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Optimizing examination time and diagnostic performance of the histamine-induced axon-reflex flare response in diabetes

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Abstract

Introduction/Aims: The axon-reflex flare response is a reliable method for functional assessment of small fibers in diabetic peripheral neuropathy (DPN), but broad adoption is limited by the time requirement. The aims of this study were to (1) assess diagnostic performance and optimize time required for assessing the histamine-induced flare response and (2) associate with established parameters.

Methods: A total of 60 participants with type 1 diabetes with ($n = 33$) or without ($n = 27$) DPN participated. The participants underwent quantitative sensory testing (QST), corneal confocal microscopy (CCM), and flare intensity and area size assessments by laser-Doppler imaging (FLPI) following an epidermal skin-prick application of histamine. The flare parameters were evaluated each minute for 15 min, and the diagnostic performance compared to QST and CCM were assessed using area under the curve (AUC). Minimum time-requirements until differentiation and to achieve results comparable with a full examination were assessed.

Results: Flare area size had better diagnostic performance compared with CCM (AUC 0.88 vs. 0.77, $p < 0.01$) and QST (AUC 0.91 vs. 0.81, $p = 0.02$) than mean flare intensity, and could distinguish people with and without DPN after 4 min compared to after 6 min (both $p < 0.01$). Flare area size achieved a diagnostic performance comparable to a full examination after 6 and 7 min (CCM and QST respectively, $p > 0.05$), while mean flare intensity achieved it after 5 and 8 min (CCM and QST respectively, $p > 0.05$).

Discussion: The flare area size can be evaluated 6–7 min after histamine-application, which increases diagnostic performance compared to mean flare intensity.

KEYWORDS
diabetic peripheral neuropathy, neurogenic inflammation

Abbreviations: AUC, area under the curve; CCM, corneal confocal microscopy; CDT, cold detection threshold; CNFL, corneal nerve fiber length; DPN, diabetic peripheral neuropathy; FLPI, full-field laser speckle perfusion imaging; IENFD, intra-epidermal nerve fiber density; IQR, interquartile range; LDI_{FLARE}, laser-Doppler imaging flare; MNSI, Michigan Neuropathy Screening Instrument; MEDON, Methods of Early Detection and grading Of diabetic Neuropathy; NPV, negative predictive value; PDPN, painful diabetic peripheral neuropathy; PPV, positive predictive value; PU, perfusion units; QST, quantitative sensory testing; ROC, receiver operating characteristics; ROI, region of interest; T1DM, type 1 diabetes mellitus.

1 | INTRODUCTION

Early and adequate assessment of small nerve fibers is particularly important for early detection of DPN.[1] However, the most commonly used methods (skin biopsies and corneal confocal microscopy [CCM]) only assess the extent of morphological nerve fiber damage without providing any information on their remaining function.[2]

Several methods for assessment of small nerve fiber function are currently available including (but not limited to) laser- or heat contact evoked potentials, electricity- or laser-evoked cutaneous silent periods, microneurography, electrochemical sweat conductance or assessment of the axon-reflex flare response.[3–5]

The latter is a method to evaluate small nerve fiber function through a provoked flare response assessed using either laser-Doppler imaging flare (LDI_{FLARE}) or full-field laser speckle perfusion imaging (FLPI) following approximately 30 min of local heating.[6,7] The technique provides a non-invasive assessment of C-fiber function and show strong correlations with structural measures like CCM and intraepidermal nerve fiber density (IENFD), but a wide adaptation of the technique is limited by complexity and time requirement.[8,9]

