10] and, although they are indirect methods, since they rely on cardiovascular reflexes, they are considered the gold standard in autonomic testing [7,8]. However, these tests cannot be easily performed and therefore have limited practical utility in daily routine practice. Thus, an easy and faster-to perform screening test, which would be able to identify those patients who must be referred for a definitive DAN/CAN diagnosis with CARTs, would be extremely useful.

One of the first detectable abnormalities in autonomic neuropathy is dysregulation of the sudomotor system [11]. While the parasympathetic system has minimal influence on sudomotor function, the sympathetic system has a more profound effect through postganglionic sympathetic cholinergic fibers that innervate exocrine glands of the skin that regulate sweat function [12–14]. Dysfunction of the sudomotor system due to DAN has an impact on sweat production, thus leading in disruption of microvascular skin blood flow and loss of sweat with a “stocking and glove” distribution. These changes can be detected with an easy-to-perform Indicator Plaster (IP) test of sudomotor function called Neuropad. Because DAN occurs usually first in longer fibers, dysregulation of sudomotor function in the feet may constitute an early indicator of autonomic system dysfunction throughout the body.

Previous studies in patients with type 1 and type 2 DM have found that the IP test after 10 min of application has a sensitivity of 65–87% and a specificity of 47–66% for the detection of distal symmetric polyneuropathy [15–17]. However, there is still insufficient evidence on the potential effectiveness of the same test for the diagnosis of autonomic neuropathy. The purpose of this study was to evaluate the diagnostic performance (specificity, sensitivity and accuracy) of the Neuropad test as a screening tool to detect DAN.

2. Materials and Methods

We recruited a total of 180 consecutive patients with DM, from two academic diabetes centers in northern Greece (1st and 2nd Propaedeutic Department of Internal Medicine, Aristotle University of Thessaloniki, Greece) from June 2022 to July 2023. Exclusion criteria were the following: age > 75 years, estimated creatinine clearance rate (using the Cockcroft-Gault formula) < 30 mL/min/1.73 m^2, history of limp amputation, causes of neuropathy other than diabetes, psychiatric antidepressant drugs that can affect perspiration such as selective serotonin reuptake inhibitors and serotonin and norepinephrine reuptake inhibitors, and skin disorders that could affect the result. Individuals with perspiration disorders as well as extreme dehydration (abnormal skin turgor) were clinically excluded.

Neuropad (Trigocare International GmbH, Wiehl-Drabenderhöhe, Germany) examination was performed in all patients, after 10 min of rest without socks in a room with well-controlled conditions (temperature). The Neuropad test is an adhesive patch for the

evaluation of skin hydration. It contains the blue complex salt anhydrous cobalt II chloride, which in the presence of water absorbs six water molecules and its color changes from blue to pink [18,19]. The Neuropad test is simple and can be applied even by patients themselves to diagnose the loss of sweat on the foot, as the result is visualized [13]. The test is performed at room temperature (20–25 °C), after the patient has removed socks and shoes for a 10-min acclimatization period. The Neuropad is applied to the plantar aspect between the first and second metatarsal heads in a callus-free area and the color is assessed after 10 min. The results are classified as normal if there is a complete color change from blue to pink and abnormal if there is incomplete or no color change [16].

DAN was assessed in all patients with the following four CARTs using age related reference values [8,20]: (1) R-R variability during 6 cycles of deep breathing [assessed by, Mean Circular Resultant (MCR, vector analysis) and Standard Deviation (SD)], (2) R-R variability during Valsalva maneuver with an intrathoracic pressure of at least 40 mm Hg (expressed as the ratio of the longest R-R interval after the maneuver to the shortest R-R during the maneuver; a result equal to or greater than 1.21 was considered normal), (3) R-R variability after a rapid change from lying to standing position [expressed as the ratio of the R-R interval at the 30th beat after standing up to the one at the 15th beat after standing up (30:15 ratio) and a result equal to or greater than 1.04 was considered normal], and (4) postural (orthostatic) hypotension (expressed as a fall of systolic pressure more than 20 mmHg within 2 min from lying to standing position). The presence of DAN was established if $\geq$2 CARTs were abnormal. One abnormal test was considered as early DAN.

