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Perception threshold tracking: validating a novel method for assessing function of large and small sensory nerve fibers in diabetic peripheral neuropathy with and without pain

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Abstract

It remains unknown why some people with diabetes develop painful neuropathies while others experience no pain. This study aimed to validate a novel method for assessing the function of small sensory nerves in diabetes to further elucidate this phenomenon.

The function of large and small nerves was assessed using a novel perception threshold tracking technique in three well-characterized groups (n=60) with type 1 diabetes and 1) painful diabetic peripheral neuropathy (T1DM+PDPN), 2) painless diabetic peripheral neuropathy (T1DM+DPN), 3) no neuropathy (T1DM-DPN), and 4) healthy controls (n=20). Electrical currents with different shapes, duration, and intensities were applied by two different skin electrodes activating large- and small fibers, respectively. The minimal current needed to activate the fibers were analyzed as the rheobase of the stimulus-response function. Nerve fiber selectivity was measured by accommodation properties of stimulated nerves.

The rheobase of both fiber types were highest for T1DM+PDPN, followed by T1DM+DPN, T1DM-DPN and healthy controls, indicating that the nerve properties are specific in individuals with diabetes and pain. There was an overall significant difference between the groups ($p<0.01$). The accommodation properties of stimulated fibers were different between the two electrodes ($p<0.05$) apart from in the group with T1DM+PDPN, where both electrodes stimulated nerves displaying properties similar to large fibers.

Perception threshold tracking reveals differences in large- and small nerve fiber function between groups with and without diabetes, DPN, and pain. This indicates that the methods have potential applications in screening DPN and explore further the features differentiating painful from non-painful DPN.

Keywords: diabetes mellitus, diabetic peripheral neuropathy, microvascular complications, neuropathic pain, nerve function, small fiber neuropathy, large fibers

1. Introduction

Diabetic peripheral neuropathy (DPN) is the most common complication to diabetes mellitus and is associated with severe conditions like neuropathic pain and diabetic foot ulcers with subsequent risk of amputation and premature death[29,31]. However, diagnosing the nerve conditions reliably at an early stage remains a major challenge[30]. DPN has historically been screened for using either the monofilament or the vibration perception threshold and if needed confirmed by conventional nerve conduction studies, all methods assessing the integrity of the large sensory nerve fibers. However, as research in the latest decades has provided insight into the importance of small nerve fibers due to their early signs of damage that potentially precedes those of the large fibers in DPN, the search for methods to assess small nerve fiber integrity has become increasingly important[3,5,21,36,40,44]. This has led to the development of several new methods with corneal confocal microscopy (CCM) and skin biopsies emerging as the two primary options[9,16,23,34]. Both methods have different strengths and weaknesses but ultimately suffer the inherent issue of only measuring the extend of structural nerve fiber damage without providing any information regarding the function of the remaining fibers and associations to pain. This problem justifies the need for a new, reliable, and sensitive method for measuring the function of the small fibers.

One potential method is the perception threshold tracking[8]. The method is based on the foundation of conventional threshold tracking, but excels due to its likely ability to selectively

stimulate small sensory nerve fibers without activating the large fibers and vice versa[2,8]. This preferential activation of small sensory fibers (Aδ and C) is achieved by applying a current that is predominantly present where the small nerve fibers terminate in the epidermis, without reaching the deeper termination of the large fibers (Aβ) in the dermis[27,42].

The method has been validated in several studies in healthy individuals, but is yet to be validated in a large clinical setting in people painful and non-painful neuropathy[11,25,41].

The aims of this study were: 1) to validate if perception threshold tracking can distinguish between different groups with and without DPN and 2) distinguish between people with and without painful diabetic neuropathy (PDPN).

2. Methods

2.1 Study Design and Participants

This was an observational, cross-sectional, cohort study conducted between August 2019 and April 2021. The study population is described in detail elsewhere, but in short, the cohort consisted of 80 participants aged 18-70 years[28]. The participants were divided into four groups consisting of: 20 with type 1 diabetes mellitus (T1DM) and PDPN (T1DM+PDPN), 20 with T1DM and painless DPN (T1DM+DPN), 20 with T1DM and no DPN or pain (T1DM-DPN) and 20 healthy controls (HC). Each participant was matched on age (+/- 2 years) and sex with one participant from each of the other groups. The diagnosis of PDPN and the presence or absence of DPN were determined by a specialist in diabetes and later confirmed at screening by another doctor. This assessment was further supported by a Douleur Neuropathique 4 Questions (DN4)-score $\geq$ 4 (for PDPN) and a vibration perception threshold above or below 25V as well as probable neuropathy according to the Toronto consensus on diabetic neuropathies (for the presence of DPN)[37,39]. Potential participants