We recently reported that a simple epidermal skin-prick application of histamine could reliably induce an axon-reflex flare response on the foot in people with and without type 1 diabetes mellitus (T1DM) and neuropathic complications.[10] The method utilizes the fact that an activation of histaminergic C-fibers causes a spreading vasodilation by a neurogenic release of peptides, which is known as a "flare response."[11,12] Our method reduced the examination time and complexity of the examination but still required 15 min for evaluation. In our initial proof-of-concept study, we found a difference between groups of people with T1DM with and without DPN and theorized that the groups could be distinguished at an earlier point during the examination. Also, we previously only evaluated the intensity of the flare response without assessing the flare area size, which is important for a direct comparison to established methods.[7] Therefore, the present study aimed to (1) compare the FLPI-assessed, quantitative measures of mean flare intensity with a measurement of flare area size, (2) evaluate how long each method needs to distinguish people with T1DM and established DPN from people with T1DM without DPN, and (3) evaluate the diagnostic performance of the two methods compared to established measures (CCM and quantitative sensory testing [QST]).

2 | METHODS

2.1 | Study design and participants

The present study was conducted at Steno Diabetes Center North Denmark, Aalborg University Hospital, Denmark, between August 2019 and February 2022. The cohort used for the study was derived from the "Methods of Early Detection and grading Of diabetic Neuropathy (MEDON)"-cohort, which is described in detail elsewhere.[10,13–17] The participants with T1DM were divided into those with or without DPN. The presence of DPN was defined as per the Toronto consensus for definite neuropathy.[18] Causes of neuropathy other than DPN were excluded from the cohort. The exclusion criteria included vitamin deficiencies, hematologic or immune diseases, thyroid, or parathyroid disease, chronic kidney disease, previous alcohol or drug abuse, previous chemotherapy, severe or chronic viral infection, severe skin disease, and active cancer. The study received approval from the local ethics committee (N-20190003) and was prospectively registered on clinicaltrials.gov (NCT04078516). All participants gave informed consent prior to participation.

2.2 | Neuropathy assessment

Participants underwent a clinical examination including a neurological evaluation according to the Michigan Neuropathy Screening Instrument (MNSI).[19] Conventional nerve conduction studies were performed following usual clinical standards using superficial recordings as previously described and reported with evaluation of the median, ulnar, radial, tibial, peroneal, and sural nerves.[10,13] QST was performed according to the full protocol provided by the German Research Network on Neuropathic Pain as previously described and reported.[10,20]

In-vivo CCM was performed in all eligible participants (people with active eye infections, corneal disease or abrasions, a history of bilateral refraction surgery or anterior segment trauma were excluded) using a Heidelberg Retinal Tomograph III Rostock Cornea Module (Heidelberg Engineering GmbH, Heidelberg, Germany) following established guidelines. A volume scan of the corneal apex was performed, and 100 images with a resolution of 400 × 400 µm were obtained from each participant. Subsequent image selection was performed by two different blinded authors (J.R. and S.S.C.), who each selected three to four representative images based on criteria including good image contrast between background and nerves, limited motion artifacts, limited pressure lines, limited image overlap, en-face alignment, and proper focus.[21] Manual morphometric analysis was conducted using CCMetrics (M.A. Dabbah, Imaging Science and Biomedical Engineering, University of Manchester, Manchester, U.K.). Corneal nerve fiber length (CNFL), corneal nerve branch density, and corneal nerve fiber density were obtained following previously established definitions.[22,23] Cutoffs for abnormal values were determined as the lower fifth quantile from the published normative dataset.[24] Contact lenses were removed prior to examination if present.

The axon-reflex flare response was evoked by an epidermal application of one drop of 1% weight by volume histamine (Lofarma, Milano, Italy) applied in an area approximately 2–3 cm proximal to the second toe on the dorsum of the right foot. The application was followed by the application of a simple, handheld, skin-prick lancet (Aalborg University, Aalborg, Denmark) with a standardized 85 g pressure to allow the histamine to penetrate the skin.[25] Images of the dermal blood flow were captured using an FLPI-device (Moor Instruments, Axminster, UK) and analyzed using appropriate software (moorFLPI-2 Review V5.0, Moor Instruments, Axminster, UK). Images were obtained at baseline (before application) and each minute for 15 min (after application). All images were subsequently screened

