**Spinal inhibitory dysfunction in patients with painful or painless diabetic neuropathy**

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ABSTRACT

Objective

Impaired rate dependent depression of the Hoffman reflex (HRDD) is a marker of spinal inhibitory dysfunction and has previously been associated with painful neuropathy in a proof-of-concept study in patients with type 1 diabetes. We have now undertaken assessment of HRDD in patients with type 1 or type 2 diabetes.

Research Design and Methods

A total of 148 participants, including 34 healthy control subjects, 42 patients with painful diabetic neuropathy and 62 patients with diabetic neuropathy without pain, underwent assessment of HRDD and detailed assessment of peripheral neuropathy including nerve conduction studies (NCS), corneal confocal microscopy (CCM) and thermal threshold testing.

Results

We found, compared to healthy controls (p<0.001) and patients without pain (p<0.001), that HRDD is impaired in patients with both type 1 or type 2 diabetes with neuropathic pain. These impairments are unrelated to diabetes type and the presence or severity of neuropathy. In contrast, patients without neuropathic pain (p<0.05) exhibited enhanced HRDD compared to control subjects.

Conclusions

We suggest that loss or impairment of HRDD may help to identify a sub-population of patients with painful diabetic neuropathy mediated by impaired spinal inhibitory systems who may respond optimally to therapies that target spinal or supraspinal mechanisms. Enhanced RDD in patients without pain may reflect engagement of spinal pain suppressing mechanisms.

INTRODUCTION

Painful diabetic peripheral neuropathy can affect up to one-third of people with diabetes and results in somnipathy, depression and a poor health-related quality of life [1-3]. The majority of patients achieve limited pain relief with considerable side effects when using first line anti-neuropathic pain medication at maximum tolerated doses or in combination [4]. There has been a resurgence of interest in identifying new drug targets or predictive biomarkers for specific pain mechanisms that may be more effectively targeted using existing therapies [5-7]. One potential mechanism is spinal disinhibition, where decreased tonic spinal inhibitory processes results in lack of suppression or even amplification of painful and non-painful peripheral signaling [8].

In rodent models ofType 1 diabetic rats, behavioral indices of neuropathic pain are driven by spinal disinhibition [9, 10]. Despite impaired inhibition/increased excitability in the dorsal spinal cord, diabetic rats exhibiting tactile allodynia and exaggerated hyperalgesia show an increase in both basal and evoked spinal levels of the inhibitory neurotransmitter GABA [11]. It has been suggested that this paradoxical finding reflects a switch of GABAA receptor function, so that it is no longer inhibitory and becomes pro-nociceptive [9]. In support of this, the potassium/chloride co-transporter KCC2, which is critical in determining intracellular chloride levels and the direction of ion flow through the ionotropic GABAA receptor, is reduced in the dorsal spinal cord of diabetic rats [9, 10, 12].

A biomarker of altered spinal inhibition is rate dependent depression of the Hoffmann-reflex (H-reflex) [13]. The stimulation protocol for the H-reflex evokes two waveforms: a direct nerve to muscle M-wave and the longer latency trans-spinally mediated H-wave. Originally thought to represent a purely monosynaptic trans-spinal reflex, it is now accepted that the H-reflex arc is modulated by oligosynaptic connections [13-15]. The H-reflex has several properties that can be used to evaluate spinal function. HRDD is the measure of the change in amplitude of the H-reflex component over consecutive stimulations and can be measured non-invasively in humans using a modification of standard nerve conduction studies. Loss of inhibitory function in the spinal cord results in reduced depression of the H-reflex amplitude during successive stimulations. For example, the impairment of HRDD has been used as a marker of disinhibition of spinal sensory processing caused by spinal cord injury in both animals and humans [16-18] and has been linked to loss of GABAergic inhibition [16, 19].

Accumulating pre-clinical evidence in animal models of type 1 diabetes demonstrate that behavioral indices of painful neuropathy arising from loss of spinal GABAergic inhibitory function are associated with a loss of HRDD [9, 10]. The potential of these findings to translate to clinical population was indicated in our exploratory study in patients with type 1 diabetes in which we identified impaired HRDD in around half of patients with painful diabetic neuropathy [12]. Whilst preclinical studies in a rat model of type 2 diabetes have identified loss of HRDD [12], it is not known if this extends to patients with type 2 diabetes who represent the majority of people with painful diabetic neuropathy [20]. We have therefore assessed HRDD in conjunction with detailed peripheral structural and functional phenotyping of neuropathy in a large cohort of patients with type 1 or type 2 diabetes with and without neuropathic pain.

RESEARCH DESIGN AND METHODS

Research Ethics Committee approval was granted (East Midlands – Leicester South Research Ethics Committee reference 17/EM/0076) and written informed consent obtained from each participant. Study conduct adhered to the tenets of the Declaration of Helsinki.