were pre-screened prior to their scheduled visit in the outpatient clinic, and if they seemed to fulfill the inclusion criteria, they were invited for a screening visit. For each set of four participants (one from each group), we initially identified a person with T1DM+PDPN and matched the others based on age and sex. To only include diabetes-induced neuropathy, participants were screened for other causes for neuropathy, including vitamin- or immune-deficiencies, hematologic diseases, abnormalities in the thyroid or parathyroid metabolism and impaired liver or kidney function. Individuals with ongoing or previous alcohol or drug abuse, impaired cognitive function, previous chemotherapy, severe or chronic viral infections, severe skin diseases, active cancer, known lower-extremity ischemia, and known pregnancies were also excluded. The exclusion criteria applied for all participants. The study received approval from the local ethics committee (N-20190003) and was prospectively registered on clinicaltrials.gov as NCT04078516 ("*Methods for Early Detection Of Diabetic peripheral Neuropathy (MEDON)*").

2.2 Examinations

During screening the participants underwent a thorough clinical examination, blood samples, vibration perception threshold using biothesiometry, and answered the DN4 questionnaire[7,37]. If included, participants then underwent conventional nerve conductions studies, quantitative sensory testing, and perception threshold tracking.

Conventional nerve conduction studies were performed at the right leg and arm by the local Department of Neurophysiology at Aalborg University Hospital following usual clinical practice with standardized skin temperature.

To ensure participants with PDPN had neuropathic- and not ischemic pain, the participants also underwent peripheral blood pressure measurements (Periflux 6000, Perimed, Sweden).

Quantitative sensory testing was performed in accordance with the standardized protocol defined by the German Research Network of Neuropathic Pain (DFNS) using an "Advanced Thermosensory Stimulator" (TSA), Advanced Medical Systems (MEDOC), Israel[33]. The examinations were conducted using the standardized instructions (translated to Danish) in a room with standardized room temperature in a predefined area located 2-3 centimeters proximal to the second toe on the dorsum of the right foot.

2.3 Perception Threshold Tracking

Given the importance of assessing large (touch, vibration, and proprioception) and small (temperature, pain) nerve fibers individually, the participants were electrically stimulated in the same area as for quantitative sensory testing using two different electrodes: A patch electrode (for evaluation of the large fibers) and a pin electrode (for evaluation of the small fibers). The patch electrode was an Ag-AgCl surface electrode (Neuroline 700, Ambu A/S, Denmark) used as cathode, and a 4 x 6.4 cm Pals Neurostimulation Electrode (Axelgaard, CO., United States) used as anode. The pin electrode was a custom-designed concentric stainless-steel ring electrode (area: 8.8 cm^2) serving as the anode, surrounding a printed circuit board in which 16 stainless steel pin electrodes placed in a circle to serve as the cathodes. The pins were blunted with a diameter of 0.2 mm[8,20,27]. The electrical stimuli were delivered using a DS5 electrical stimulator (Digitimer Ltd, UK). The participants used a custom-made handheld response button to indicate when they felt the electrical stimulations (Inventors Way, Denmark). A personal computer and a data acquisition card (LabBench Io, Inventors Way, Denmark) were used to collect the responses from the subjects and control the electrical stimulator. The electrical stimulations were controlled by a protocol implemented in a custom made program (LabBench Io, Inventors Way, Denmark)[8]. The perception threshold was estimated using square impulses with varying durations (0.1ms, 1 ms, 50

ms) by slowly increasing the intensity of each stimulus until the participant pressed the button indicating that the stimuli were perceived, and the perception threshold reached. The intensity was then increased by 20 % to ensure a suprathreshold stimuli and subsequently lowered until the participant relieved the button indicating that the stimulus was no longer felt. This was repeated three to five times for each impulse to increase precision (method of limits). The setup is depicted in supplementary figure S1 (available at http://links.lww.com/PAIN/B722).

Based on the perception thresholds obtained for the square pulses, a strength-duration curve was plotted for each participant. From this curve, the rheobase (the lowest current intensity of infinite duration that results in a depolarization of the nerve membrane) and the chronaxie (the minimal pulse duration required to double the strength of the rheobase) were derived[14]. A strength-duration curve is depicted in supplementary *figure S2* (available at http://links.lww.com/PAIN/B722).