The tests were carried out in the early morning, in a quiet room, without interruption with HOKANSON ANS 2000 (Hokanson Inc., Bellevue, WA, USA). We used a computerized system based on measurements of heart rate variation (RR interval) during rest, deep breath, Valsalva maneuver, and finally at orthostatic test. All patients were advised to fast overnight and avoid smoking and coffee at least 3 h prior to testing. Insulin-treated patients were advised not to inject their morning dose.

Plasma biochemical parameters, including fasting plasma glucose (FPG), total cholesterol, triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol and vitamin B12 levels, were measured with a Cobas-e 602 analyzer with electrochemiluminescence (ECLIA) (Roche, Basel, Switzerland) in the laboratories of the 2 hospitals. Glycated hemoglobin (HbA1c) was measured by an automated latex immunoagglutination inhibition assay (DCA2000, Bayer Diagnostics Europe Ltd., Dublin, Ireland). Estimated glomerular filtration rate (eGFR) was calculated according to the Gault-Cockroft equation (eGFR (mL/min/1.73 m^2) = [(140Age) $\times$ Body Weight (in kg)]/[72 $\times$ Serum creatinine (in mg/dL)] $\times$ (0.85 if female) [21].

2.1. Statistical Analysis

The normality of the distributions was assessed using the Kolmogorov-Smirnov test. Continuous data are presented as mean $\pm$ 1 SD (standard deviation) and categorical data as absolute numbers and percentages. Comparisons between 2 groups were performed by the independent *t*-test and the Mann-Whitney U test for normal and non-normal distributions, respectively. Comparisons between 3 groups were performed by the one-way ANOVA and the Kruskal Wallis test for normal and non-normal distributions, respectively. Where applicable the Bonferroni correction for the post hoc analysis was used [22]. The Chi-square test was used to compare categorical variables between groups. Pearson's correlation coefficient was utilized for normally distributed continuous variables, while Spearman's rho was employed to correlate categorical variables with continuous variables. In all cases, a 2-tailed *p*-value less than 0.05 was considered statistically significant. Post hoc power analysis was also performed along with testing of the research hypothesis with binomial

test. Data were analyzed using the SPSS statistical package [23] (version 29; SPSS Inc., Chicago, IL, USA) and Jupyter notebook with Python v.3.12 and packages scipy 1.11.4, numpy 1.26.4, statsmodels.stats.power 0.14.0, scipy.stats 1.11.4, matplotlib.pyplot 3.8.0. seaborn 0.12.2, sklearn.metrics 1.2.2.

2.2. Ethical Considerations

The study was carried out according to the principles of the Declaration of Helsinki and its subsequent amendments. All study participants gave their informed written consent before enrolment in the study and the study protocol was approved by the Scientific Committee of University General Hospital "AHEPA" with registration number: 16162/24.3.2022 and was registered in Clinicaltrials.gov with registration number: NCT06734143.

3. Results

A total of 180 patients with DM (82 with DM type 1, 92 women) were included in the analysis, with a mean age of 49.5 ± 16 years and a mean duration of diabetes of 17.2 ± 7.6 years. Neuropad was abnormal in 94 patients. DAN was diagnosed in 85 of the patients (group A), while 95 of the patients did not had DAN (group B). The patients in the latter group presented with none (n = 57) or only one abnormal CART (n = 38 early DAN). Thus, in the whole population, established DAN was diagnosed in 47.2% of the patients. Regarding CARTs in the whole population, 51 patients had 2 abnormal CARTs, 24 patients 3 abnormal and 10 patients had 4 abnormal CARTs respectively. The characteristics of the patients in the two groups are shown in Table 1. Patients with DAN were older and had longer DM duration compared to participants without DAN. There was no difference in lipid profile, eGFR, HbA1c, FPG, smoking and alcohol consumption between the two groups. All patients with hypertension were on ACE inhibitors and 30% were also receiving calcium channel blockers. A stratified analysis according to the number of the abnormal CARTS is presented in the Supplementary Materials. Also, in this analysis a greater number of abnormal CARTs is associated with a higher age, longer diabetes duration, type 2 diabetes and the presence of hypertension. Except for the type of diabetes, all other associations remain significant even after correction for multiple testing (Tables 2–4, Supplementary Materials).