for poor quality (see supplementary material for examples) by two individual authors (J.R. and S.S.C.). The process resulted in 26 images being excluded (2%) with full agreement between the two raters. All remaining images were analyzed to obtain mean flare intensity and flare area size. Mean flare intensity was evaluated in a predefined, circular, region of interest (ROI) with an area of 450 mm^2. The parameter was evaluated as a change from baseline by subtracting the baseline image (image taken before the application of histamine) from each subsequent image. Flare area size was determined using the volumetric function in moorFLPI-2 Review V5.0 (Moor Instruments, Axminster, UK). Mean flare intensity was expressed in perfusion units (PU), while flare area size was expressed in mm^2.[26] Examples of a normal and an abnormal flare response are shown in Figure S1 in Data S1. The two methods for analysis and an example of a poor quality image are shown in Figure S2 in Data S1.

All examinations were conducted in a room with standardized temperature (23°C) and lighting. Antihistamine was prohibited 24 h prior to the examination. CNFL was selected as the primary CCM parameter for comparison, while cold detection threshold (CDT) was selected as the primary QST parameter for comparison, as these parameters have consistently displayed the lowest variability across different studies.[24,27]

2.3 | Statistical analysis

Categorical variables are expressed as percentages and compared using Chi^2 or Fisher's exact tests. Continuous data are expressed as mean ± standard deviations (SD) or as medians with interquartile ranges (IQR) depending on their distribution. Normality was assessed visually (histograms and QQ-plots) and statistically (Shapiro–Wilk tests). A parametrical comparison was done by paired student's *t*-tests or ANOVA. A non-parametrical comparison was done by Kruskal-Wallis H test potentially followed by pairwise Mann–Whitney U tests. Bonferroni-corrections were applied where relevant. The significance level was set to $p = 0.05$. Receiver operating characteristic (ROC)-curves were created using logistic regression and used to estimate the area under the curve (AUC), and calculate sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Spearman's rank correlation coefficient was used to determine correlations. ROC-curves were compared using the approach proposed by Delong.[28]

Mean flare intensity and flare area size for each participant were fitted as inverse exponential decay ($Y = A * \left(1 - e^{(-b*t)}\right)$ using least-squares linear regression and a time-constant (timepoint of approximately 63.2% maximum mean intensity/area size) restricted between zero and 15 min.[10] The fitting was done to mitigate cases where the same participant had multiple bad quality images. Following this procedure, the maximal value for each parameter (mean flux intensity and flare area) could be derived as the constant *A* with a corresponding time constant ($\frac{1}{b}$).

All analyses were performed using Stata/MP, Stata Statistical Software: Release 16.1 (StataCorp LLC, College Station, TX:).

3 | RESULTS

3.1 | Demographics

There were 60 persons with T1DM: 33 with and 27 without DPN. Nine participants did not undergo CCM due to previous refraction surgery. All participants completed all other examinations. A total of 15 participants received pain medication with duloxetine being the most common drug (50% of participants with pain), followed by opioids (36%) and anticonvulsants (21%). Those receiving pain medication was not statistically

TABLE 1 Demographics and test results

Variable	T1DM-DPN (*n* = 27)	T1DM + DPN (*n* = 33)	*P*-Value
Age, y	47.0 [44.0; 57.0]	52.0 [46.0; 57.0]	ns
Sex, % male	44.0%	55.0%	ns
BMI, kg/m^2	27.8 [25.6; 30.2]	26.9 [24.2; 31.0]	ns
HbA1c, %	8.1 [7.3; 8.8]	8.8 [8.0; 9.5]	>.01
Diabetes duration, y	25.0 [17.0; 34.0]	34.0 [28.0; 41.0]	>.01
NCV, m/s	46.0 [32.0; 48.0]	6.0 [0.0; 38.0]	>.01
NCA, μV	4.8 [1.9; 7.7]	0.9 [0.0; 2.4]	>.01
CDT, °C	27.9 [19.4; 30.2]	17.8 [7.4; 22.8]	>.01
HDT, °C	40.4 [37.5; 44.0]	45.3 [43.1; 49.2]	>.01
CNFD, $no./mm^2$	15.6 [12.5; 18.7]	10.0 [6.25; 12.5]	>.01
CNBD, $no./mm^2$	37.5 [31.2; 48.7]	17.2 [10.4; 21.9]	>.01
CNFL, mm/mm^2	16.3 [14.4; 17.8]	9.0 [7.3; 11.0]	>.01