To test if the method indeed did activate the small and large nerve fibers individually, we also measured accommodation (the ability to adapt to a slowly rising pulse by increasing the excitation threshold for increasing pulse widths)[10,41]. This was done as previous studies have shown that probable differences in ion-channel composition of large- and small nerve fibers result in different accommodation properties, where large fibers accommodate while small fibers do not[41,42]. This was done by applying a 100ms ramp-shaped, that would cause accommodation in the large sensory nerve fibers[18]. Accommodation is caused by the transient voltage-gated sodium channels becoming refractory and thus increased the excitation threshold of these fibers[1]. Persistent voltage-gated sodium channels that are exclusively expressed in the small nerve fibers remain open and thus triggered an action potential, so that accommodation will be prohibited, and the threshold will therefore remain unchanged[6,41].

2.4 Re-grouping

In accordance with the analysis plan supplied for the pre-registration (Clinicaltrials.gov: NCT04078516), two rounds of analyses were performed. Initially, the four clinically defined groups were analyzed as outlined below. Then, each participant with diabetes were re-grouped for a secondary analysis based on them having zero, one or two abnormalities in small fiber function rated by heat- and cold detection thresholds outside the 95% confidence interval of the age and sex-specific normative values provided by the DFNS [32]. If participants had abnormal cold- AND heat detection threshold they were classified as having small fiber involvement (+SFN), if participants had only one of either abnormal cold- OR heat detection threshold they were classified as possible small fiber involvement (pSFN), and if the participants had both normal cold- AND heat detection threshold they were classified as not having small fiber involvement (-SFN). This way, three new groups were created: (+SFN) "definite small fiber involvement" (33 participants), (pSFN) "possible small fiber involvement" (15 participants), and (-SFN) "no small fiber involvement" (12 participants). This re-grouping was performed as the initial grouping mainly relied on examinations regarding the presence of large, rather than small, fiber involvement, which might blur otherwise relevant findings. Characteristics for the new groups are depicted in *supplementary table S3* (available at http://links.lww.com/PAIN/B722).

2.5 Statistical Analyses

As both the rheobases and chronaxie-estimates were non-normally distributed within the groups (even after log-transformation), statistical differences between groups were initially calculated using Kruskal–Wallis H-test followed by pairwise Mann-Whitney U-tests with Bonferroni-

corrections. To eliminate the possible bias induced by within-group difference in age and sex, we also conducted Bonferroni-corrected Wilcoxon signed-rank tests. Other variables were analyzed using Bonferroni-corrected, pairwise t-tests, or Mann-Whitney U-tests. A Bonferroni-corrected Spearman's correlation was used to assess the relationship between the rheobase of large and small nerve fibers and measurements of neuropathy severity, peripheral damage, and known risk factors. Logistic regression was used to produce a receiver operating characteristic (ROC) curve and to estimate sensitivity and specificity of the rheobase of large and small fibers in detecting neuropathy based on the thermal measurements from QST and the sural nerve conduction velocity and amplitude from conventional nerve conduction studies, respectively. The analyses were performed using Stata/MP, Stata Statistical Software: Release 16.1. College Station, TX: StataCorp LLC.

3. Results

3.1 Demographical Characteristics

A total of 87 participants were screened with seven not being included predominantly due to unregistred alcohol or drug abuse. There was a significant difference in diabetes duration, nerve conduction velocity and amplitude, heat and cold perception threshold, ankle-brachial index, Michigan Neuropathy Screening Instrument-score, and PainDETECT/DN4-scores between the four groups (all $p<0.05$). A full overview of the different characteristics can be found in *table 1*.

(table 1)

3.2 Perception threshold tracking of large fibers

Rheobase: The rheobase of the large fibers was highest for T1DM+PDPN: 3.94 mA [IQR 1.99-25.0], followed by T1DM+DPN: 2.49 mA [IQR 1.74-4.09], T1DM-DPN: 1.68 mA [IQR 1.16-1.89] and HC: 1.09 mA [IQR 1.02-1.36], (*Figure 1a*). There was an overall significant difference in the rheobase between the four groups: $X^2(3)=28.785$ ($p<0.001$). The pairwise (unpaired) comparison showed a difference between all pairs of groups ($p<0.001$ for T1DM+PDPN versus T1DM-DPN, T1DM+PDPN versus HC and T1DM+DPN versus HC, $p=0.042$ for T1DM+DPN versus T1DM-DPN), apart from between T1DM+PDPN and T1DM+DPN ($p=0.696$) and between T1DM-DPN and HC ($p=0.288$), where statistical significance was lost after Bonferroni-correction.