Table 1. Characteristics of patients with established and not established DAN.

Parameters	Group A (Established DAN)	Group B (Not Established DAN)	*p*
N	85	95	
Sex (m/f)	37/48	51/44	0.113
Type 1 DM (n, %)	31 (36.5%)	51 (53.7%)	**0.025**
DM duration (years)	19.4 ± 7.9	15.1 ± 6.7	**<0.001**
Age (years)	54.5 ± 13.6	45.1 ± 16.7	**<0.001**
HbA1c (%)	7.7 ± 1.3	7.3 ± 1.4	**0.042**
FPG (mg/dL)	151 ± 27	145 ± 38	0.66
Cholesterol (mg/dL)	189 ± 37	192 ± 51	0.35
HDL-C (mg/dL)	47 ± 9	45 ± 10	0.73
LDL-C (mg/dL)	108 ± 25	119 ± 48	0.58
Triglycerides (mg/dL)	179 ± 71	170 ± 58	0.37
Creatinine (mg/dL)	0.97 ± 0.2	0.98 ± 0.23	0.98
eGFR (mL/min/1.73 m^2)	111.0 ± 33.5	109.7 ± 35.6	0.84
Vitamin B12 (pg/mL)	545 ± 150.5	511 ± 114.8	0.088
Alcohol consumption (n, %)	5 (5.9%)	12 (12.6%)	0.135
Smoking (n, %)	9 (10.6%)	19 (20%)	0.100

Table 1. *Cont.*

Parameters	Group A (Established DAN)	Group B (Not Established DAN)	*p*
Hypertension (n, %)	53 (62.4%)	77 (81.1%)	**0.007**
Vibration Perception Threshold (V)	27.9 ± 13	13.3 ± 7.1	**<0.001**
Abnormal Neuropad test (n, %)	74 (87.1%)	20 (21.1%)	**<0.001**

Abbreviations: DAN: Diabetic autonomic neuropathy; HbA1c: glycated hemoglobin; FPG: fasting plasma glucose; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; eGFR: estimated glomerular filtration rate; m: males; f: females. Significant results are presented with bold. Alcohol consumption was defined as consumption of more than 1 alcohol drink daily. Definition of 1 alcohol drink as per the Centers for Disease Control and Prevention (CDC) is a half-ounce or 13.7 g pure alcohol which is the amount of alcohol present in: (i) 12 oz beer (5% alcohol), (ii) 8 oz malt liquor (7% alcohol), (iii) 5 oz wine (12% alcohol), (iv) 1.5 oz 80-proof "hard-liquor" (40% alcohol) [24]. Hypertension was defined according the American Diabetes Association guidelines or already taking antihypertensives [25].

The presence of DAN was positively correlated with age ($p < 0.001$, r = 0.3), DM duration ($p < 0.001$, r = 0.28), HbA1c level ($p = 0.039$, r = 0.15), hypertension ($p = 0.005$, r = 0.21) and abnormal Neuropad result ($p < 0.001$, r = 0.68). On the contrary, there was no correlation with alcohol consumption and smoking. This is also shown in a more detailed analysis is presented in Supplementary Materials Table S1, which presents the distribution of the patient characteristics depending the number of abnormal CARTs.

Patients with type 1 DM were younger, but had longer disease duration than participants with type 2 DM. There were no differences in HbA1c levels (Table 2).

Table 2. Comparisons between type 1 and type 2 DM groups.