Abbreviations: BMI, body mass index, CDT, cold detection threshold, CNBD, corneal nerve branch density, CNFD, corneal nerve fiber density, CNFL, corneal nerve fiber length, DPN, diabetic peripheral neuropathy, HbA1c, glycated hemoglobin A1c, HDT, heat detection threshold, MNSI, Michigan Neuropathy Screening Instrument, NCA, nerve conduction amplitude (sural nerve), NCV, Nerve conduction velocity (sural nerve), PDPN, painful diabetic peripheral neuropathy, T1DM, type 1 diabetes mellitus.

different from other participants with DPN ($p > 0.05$). The participants with DPN generally had higher hemoglobin A1c, and longer diabetes duration, than those without DPN (all $p < 0.01$). Participant characteristics and test results from QST and CCM can be found in Table 1 and Figure S3 in Data S1.

3.2 | Association between mean flare intensity and flare area size

There was a strong linear positive correlation between the maximum mean flare intensity and the maximum flare area size ($p = 0.77$, $p < 0.001$). The results are depicted in Figure 1.

3.3 | Examination time needed to distinguish people with and without neuropathy

The time required before mean flare intensity and flare area size were able to differentiate between participants with T1DM + DPN and T1DM-DPN differed ($p < 0.01$). The measurements of mean flare intensity were able to differentiate the two groups after 6 min ($p < 0.01$), while the measurements of flare area size were able to differentiate the two groups after only 4 min ($p < 0.01$). The results are displayed in Table 2. Comparable data from healthy controls are displayed in Table S4 in Data S1.

3.4 | Diagnostic performance

3.4.1 | The axon-reflex flare response versus corneal confocal microscopy

The diagnostic performance of flare area size with CNFL as reference was highest after 6–15 min. The calculated max flare area size had a similar performance (Tables 3 and 4).

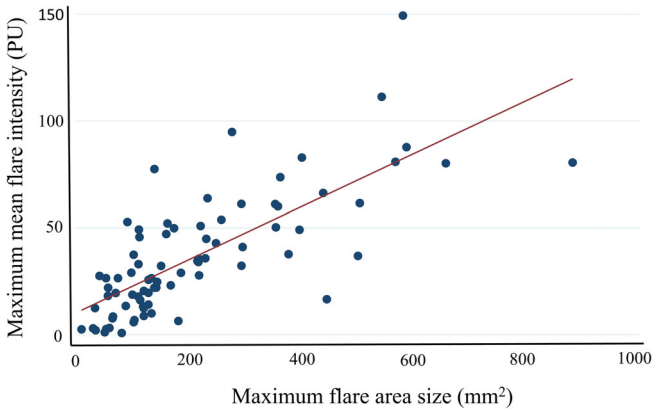

FIGURE 1 Relationship between maximum flare area size and maximum mean flare intensity

TABLE 2 Observation time until flare area size and mean flux can differentiate between people with type 1 diabetes with and without diabetic peripheral neuropathy