In the paired analysis (not affected by within-group differences in age and sex) there was a significant difference between all pairs of groups ($p=0.006$ for T1DM+PDPN versus T1DM-DPN, $p<0.001$ for T1DM+PDPN versus HC, $p=0.038$ for T1DM+DPN versus T1DM-DPN, and $p<0.001$ for T1DM+DPN versus HC, respectively) apart from between T1DM+PDPN and T1DM+DPN ($p=0.060$), and T1DM-DPN and HC ($p=0.630$) where statistical significance was lost after Bonferroni-correction. These results are depicted in *figure 1a*.

Chronaxie: The chronaxie of the large fibers was highest for T1DM+PDPN: 1.31 ms [IQR 0.92-2.03], followed by T1DM+DPN: 1.14 ms [IQR 0.70-1.54], HC: 0.85 ms [IQR 0.72-0.99], and T1DM-DPN: 0.70 ms [IQR 0.57-0.97]. There was an overall significant difference in the chronaxie between the four groups: $X^2(3)=15.810$ ($p=0.001$). The pairwise (unpaired) comparison revealed a difference between T1DM+PDPN and T1DM-DPN ($p<0.001$) and HC ($p=0.012$), while there were no differences between the rest of the groups (all $p>0.05$). In the paired analysis (not affected by within-group differences in age and sex) no differences were found (all $p>0.05$).

3.3 Perception threshold tracking of small fibers

Rheobase: The rheobase of the small nerve fibers was highest for T1DM+PDPN: 1.09 mA [IQR 0.52-25.0], followed T1DM+DPN: 0.78 mA [IQR 0.19-1.17], T1DM-DPN: 0.25 mA [IQR 0.14-0.45] and HC: 0.14 mA [IQR 0.08-0.24], see Figure 1b. There was an overall significant difference in the rheobase between the four groups: $X^2(3)=24.136$ ($p<0.001$). The pairwise (unpaired) comparison showed a difference between T1DM+PDPN and T1DM-DPN ($p=0.012$), between T1DM+PDPN and HC ($p<0.001$), and between T1DM+DPN and HC ($p=0.006$). After Bonferroni-correction, there was no statistically significant difference between T1DM+PDPN and T1DM+DPN ($p=0.122$), between T1DM+DPN and T1DM-DPN ($p=0.276$), and between T1DM-DPN and HC ($p=0.408$).

In the paired analysis (not affected by within-group differences in age and sex) there was a significant difference between all pairs of groups ($p=0.047$ for T1DM+PDPN versus T1DM+DPN, $p=0.032$ for T1DM+PDPN versus T1DM-DPN, $p<0.001$ for T1DM+PDPN versus HC, and $p<0.001$ for T1DM+DPN versus HC, respectively), apart from between T1DM-DPN and HC ($p=0.096$) and between T1DM+DPN and T1DM-DPN ($p=0.268$), where statistical significance was lost after Bonferroni-correction. These results are depicted in *figure 1b*.

Chronaxie: The chronaxie of the small fibers was highest for HC: 2.25 ms [IQR 1.40-2.98], followed by T1DM+DPN: 1.96 ms [IQR 1.40-3.75], T1DM-DPN: 1.64 ms [IQR 1.11-4.13], and T1DM+PDPN: 1.57 ms [IQR 1.52-5.69]. There was no overall difference in the chronaxie between the four groups: $X^2(3)=1.440$ ($p=0.696$).

(Figure 1)

3.4 Results for small nerve fibers after re-grouping

Rheobase: The rheobase was highest for +SFN: 1.03 mA [IQR 0.51-6.62], followed by -SFN: 0.25 mA [IQR 0.11-0.32], and pSFN: 0.20 mA [IQR 0.15-0.94]. There was an overall difference in the rheobase between the three small fiber neuropathy groups: $X^2(2)=14.029$ ($p<0.001$). The pairwise comparison showed a difference between +SFN and -SFN ($p=0.003$), and between +SFN and pSFN ($p<0.001$), while there was no difference between pSFN and -SFN ($p=0.337$). The results are depicted in *figure 2*.