Parameters	All Patients	T1DM	T2DM	*p*
n	180	82	98	
Sex (m/f)	88/92	40/42	48/50	0.98
DM Duration (years)	17.2 ± 7.6	18.5 ± 8.4	16 ± 6.7	**0.028**
Age (years)	49.5 ± 16	36.1 ± 12	60.8 ± 8.5	**<0.001**
HbA1c (%)	7.5 ± 1.4	7.6 ± 1.3	7.4 ± 1.4	0.516
Alcohol (%)	17 (9.4%)	7 (8.5%)	10 (10.2%)	0.801
Smoking (%)	28 (15.6%)	12 (14.6%)	16 (16.3%)	0.838
Hypertension (%)	50 (27.8%)	10 (12.2%)	40 (40.8%)	**<0.001**
Neuropad abnormal (%)	94 (52.2%)	29 (35.4%)	65 (66.3%)	**<0.001**
DAN present (%)	85 (47.2%)	31 (37.8%)	54 (55.1%)	**0.025**

Abbreviations: DM: Diabetes mellitus; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; HbA1c: glycated hemoglobin; DAN: Diabetic autonomic neuropathy; m: males; f: females. For the definitions of alcohol consumption and hypertension please refer to Table 1. Significant results are presented with bold.

The results of Cardiovascular Autonomic Reflex Tests are presented in Table 3 for both groups. As per the definition of the diagnosis of DAN expected, all values were significantly different between individuals with established DAN and individuals without DAN.

We calculated the sensitivity, specificity and accuracy of Neuropad to detect DAN in all study participants and separately in the type 1 and type 2 DM groups. The results are depicted in Table 4. Neuropad results were reversely correlated with CARTs: MCR (r = −0.632, $p < 0.001$), SD of deep breathing test (r = −0.527, $p < 0.001$), Valsalva (r = −0.501, $p < 0.001$), postural index (r = −0.552, $p < 0.001$), and positively with orthostatic hypotension (r = 0.748, $p < 0.001$). Receiver Operating Charasteristic (ROC) curve for the diagnostic performance is presented in Figure 1. The area under the curve was 0.846 (95% CI 0.787, 0.905).

Table 3. Cardiovascular Autonomic Reflex Tests (CARTs) results.

	Group A (Established DAN)	Group B (Not Established DAN)	*p*
MCR	13 ± 7.9	42.6 ± 28.6	**<0.001**
SD of deep breathing test	22.7 ± 13	62.6 ± 48	**<0.001**
PO Postural Index (30:15)	1.04 ± 0.04	1.1 ± 0.08	**<0.001**
Valsalva	1.2 ± 0.09	1.4 ± 0.2	**<0.001**
OH (Orthostatic Hypotension) (mmHg)	22.1 ± 9.9	1.6 ± 5.6	**<0.001**

Abbreviations: MCR: Mean Circular Resultant, OH: Postural (orthostatic) hypotension (expressed as fall of systolic pressure within 2 min from lying to standing position). Significant results are presented with bold.