Group	Min 3	Min 4	Min 5	Min 6	Min 7	Min 8	Min 9	Min 10	Min 11	Min 12	Min 13	Min 14	Min 15
Flare area size													
DPN+ (mm^2)	4 [0–37]	20 [2–73]	40 [7–88]	75 [22–99]	66 [37–101]	84 [42–110]	103 [35–125]	90 [48–128]	103 [62–121]	97 [42–114]	88 [57–121]	86 [48–120]	84 [44–112]
DPN− (mm^2)	22 [4–55]	55 [29–90]	68 [44–161]	132 [66–222]	180 [66–310]	168 [121–310]	191 [123–352]	205 [121–400]	255 [132–407]	202 [130–363]	194 [121–376]	176 [134–343]	176 [128–374]
p-value	.16	**.04**	**.04**	**<.01**	**<.01**	**<.01**	**<.01**	**<.01**	**<.01**	**<.01**	**<.01**	**<.01**	**<.01**
Mean flare intensity													
DPN+ (PU)	4 [1–6]	6 [2–13]	6 [3–20]	10 [6–18]	12 [7–22]	11 [8–22]	11 [7–26]	13 [6–25]	14 [8–28]	11 [7–28]	13 [6–23]	13 [8–32]	14 [7–29]
DPN−(PU)	6 [1–17]	7 [5–25]	12 [3–23]	25 [13–35]	18 [10–39]	25 [16–42]	25 [14–46]	27 [16–53]	33 [15–47]	35 [16–46]	27 [16–70]	38 [21–52]	38 [57–21]
p-value	.16	.30	.27	**.04**	**.03**	**.03**	**.03**	**<.01**	**.02**	**<.01**	**<.01**	**<.01**	**<.01**

Note: Flare area size is measured as total flare area in square millimeter (mm^2), while mean flare intensity is measured as mean flare intensity in perfusion units (PU). Grey shades marks timeslots where with statistically insignificant differences between the two groups.

The diagnostic performance of mean flare intensity with CNFL as reference was highest after 5–15 min. The calculated max flare intensity had a similar performance (Tables 3 and 4). The calculated max flare area size had a better diagnostic performance than the calculated max mean flare intensity ($p < 0.01$) and performed better at every single timepoint from minute 7 and onward (all $p < 0.05$).

3.4.2 | Axon-reflex flare response versus quantitative sensory testing

The diagnostic performance of flare area size with the cold detection threshold as reference was highest after 7–15 min. The calculated max flare area size had a similar performance (Tables 3 and 4).

The diagnostic performance of mean flare intensity with the cold detection threshold as reference was highest after 8–15 min. The calculated max flare intensity had a similar performance (Tables 3 and 4). The calculated max flare area size had a better diagnostic performance than mean flare intensity ($p = 0.02$) and

TABLE 4 Diagnostic performance of calculated max mean area size and calculated max flare intensity

Diagnostic performance	Corneal confocal microscopy (CNFL)	Quantitative sensory testing (CDT)
	Flare area size	
Sensitivity	79% [63%–89%]	80% [64%–92%]
Specificity	80% [80%–96%]	83% [70%–92%]
PPV	80% [64%–91%]	85% [69%–96%]
NPV	78% [62%–89%]	77% [63%–89%]
AUC	0.88 [0.80–0.96]	0.91 [0.84–0.97]
	Mean flare intensity	
Sensitivity	70% [54%–83%]	75% [60%–87%]
Specificity	74% [57%–87%]	74% [57%–88%]
PPV	74% [57%–87%]	79% [63%–90%]
NPV	70% [54%–83%]	70% [53%–84%]
AUC	0.77 [0.66–0.88]	0.81 [0.72–0.91]

Note: Abnormal corneal nerve fiber length (CNFL) and abnormal cold detection threshold (CDT) are used as a reference.