Chronaxie: The chronaxie was highest for +SFN: 1.83 ms [IQR 1.52-5.49], followed by -SFN: 1.64 ms [IQR 1.12-4.13], and pSFN: 1.49 ms [IQR 1.12-3.32]. There was no overall difference in the chronaxie between the three groups: $X^2(2)=2.672$ ($p=0.263$).

(Figure 2)

3.5 Accommodation

We were unable to elicit a response in 5 participants with T1DM+PDPN and in 1 participant with T1DM+DPN. In these cases, the highest current intensity applied within the safety limit was used as the result. There was a significant difference between the accommodation properties of the nerves stimulated by the two different electrodes within all groups (all $p<0.05$) apart from within the group of people with T1DM+PDPN ($p=0.47$). This indicates plausible stimulation of different fiber types using the two different electrodes in three of the four groups. The results are depicted in *figure 3*.

(Figure 3)

3.6 Correlation analyses

There were statistically significant correlations between the rheobase of both large and small fibers and all other included parameters (HbA1c, Michigan Neuropathy Screening Instrument, sural nerve conduction velocity and amplitude, the vibration perception threshold, and the cold- and heat perception thresholds) (all $p<0.01$). The results from each analysis are depicted in *table 2*.

(Table 2)

3.7 ROC-curves, sensitivities, and specificities

Large nerve fibers: The ability of the rheobase of the large nerve fibers to detect neuropathy defined by abnormal sural nerve conduction velocity and amplitude were very good. The area under the curve (AUC) from the ROC-curve was excellent at 0.89 with a corresponding sensitivity of 90% and a specificity of 89%.

Small nerve fibers: The ability of the rheobase of the small nerve fibers to detect neuropathy defined by abnormal heat and cold perception threshold (based on the age- and sex-specific 95% CI provided by the DFNS) were very good. The AUC from the ROC-curve was excellent at 0.84 with a corresponding sensitivity of 82% and a specificity of 82% *(ROC-curves are available as supplementary figures S4 and S5*, available at http://links.lww.com/PAIN/B722).

4. Discussion

This study is the first to validate perception threshold tracking in characterizing the function of both large and small sensory nerve fibers in people with painful and non-painful diabetic polyneuropathy. The technique successfully differentiated people with diabetes from controls and PDPN from non-painful DPN, although a rather big overlap was seen between the latter two. This indicates different subtypes of PDPN within the cohort, and the method might only be suitable for distinguishing one distinct subtype of PDPN from painless DPN. As such, perception threshold tracking technology may provide further understanding of the fundamental differences in sensory nerve fiber properties in subgroups of people with painful and non-painful DPN. This may give new insight to pharmacological targeting pain in subtypes of people with PDPN.

4.1 Conventional threshold tracking in diabetes

Conventional threshold tracking has previously been used to examine nerve excitability properties in people with DPN but has been limited to accessing the integrity of large fibers. In one such study of 106 persons with type 2 diabetes and 33 controls, the authors found increased electrical stimulus intensity for the 50% sensory nerve action potential and shortened strength-duration time constant (chronaxie) in all groups with diabetes compared to those without[38]. In that same study, the authors also observed a worsening of all examined parameters as the severity of neuropathy increased. This is in line with the findings from the present study, where an increase in current intensity needed to reach the perception threshold (rheobase) was observed in the group with DPN compared to those without. However, in the present study, we only observed an increase in chronaxie for T1DM+PDPN when compared to T1DM-DPN and HC, while T1DM+DPN did not

differ significantly from either group. Also, we observed no such difference when measuring the chronaxie of small nerve fibers, which could either indicate preserved nodal sodium permeability or simply reflect the fact that we probably ended up predominantly measuring the large fibers in those with T1DM+PDPN as discussed later. Otherwise, these different findings could be due to differences in the pathogenesis of type 1 and type 2 diabetes, or due to varying populations with different age- and sex-composition, varying methodology and stimulation site (hand vs foot), and markedly different diabetes duration. However, not all studies support changes in the chronaxie in DPN, with one recent large study in 111 persons with type 2 diabetes and 60 controls reporting no difference in the strength-duration time-constant between people with type 2 diabetes and no/probable or confirmed DPN and healthy controls[17]. The same study also reported minimal changes to the rheobase, with only those with confirmed DPN being significantly different from those without diabetes[17].