Study Participants

Patients with type 1 diabetes (n=47), type 2 diabetes (n=81) and control subjects (n=36) underwent nerve conduction studies and assessment of HRDD. The H-reflex was absent in 24 patients, 10 with type 1 and 14 with type 2 diabetes as well as 2 control subjects and these subjects were excluded from the study (Figure 1). Participants underwent further assessment of neuropathic symptoms, signs, thermal threshold testing and corneal confocal microscopy. Detailed demographic data, medical history and current medications, age, gender, ethnicity, type and duration of diabetes, co-morbidities, height, weight, blood pressure, HbA1c, lipids and renal function were documented. Other common causes of neuropathy were excluded based on a family history as well as testing for serum B12, folate, immunoglobulins, electrophoresis and anti-nuclear antibody. The modified Toronto Diabetic Neuropathy Expert Group recommendations [21] were used to allow for the inclusion of small fibre abnormalities [22] to diagnose diabetic peripheral neuropathy (DPN) based on the presence of symptoms and signs and an abnormality in either nerve conduction studies or corneal confocal microscopy. The Neuropathy Symptom Profile (NSP) questionnaire and Visual Analogue Scale (VAS) pain scores recording current, average and maximum pain ratings over the previous 24 hours were documented. Patients were stratified into painful (DPN+) and painless (DPN-) cohorts based on the Toronto consensus that ‘the symptoms are distal, symmetrical, often associated with nocturnal exacerbations, and commonly described as prickling, deep aching, sharp, like an electric shock, and burning with hyperalgesia’[21] for greater than 3 months which is consistent with the requirement of pain chronicity defined by the International Association for the Study of Pain (IASP) [23]. All patients with a current, average or maximum VAS pain score > 0 were placed in the pain cohort. The pain group was further subdivided into mild (VAS <4) and moderate/severe (VAS 4-10) (Figure 1A) [24].

Experimental Procedures

Vibration and Thermal Detection

Vibration detection threshold (VDT) was evaluated using a Rydel Seiffer 64Hz tuning fork with fixed weights on the first metatarsophalangeal joint of the right foot. The vibration amplitude of the tuning fork at the point of sensation loss is used to assess VDT. The scale reading moves from 0 to 8, exponentially with decreasing vibration amplitude and a low value indicates a loss of vibration sensation at high vibration amplitude. Cold (CDT) and warm (WDT) detection thresholds were recorded on the dorsum of the right foot using a TSA-II NeuroSensory Analyser (Medoc, Ltd., Ramat-Yishai, Israel).

Corneal Confocal Microscopy

Corneal confocal microscopy (CCM) using a laser scanning Heidelberg Retinal Tomograph III Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany) was undertaken in both eyes using an established protocol [25]. Corneal nerve fibre density (CNFD – total number of main nerves per square millimetre (no./mm2)), corneal nerve fibre length (CNFL – total length of main nerves and nerve branches per square millimetre (mm/mm2)) and corneal nerve branch density (CNBD – total number of branches per square millimetre (no./ mm2)) were quantified.

Nerve conduction and H-reflex studies

Nerve conduction and H-reflex studies were performed using a DANTEC Keypoint system (Dantec Dynamics Ltd, Bristol, UK). Participants were sat semi-recumbent at 45° with limb temperature maintained between 32-35°. Sural sensory amplitude (SNAP) and conduction velocity (SNCV) along with peroneal motor nerve amplitude (PMNAP) and conduction velocity (PMNCV) were recorded. For H-reflex studies, tibial nerve stimulation was performed using 1ms square wave monophasic pulses delivered using surface silver-silver chloride electrodes, to the popliteal fossa. Surface silver-silver chloride recording electrodes with a diameter of 9mm were placed on the long axis of soleus. H-reflex recruitment curves were obtained to determine peak-peak H-reflex maximal amplitude (Hmax) by incrementing stimulation current by 1mA. A minimal inter-stimulation interval of 10 seconds was observed. For HRDD, a submaximal stimulus strength (to achieve a response of 75% of max) was used. The HRDD measurement consists of H wave responses to trains of 10 stimuli delivered at 1 Hertz. HRDD was calculated as the mean of responses 2-10 (meanH2-10) of a 1Hz stimulus train, expressed as a percentage of response number 1. Therefore, a higher value of HRDD indicates a smaller degree of depression than a lower value and *vice versa*. The average of stimulus responses 2-10 was used to mitigate against random and time course fluctuations.