TABLE 3 Diagnostic performance of flare area size and mean flare intensity

Diagnostic performance	Min 3	Min 4	Min 5	Min 6	Min 7	Min 8	Min 9	Min 10	Min 11	Min 12	Min 13	Min 14	Min 15
Corneal confocal microscopy (Corneal nerve fiber length)													
Flare area size													
Sensitivity	70%	66%	71%	80%	80%	**82%**	79%	79%	79%	74%	74%	79%	80%
Specificity	72%	67%	67%	79%	79%	**80%**	79%	80%	79%	76%	76%	82%	79%
PPV	72%	66%	68%	80%	80%	**80%**	80%	79%	79%	76%	76%	81%	79%
NPV	70%	67%	70%	79%	79%	**82%**	79%	80%	80%	74%	74%	80%	78%
AUC	0.76	0.77	0.81	0.85	0.86	**0.88**	0.88	0.88	0.88	0.87	0.86	0.85	0.86
Mean flare intensity													
Sensitivity	63%	66%	66%	66%	70%	71%	72%	72%	**74%**	72%	74%	63%	70%
Specificity	62%	69%	68%	67%	72%	79%	74%	71%	**77%**	74%	74%	67%	72%
PPV	63%	68%	68%	66%	72%	67%	74%	72%	**76%**	74%	74%	65%	72%
NPV	62%	68%	66%	67%	70%	74%	72%	71%	**75%**	72%	74%	65%	70%
AUC	0.64	0.69	0.74	0.75	0.77	0.75	0.77	0.77	**0.79**	0.79	0.78	0.73	0.72
Quantitative sensory testing (Cold detection threshold)													
Flare area size													
Sensitivity	61%	70%	67%	74%	68%	72%	74%	74%	**79%**	79%	77%	76%	80%
Specificity	72%	77%	69%	72%	75%	78%	77%	78%	**81%**	78%	77%	78%	83%
PPV	73%	79%	73%	76%	77%	80%	80%	80%	**83%**	81%	80%	80%	85%
NPV	60%	68%	63%	70%	66%	70%	71%	72%	**76%**	76%	73%	74%	76%
AUC	0.73	0.80	0.80	0.83	0.85	0.87	0.87	0.88	**0.90**	0.89	0.88	0.89	0.90
Mean flare intensity													
Sensitivity	61%	63%	64%	63%	66%	69%	70%	72%	74%	71%	72%	67%	**68%**
Specificity	61%	63%	68%	69%	69%	72%	69%	74%	74%	68%	71%	69%	**69%**
PPV	66%	68%	71%	71%	73%	74%	73%	78%	78%	67%	76%	72%	**73%**
NPV	56%	58%	61%	60%	63%	67%	65%	68%	70%	72%	68%	64%	**64%**
AUC	0.70	0.71	0.69	0.76	0.73	0.80	0.77	0.80	0.80	0.75	0.80	0.80	**0.82**

Note: Abnormal corneal nerve fiber length (CNFL) or abnormal cold detection threshold (CDT) are used as a reference. Grey shades marks timeslots that had significantly worse diagnostic performance than the optimal timeslot (marked in bold).

performed better at every single timepoint from minute 11 and onward (all $p < 0.05$).

4 | DISCUSSION

The study provides evidence that measurements of flare area size are superior to mean flare intensity, with faster examinations for differentiation between T1DM with and without DPN and better agreement with established methods (QST and CCM). In addition, the study showed that flare area size had the highest diagnostic performance 7–15 min after application of histamine, and that this interval was non-inferior to a full 15 min examination. This indicates that only 7 min of examination time may be sufficient for future use/studies in DPN.

4.1 | Histamine for evoking the axon-reflex flare response

Few studies have previously explored histamine as a mediator for an axon-reflex flare response in diabetes. In a small study, the authors found a diminished response to both histamine, capsaicin, and substance P in people with DPN compared to controls.[29] Likewise, we previously reported a diminished response in people with T1DM and DPN compared to people without DPN and healthy controls.[10] Studies using local heating have repeatedly validated the axon-reflex flare response technique against QST, CCM, and skin biopsies, and although strong longitudinal datasets are yet to be published, small studies and preliminary results have indicated that the technique might be suitable to assess the severity of small fiber neuropathy.[6,8,9,30]