Only one study has previously used nerve excitability testing in T1DM, although the aim of this study was slightly different compared to ours[19]. The authors reported that in a cohort of 30 persons with T1DM without DPN, they observed multiple abnormalities in large sensory axons including sub-excitability during the recovery cycle and during hyperpolarizing threshold electrotonus. These findings are not comparable with data from the present study, but interestingly, the authors claim that their findings could resemble changes in nodal sodium- and potassium-channels with reduced function of the sodium-potassium-pump, which has previously been suggested from animal models of T1DM and could be further examined utilizing the present threshold tracking technique. Finally, recent studies have shown a relationship between small fiber structure and motor nerve excitability, although these are not comparable with the method presented in the current study[43,45].

4.2 Painful and painless neuropathy

The present study also examined differences between painful and painless DPN. The rheobase of small nerve fibers were significantly greater in those with PDPN compared to those with painless DPN, which would indicate more severe small fiber damage in those with pain. Furthermore, there appears to be a trend towards higher rheobases of large fibers in T1DM+PDPN compared to T1DM+DPN, although this trend lost statistical significance after correction for multiple testing. However, the high current needed to active the fibers stimulated by both electrodes in participants with the most severe DPN (mainly T1DM+PDPN) probably limited the nerve fiber selectivity, as the ability to produce a current specifically located in the epidermis declines as the intensity of the stimuli increases. This would cause activation of a mixture of fiber types or maybe even predominantly large nerve fibers, as their size, myelination, and ion-channel composition in the nodes of Ranvier cause them to be significantly more excitable than small nerve fibers[13,26]. From our accommodation findings (*figure 3*) it appears, that the selectively of nerve fiber stimulation is only preserved in healthy controls, T1DM-DPN, and T1DM+DPN, as the accommodation properties vary between the stimuli from the different electrodes indicating activation of different nerve fibers. Meanwhile, this is not the case for T1DM+PDPN, where the two different electrodes activate nerves with almost identical accommodation properties. This means that the measurements from this group probably reflects a mixture of fiber types, probably due to severe dying-back of the small nerve fibers, as previously suggested from studies on intra-epidermal nerve fiber density[15]. More severe small fiber nerve damage in those with pain has also previously been reported[4]. While the above-mentioned theory is probably the most likely explanation, it is also possible that our findings instead represents a shift in ion-channel composition in the nerves of those with PDPN. While this explanation is not the most likely scenario, the fact that many of the other measurements

appear similar between the two groups (cold detection threshold, heat perception threshold, sural nerve conduction velocity, sural nerve conduction amplitude etc.) make it an interesting proposition, which will require further exploration in future studies.

4.3 Limitations

The present study validates perception threshold tracking as a novel method in type 1 diabetes with and without DPN and PDPN. However, due to its nature, it does suffer several inherent issues. Firstly, the study is the first to ever use this perception threshold tracking technique in a clinical setting, which vastly limited our ability to conduct a sample size estimation before initiation. This meant that we likely underestimated the number of participants needed for proper sub-group analyses, although it did not impact the overall comparisons or the goal of validation. Secondly, prolonged usage of the pin-electrode (small nerve fibers) sometimes caused an unspecific, tingling or burning feeling, that would persist between stimulations[24]. This is thought to be caused by activation of C-fibers (in contrast to the predominant activation of Aδ-fibers otherwise caused by the method), which could confuse the participants without sufficient instruction. Possible shunting of the electrical signal through the epidermis via hair fascicles is also something to consider when using the pin electrode (small fibers), although this would require an electrode placement directly on top of a hair fascicle or sweet duct[35]. Also, like other measurements of DPN, the results from our perception threshold tracking technique appear to be influenced heavily by age and sex. Unfortunately, our data was not normally distributed and displayed uneven variance, which limited our ability to adjust for these parameters in our statistical analyses. However, our design with participants matched 1:1 on age and sex allowed us to circumvent this problem, as our paired analysis removed the bias provided by these two factors. The fact that bias was present due to the

two factors means that age- and sex-specific normative values must be developed for the method to be applicable for future screening on unselected (in contrast to the highly selected groups in the present study) participants. Finally, the current study does not include skin biopsies. As our perception threshold tracking method is meant as a (non-invasive) functional nerve fiber test to either supplement or even replace the current gold standard, the study would have benefitted from a direct comparison of the two methods, which is something that will be included in future studies utilizing the technique.