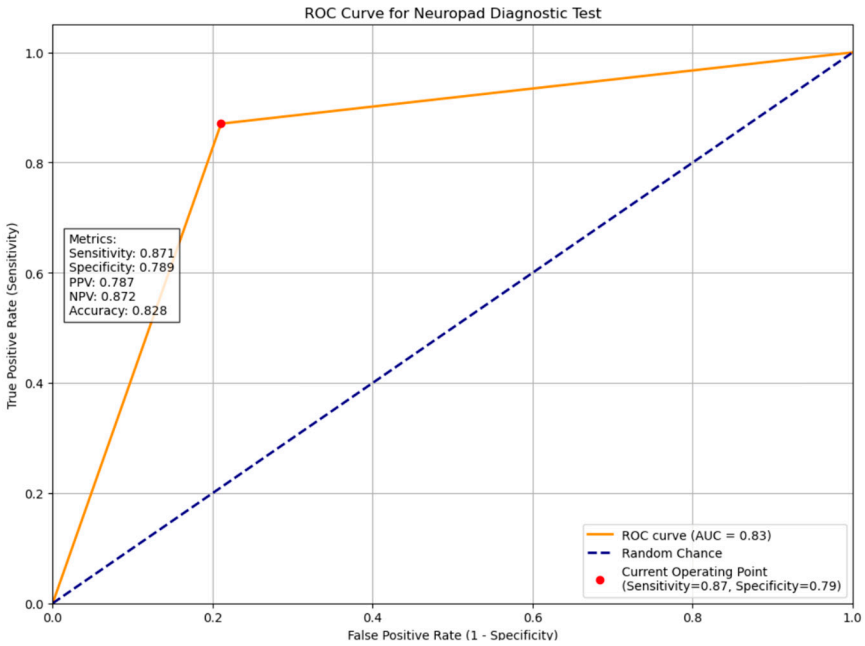

Figure 1. Receiver Operating Characteristic (ROC) curve of Neuropad to detect DAN. Abbreviations: PPV: positive predictive value, NPV: negative predictive value.

Table 4. Diagnostic performance of the Neuropad test to detect established DAN.

	All Patients (n = 180)	T1DM (n = 82)	T2DM (n = 98)
Sensitivity	87.1%	83.9%	88.9%
Specificity	78.9%	94.1%	61.4%
Disease prevalence	52.2%	35.4%	66.3%
Positive Predictive Value	78.7%	89.7%	73.8%
Negative Predictive Value	87.2%	90.6%	81.8%
Accuracy	82.3%	90.2%	76.5%

Abbreviations: T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus.

The sensitivity, specificity and accuracy of the IP test to detect established DAN were 87%, 78.9% and 82.3% respectively. Sensitivity and accuracy increase with increasing number of abnormal CARTs (Table 5). MCR was abnormal in 120 patients and postural hypotension in 75. The sensitivity, specificity and accuracy of the IP test to detect an abnormal MCR was 73.3%, 90% and 78.9%, respectively, and to detect (abnormal) Postural Hypotension 93.3%, 77.1% and 83.9% respectively (Table 5).

Table 5. Diagnostic performance of Neuropad to detect established DAN.

Method	Sensitivity	Specificity	Accuracy
Established DAN (≥2 CARTs)	87.1%	78.9%	82.3%
2 CARTs	82.4%	78.9%	80.1%
3 CARTs	91.7%	78.9%	81.5%
4 CARTs	100%	78.9%	80.9%
Abnormal MCR	73.3%	90%	78.9%
Postural hypotension	93.3%	77.1%	83.9%

Abbreviations: DAN: Diabetic autonomic neuropathy, MCR: Mean Circular Resultant.

4. Discussion

Diabetic autonomic neuropathy (DAN) is often overlooked, because particularly in the early stages of the disease it is either completely asymptomatic or has very moderate symptoms that easily escape attention (such as resting tachycardia). In addition, setting the diagnosis of CAN can be challenging [1,2]. The most widely accepted diagnostic tests are the Cardiovascular Autonomic Reflex Tests (CARTs) proposed by the Toronto panel; More than 2 abnormal CARTs are considered to be diagnostic of established CAN [8,9]. It should be noted however that performing CARTs requires the appropriate equipment and some experience, thus making them not easily applicable in the routine clinical practice. Yet, detection of CAN as early as possible is extremely important, since it confers high risk for cardiovascular morbidity and mortality. It follows that a well-performing screening tool will greatly help in accomplishing this challenge. One of the tools that can be used to this purpose is the indicator plaster Neuropad. This patch detects decrease sweating of the feet, which is at least partly regulated by the sympathetic system (postganglionic sympathetic cholinergic fibers). The patch test is performed easily and provides fast visible results, which thus can be read by the patients themselves [19,26,27].

In a previous study, our group tested the performance of Neuropad compared to Michigan screening tools and single functional tests in detecting diabetic peripheral neuropathy (DPN) [25]. The patch demonstrated sensitivity, specificity and accuracy over 75% against all methods, indicating that it is useful for the identification of patients with DPN and at risk for diabetic foot ulcers. In that study, however, we did not perform systematic CART testing and detailed data on DAN were missing. Thus, in the present study, we assessed the performance of the Neuropad screening test compared to CARTs for detecting DAN. Because of our previous study, we focused on CARTs and did not perform simultaneously the whole panel of functional tests.