The finding that flare area size is superior to mean flare intensity is in line with most studies using local heating, although the reasoning is not the same. When using local heating, a heating probe with a set temperature of 44°C is applied to the skin for approximately 30 min, which causes increased blood flow irrespective of the function of small cutaneous C-fibers.[6,31] This interaction was confirmed in a study using capsaicin to attenuate the axon-reflex flare response prior to local heating, which caused the flare area size, but not the maximal flare intensity, to diminish.[31] Similarly, in studies using iontophoresis of acetylcholine, the maximal flux is also considered an expression of microvascular function rather than a neurogenic response, which is again due to a direct interaction between a prolonged electrochemical stimulus and the endothelium.[32,33] This interaction is not present when using a skin-prick application of histamine, as the axon-reflex flare response generated is thought to be entirely neurogenic. Despite this, the flare area size still seems to be a superior measurement also following a skin-prick application of histamine.

4.2 | Diagnostic performance of the axon-reflex flare response

Studies assessing the diagnostic performance of the axon-reflex flare response are variable and sparse, but generally indicate good results when assessing DPN. One study found a sensitivity of 86% for diagnosing mixed-fiber neuropathy in diabetes using local heating, while another found a sensitivity of 79% with a corresponding AUC of 0.75 using the same method.[34,35] Both results are in line with data from our present study, although differences in methodology make direct comparison across studies impossible. This issue is also apparent from a recent meta-analysis of the heat-induced axon-reflex flare response, where the authors fail to prove the diagnostic ability to distinguish between people with different degrees of neuropathy due to very few studies being directly comparable.[36]

Our present study also provides information about the development of the axon-reflex flare response following a skin-prick application of histamine. From our data, the diagnostic performance of the flare area size seems to steadily increase for the first 7 to 10 min, and then to plateau (Table 3). However, it appears that the optimal compromise between examination time and performance is somewhere around the 6- to-7-min mark, where the diagnostic performance is statistically similar and still allows for rapid examinations. The optimal duration is however completely reliant on the situation and goals, as smaller-sized research trials might opt to wait for the optimal time, while larger population studies or clinical applications might prefer a shorter examination time over a slightly better performance.

4.3 | Limitations

The present study does have several limitations. First, the proposed skin-prick application for delivery of histamine has so far proven safe and reliable, but the reduced examination time and simplicity come at the price that the dissemination cannot be regulated and the fear that glycosylated skin might interfere with delivery and response.[31,37] In addition, it cannot be ruled out that the skin-prick itself could causes alterations in the C-fiber response, although the impact is thought to be minimal. Therefore, a reproducibility study is warranted before the method can be claimed as a faster and equally viable option to evoke the axon-reflex flare response in diabetes. Secondly, there was no comparison to local heating, and the method also needs to be tested in a less selected cohort in which the diagnostic agreement between methods might diminish. Thirdly, our cohort is relatively small and does not include participants with pure small fiber neuropathy, which means the generalizability is somewhat limited. Finally, a comparison to IENFD would have been desirable, but was unfortunately not available in the present cohort.

5 | CONCLUSIONS

The axon-reflex flare response to histamine skin-prick parameters assessed 6–7 min after application was as sensitive as QST and CCM in distinguishing people with T1DM and DPN from those with T1DM without DPN. Flare area size was superior to flare intensity, with a more rapid examination time for differentiating groups and the best

agreement with established methods. Overall, the reduction in examination time might help facilitate future clinical use.

AUTHOR CONTRIBUTIONS

Johan Røikjer: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; software; formal analysis; project administration; data curation; resources. **Suganthiya Santhiapillai Croosu:** Conceptualization; investigation; validation; project administration. **Mette Krabsmark Borbjerg:** Investigation; project administration. **Tine Maria Hansen:** Conceptualization; methodology; validation; supervision. **Jens Brøndum Frøkjær:** Supervision; conceptualization; methodology; validation. **Lars Arendt-Nielsen:** Methodology; resources; supervision; validation. **Niels Ejskjaer:** Conceptualization; funding acquisition; methodology; validation; project administration; resources; supervision. **Carsten Dahl Mørch:** Resources; supervision; data curation; conceptualization; funding acquisition; methodology; validation; software.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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