4.4 Future perspectives

This study validated the threshold tracking technique as a tool to assess the excitability properties of the small nerve fibers in people with and without diabetes and pain. Future research utilizing the technique is warranted and should be combined with other methods to assess small fiber neuropathies like corneal confocal microscopy or skin biopsies. Due to the likely ability to detect early changes to nerve fibers, the method might also have a future role in early detection, grading, and risk stratification of DPN, although it remains unsure whether the method is useful on an individual. Before bringing the technique closer to a clinical application, more research is warranted, including the generation of (age- and sex-specific) normative values and an evaluation of both reproducibility and the impact of external factors like blood glucose, skin temperature and duration of the session. Better fundamental understanding of the neurophysiological differences between painful and non-painful diabetic polyneuropathies may pave the way for developing efficient and targeted pain management programs.

5. Conclusion

Perception threshold tracking is a suitable technique for differentiating diabetic polyneuropathies from controls and might help differentiating certain subtypes of painful diabetic polyneuropathies from non-painful.

6. Acknowledgements and article information

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Figure 1*: Boxplots of the rheobases (mA) for the large- (1a) and small (1b) fibers for each of the groups (type 1 diabetes and painful diabetic peripheral neuropathy (T1DM+PDPN), type 1 diabetes and painless diabetic peripheral neuropathy (T1DM+DPN), type 1 diabetes and no diabetic peripheral neuropathy or pain (T1DM-DPN) and healthy controls (HC)). Pairwise statistically significant differences are denoted by symbols a-c. Integers denoted by the same letter are not statistically different from each other but are statistically different from groups denoted with a different letter. Statistical differences between the groups are analyzed using pairwise Bonferroni-corrected Wilcoxon signed-rank tests. A schematic overview of the rheobase estimation can be found in supplementary figure S2* (available at http://links.lww.com/PAIN/B722).

Abbreviations: ***T1DM****: Type 1 diabetes,* ***PDPN****: Painful diabetic peripheral neuropathy,* ***DPN****: Diabetic peripheral neuropathy.*

Figure 2*: Boxplot of the rheobase (mA) for each of the new groups (type 1 diabetes and definite small fiber neuropathy (+SFN), type 1 diabetes and possible small fiber neuropathy (pSFN), type 1 diabetes and no small fiber neuropathy (-SFN). Pairwise statistically significant differences are denoted by symbols a-c. Integers denoted by the same letter are not statistically different from each other but are statistically different from groups denoted with a different letter. Statistical differences between the groups are analyzed using pairwise Bonferroni-corrected Mann-Whitney U tests.*

Figure 3*: Accommodation properties for the nerves stimulated with the patch- (large fibers, orange) and pin-electrodes (small fibers, blue) in each of the four groups (type 1 diabetes and painful diabetic peripheral neuropathy (T1DM+PDPN), type 1 diabetes painless diabetic*

*peripheral neuropathy (T1DM+DPN), type 1 diabetes and no diabetic peripheral neuropathy or pain (T1DM-DPN) and healthy controls (HC). Pairwise statistically significant differences between patch- and pin-stimulations are calculated using pairwise Bonferroni-corrected Mann-Whitney U tests. Statistically significant differences are marked by *.*

Table 1:

	T1DM+PDPN	T1DM+DPN	T1DM-DPN	Healthy Controls
Participants, n	20	20	20	20
Age, years	50.5 (43.5-57.0)	51.5 (45.5-58.5)	50.5 (44.5-57.5)	50.5 (44.0-58.5)
Sex, % male	50.0%	50.0%	50.0%	50.0%
BMI, kg/m^2	27.2 (25.1-30.4)	27.8 (24.2-30.8)	27.1 (24.6-30.2)	24.3 (23.1-27.9)
HbA1c, mmol/mol	70.0 (59.0-78.5)[a]	73.0 (65.5-78.0)[a]	64.5 (58.0-72.3)[a]	34.0 (31.8-35.0)[b]
Diabetes duration, years	33 (22.5-40.5)[a]	34.5 (29.8-38.8)[a]	25.5 (15.5-31.0)[b]	-
MNSI	4.5 (2.25-5.5)[a]	4.0 (3.0-5.0)[a]	0.0 (0.0-0.0)[b]	0.0 (0.0-0.0)[b]
Average Pain intensity	5.0 (4.0-7.5)[a]	0.0 (0.0-0.0)[b]	0.0 (0.0-0.0)[b]	0.0 (0.0-0.0)[b]
Peak pain intensity	8.0 (6.0;9.0)[a]	0.0 (0.0-0.0)[b]	0.0 (0.0-0.0)[b]	0.0 (0.0-0.0)[b]
NCV, m/sec	13.5 (0-39.3)[a]	15.5 (0-39.5)[a]	47.5 (44.8-48.5)[b]	54.5 (48.0-57.0)[c]
NCA, µV	0.4 (0-2.7)[a]	1.1 (0-3.6)[a]	5.0 (2.7-7.8)[b]	10.3 (6.7-13.3)[c]
CDT, °Celsius	20.3 (7.3-25.1)[a]	14.6 (7.3-20.9)[a]	28.1 (26.7-30.3)[b]	30.1 (25.6-30.7)[b]
HDT, °Celsius	45.3 (43.2-47.2)[a]	44.3 (40.6-49.3)[a]	40.0 (37.3-42.0)[b]	37.5 (35.5-41.1)[b]
ABI	1.12 (0.90-1.25)[a]	1.15 (1.04-1.25)[ab]	1.25 (1.17-1.31)[b]	1.28 (1.18-1.31)[b]
TBI	0.81±0.27	0.80±0.25	0.87±0.19	0.87±0.23
PainDETECT score	15.5 (11.0-19-5)[a]	0 (0-3.5)[b]	0 (0.0-0.0)[c]	0.0 (0.0-0.0)[c]
DN4 score	5.0 (4.0-6.0)[a]	0.0 (0.0-1.25)[b]	0.0 (0.0-0.0)[b]	0.0 (0.0-0.0)[b]

Table 1: Demographics and tests results for participants in each of the 4 groups T1DM+PDPN, T1DM+DPN, T1DM-DPN, and healthy controls. Data are displayed as mean ± standard deviation or as a median with interquartile ranges. Pairwise statistically significant differences are denoted by symbols a-c. Integers denoted by the same letter are not statistically different from each other but are statistically different from groups denoted with a different letter. Statistical differences between the groups are tested using Mann-Witney U or t-tests. Average and peak pain intensity are reported as average or peak over the last four weeks on a scale from 0-10, where 0 is no pain and 10 is the worst imaginable pain. To be registered, the pain had to derive from the feet, and could not be caused by trauma. In cases where the sural nerve could not be activated the values were set to 0.0.

*Abbreviations: **T1DM**: Type 1 diabetes, **PDPN**: Painful diabetic peripheral neuropathy, **DPN**: Diabetic peripheral neuropathy, **BMI:** Body Mass Index, **HbA1c:** Glycated haemoglobin A1c, **MNSI:** Michigan Neuropathy Screening Instrument **NCV**: Nerve conduction velocity (sural nerve), **NCA**: Nerve conduction amplitude (sural nerve), **CDT:** Cold detection threshold, **HDT:** Heath detection threshold, **ABI:** Ankle-brachial-index, **TBI:** Toe-brachial-index*

Table 2:

	Hba1c	mnsi	ncv	nca	vpt	cdt	hdt
Rheobase of small fibers	r_s = 0.511 p < 0.001	r_s = 0.470 p < 0.001	r_s = 0.675 p < 0.001	r_s = 0.759 p < 0.001	r_s= 0.377 p = 0.005	r_s = 0.596 p < 0.001	r_s =0.716 p < 0.001
Rheobase of large fibers	r_s = 0.491 p < 0.001	r_s = 0.508 p < 0.001	r_s = 0.709 p < 0.001	r_s = 0.750 p < 0.001	r_s = 0.429 p < 0.001	r_s = 0.630 p < 0.001	r_s = 0.595 p < 0.001

Table 2: Relationship between the rheobase of large and small nerve fibers and glycated hemoglobin A1c, the Michigan Neuropathy Screening Instrument, the nerve conduction velocity and amplitude of the Sural nerve, and peripheral heat and cold detection thresholds. All analyses were performed as Bonferroni-corrected Spearman's correlation analyses. The data are presented as Spearman's rho with corresponding p-value.

Abbreviations: ***HbA1c:*** *Glycated hemoglobin A1c,* ***MNSI:*** *Michigan Neuropathy Screening Instrument* ***NCV****: Nerve conduction velocity (sural nerve),* ***NCA****: Nerve conduction amplitude (sural nerve),* ***CDT:*** *Cold detection threshold,* ***HDT:*** *Heath detection threshold*