The prevalence of DAN was found to be 47.2% among all patients, higher in individuals with type 2 DM (55.1%) and lower in those with type 1 DM (37.8%). This difference may be attributed to the older age of type 2 DM patients, even though type 1 patients had a longer duration of diabetes. In addition, co-morbidities associated with age (such as hypertension, which was more prevalent in type 2 DM patients) may be more important for the development of DAN than the duration of the DM itself.

The Neuropad test has demonstrated high sensitivity (87%), specificity (78.9%), and accuracy (82.3%) overall. Sensitivity and accuracy steadily increased with increasing number of abnormal CARTs, i.e., with increasing severity of DAN/CAN (Table 5). However, type 1 DM patients exhibited higher specificity, while type 2 DM patients demonstrated greater sensitivity and less specificity, possibly because older type 2 DM patients may have had neuropathies also from other causes, besides DM. In addition, type 2 DM patients had more often hypertension (Table 2), for which they took ACE inhibitors. ACE inhibitors have been implicated in slightly reducing sudomotor function [6] and therefore could

in some instances produce false positive Neuropad results [28]. There are also other possible explanations of the somehow lower specificity in T2DM like small fiber neuropathy, dry skin or cholinergic medications [6,28,29]. Furthermore, specificity (overall or only in type 1 or type 2 DM patients) may be somehow even lower, if adjusted for known confounders—in the case of our population age, duration of diabetes, HbA1c, and the presence of hypertension. Such a sophisticated adjusted specificity analysis would require the use of advanced machine learning algorithms [30,31]. It is however questionable whether an "adjusted specificity" would be substantially more informative in determining the performance and usefulness of a screening test in daily clinical practice. In addition, only the "conventional" specificity of nearly all tests has been up to now published, and only this "conventional" specificity can be used for comparison to other tests. In any case, as discussed below, it is the high sensitivity, not necessarily the particular high specificity, that is desired in screening tests.

We also evaluated Neuropad's sensitivity, specificity, and accuracy against the MCR as a measure of early parasympathetic dysfunction and orthostatic hypotension as a measure of severe sympathetic dysfunction. Neuropad displayed low sensitivity but high specificity for detecting parasympathetic dysregulation. Seemingly the patch cannot detect all the most early parasympathetic lesions. In contrast, the most advanced sympathetic neuropathy was identified with high sensitivity and specificity, confirming the overall accuracy of the Neuropad test.

Previous studies have indicated that Neuropad has high sensitivity for assessing diabetic peripheral neuropathy, making it a reliable method for early detection of patients at high risk for foot ulcers [15,26,32,33]. However, there is limited data on its use for detecting autonomic neuropathy. For instance, Liatis et al. demonstrated that while Neuropad has good sensitivity and negative predictive value for diagnosing peripheral neuropathy, its sensitivity for detecting milder forms of CAN is low [34]. Similarly, Mendivil et al. reported satisfactory sensitivity (75–78%) but low specificity (33–36%) for Neuropad in identifying CAN among patients with type 2 DM [35]. In sharp contrast to these studies, our study showed high sensitivity both in type 1 and type 2 DM patients. Specificity was found to be very high (94.1%) in type 1 DM and somehow lower (61.4%) in type 2 DM, albeit markedly higher than in the aforementioned studies. In our hands, the overall precision of the test was as high as 83.4% In another study, Spallone et al. showed a strong association between Neuropad complete color change, Valsalva ratio, and cold thermal perception threshold, and reported similar to ours sensitivity and specificity, but only after 18 min after application of the test [27]. There are several potential explanations for the apparent different results compared to the present study. The most striking difference regards the composition of the study populations. Nearly half of our participants had type 1 DM, while other studies predominantly included type 2 DM patients. As mentioned before, other, besides diabetic, neuropathies are expected to be more prevalent in the older type 2 DM patients, and this may be one reason for the lower specificity of Neuropad reported in other studies. We tried to limit the cases of other neuropathies in our study population by taking a detailed history, performing a careful clinical examination and measuring vitamin B12 levels (which were not measured in other studies). Other possible reasons for the observed differences include the criteria used for the diagnosis of CAN, the number of the patients with CAN included in each study and the severity of CAN of the patients of each study. We used the only widely accepted criterion of $\geq$2 abnormal CARTs; according to this definition almost the half (47.2%) of our patients had CAN. Other studies used a "score" combining abnormal CARTs and symptoms, based on which the number of patients with CAN was much lower than in our study. In addition, since the "score" takes also symptoms into account, these studies may include more cases with advanced CAN compared to our

study [27,34]. Most of our cases had only 2 abnormal CARTs (51/180, 28.3%), indicating "less severe" disease. Mendivil et al. used ≥1 abnormal CART as diagnostic criterion [35]. The resulting overdiagnosis obviously led to lower specificity.

On the other hand, there are studies showing both high sensitivity and high specificity for Neuropad in detecting DAN. Ponirakis et al. showed that the diagnostic performance of the Neuropad is high when there is structural small fibre damage (corneal nerve fibre length) [28]. Papanas et al. [32] found a powerful association of Neuropad response with small-fibre impairment in the feet—i.e., abnormal temperature and pain sensation—and high sensitivity and specificity of neuropad for small-fibre neuropathy (99%and 78%, respectively). Although these studies used different methods for diagnosing CAN, their results are in line with the findings of our study.

Taking together, our findings, as well as the findings of all the aforementioned published studies strongly suggest that Neuropad shows a high, in some instances a very high, sensitivity for detecting DAN. Even if one can argue about the somehow lower specificity, the high sensitivity makes Neuropad suitable as screening test for identifying those patients who should be referred for a definitive diagnosis of DAN with the golden standard, CARTs. Considering the high morbidity and mortality risk of DAN, these patients will have to subsequently undergo a full, elaborate and costly evaluation. In this sense, Neuropad is probably also cost-effective [36], and because, in addition, it can be performed quickly and quite easily, in many cases from the patient himself, it may be considered a very useful screening test in daily practice. In contrast, our findings of the somehow low specificity, particularly in patients with type 2 diabetes, and of the variability of diagnostic performance between the two types of diabetes do not support the use of Neuropad as (the sole) diagnostic test or as a replacement of any other established diagnostic method of DAN/CAN.

The strength of our study lies in the strict diagnostic procedure and the higher percentage of participants with type 1 DM, resulting in a younger average age compared to previous studies that focused mainly on older populations with type 2 DM [25,29,31]. However, our findings should be considered cautiously due to significant limitations, including the study's cross-sectional design and a relatively small sample size. However, our post hoc power analysis indicated that the sample was sufficient to support our findings. Furthermore, the study population origins from 2 diabetes centers in Greece. Thus, it is formally not safe to extrapolate the results in other populations. For instance, our results may not apply to other demographic groups with more diverse ethnic backgrounds, or to other vulnerable populations (e.g., older age or having other comorbidities). These limitations highlight the need for more extensive cohort studies to confirm our results.

5. Conclusions

In conclusion, in the present study the Neuropad test has demonstrated high sensitivity and accuracy, but somehow lower specificity for detecting established DAN compared to classic CARTs. These key findings support the validity of Neuropad as a very helpful screening and possibly as a useful adjunct diagnostic tool for CAN.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/diabetology6060055/s1, Table S1: Stratified analysis Neuropad; Table S2: Comparisons between abnormal CARTs (0 abnormal vs. 1, 2, 3, 4 abnormal CARTs); Table S3: Comparison between abnormal CARTs (1 abnormal vs. 2, 3, 4 abnormal CARTs); Table S4: Comparison between abnormal CARTs (2 abnormal vs. 3, 4 abnormal CARTs and 3 vs. 4).

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Data Availability Statement: The data presented in the study are available on reasonable request.

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