Statistical methods

Statistical analyses were performed using Prism statistical software (GraphPad Software Inc, La Jolla, CA) and SPSS (Version 27.0 for Windows, IBM Corporation, New York, NY, USA). Parametric data were analysed using unpaired t-test or one-way ANOVA followed by Tukey’s multiple comparison to compare means between groups. Non-parametric data were analysed using the Kruskal-Wallis test followed by Dunn’s post-hoc for multiple comparisons. The analysis of covariance (ANCOVA) (post hoc LSD) was used to compare variables between groups, while statistically controlling for the effects of age. Correlations were calculated using Spearman’s rank test and expressed as a coefficient (r) with significance level. P < 0.05 was considered biologically relevant. Datasets are available from the corresponding author upon request.

RESULTS

Patients with type 1 and type 2 diabetes have comparable degree of HRDD

Patients with type 1 diabetes were significantly younger (p<0.001), had a longer duration of diabetes (p=0.033) and lower BMI (p=0.0029) compared to patients with type 2 diabetes. However, HbA1c and measures of both large and small fibre neuropathy did not differ significantly between patients with type 1 or type 2 diabetes (Table 1). There was no significant difference in group mean HRDD between patients with type 1 or type 2 diabetes or healthy controls (Figure 2A). There was no significant difference in group mean HRDD between patients with and without diabetic neuropathy. (Figure 2B).

Patients with painful and painless diabetic neuropathy have abnormal HRDD

Demographics and clinical characteristics are summarized in Table 2. Patients with diabetes were significantly older than control subjects and had significantly higher BMI and HbA1c. NCS, CDT, WDT, VDT and CCM parameters were significantly impaired in patients with diabetes compared to controls. There was no significant difference in demographic or neuropathy parameters between patients with or without painful diabetic neuropathy. However, HRDD was significantly impaired in patients with painful diabetic neuropathy when compared to patients without painful diabetic neuropathy (p=<0.001) and control subjects (p<0.001). In contrast, HRDD was significantly exaggerated in patients with diabetes without pain when compared to control subjects (p<0.05) (Figure 2C and 2D). There was no significant difference in HRDD between patients with mild and moderate to severe neuropathic pain.There was no significant difference in HRDD between female and male patients with or without pain or between female and male control subjects.

Correlations

There was no significant correlation across the whole patient cohort, or across the pain or no pain groups individually, between HRDD and either age, duration of diabetes, BMI, HbA1c or any of the measures of peripheral neuropathy. There was no significant correlation across the control cohort between HRDD and either age or BMI. Within the group of patients with painful diabetic neuropathy there was no correlation between HRDD and VAS pain scores.

DISCUSSION

We previously demonstrated impaired rate dependent depression of the H-reflex in a group of patients with type 1 diabetes and painful neuropathy [12]. The current study now extends this finding to a larger group of patients with type 1 diabetes and type 2 diabetes. HRDD was significantly impaired in patients with painful diabetic neuropathy when compared to patients without pain and control subjects. The impairment was not related to the presence and severity of diabetic neuropathy. A further novel finding was that HRDD was enhanced in patients without painful diabetic neuropathy when compared to control subjects.

Previous studies have shown differences in thermal thresholds [26] and we have shown greater corneal nerve loss [27] in patients with painful diabetic neuropathy. In the current study there were no significant differences in any of the markers of altered small fibre function or structure, between patients with and without painful neuropathy. This may in part relate to the relatively small number of patients studied or reflect their mild to moderate severity of neuropathy. It is well documented that H-reflex amplitudes attenuate with increasing severity of diabetic neuropathy [28, 29] and indeed are typically absent in patients with severe neuropathy [29]. Patients without an H-reflex were not included in the study resulting in a cohort of patients with predominantly mild or moderate neuropathy.

In our exploratory study of a small group of patients with Type 1 diabetes the deficits in HRDD showed a correlation with pain ratings such that greater impairment of spinal inhibitory function was associated with higher severity of clinical pain [12]. However, in this larger cohort of patients with painful diabetic neuropathy, some of whom were taking medication for neuropathic pain, the impairment of HRDD was not related to the severity of pain. Intuitively, patients with painful diabetic neuropathy and evidence of spinal disinhibition, a mechanism that is proposed to result in reduced suppression of nociceptor afferent inputs from the periphery, might be expected to suffer greater pain severity. However, there are many factors that influence the presence and severity of pain. Indeed, according to the IASP “Pain is always a personal experience that is influenced to varying degrees by biological, psychological, and social factors” [30].

Unlike in rodents where impairments in HRDD are more uniform, considerable variance of HRDD was seen in our previous study and current cohort of patients with painful diabetic neuropathy and indeed, a proportion of patients with painful diabetic neuropathy did not demonstrate impaired HRDD. This is likely to reflect the complex etiology of painful neuropathy mediated at multiple peripheral, spinal and supraspinal levels.

We propose that HRDD is a biomarker of a pain mechanism in painful diabetic neuropathy rather than of pain *per se*. In this respect, within our cohort there will likely also be patients with painful diabetic neuropathy in whom the pain is due to an alternative dominant mechanism that could cause pain of equivalent severity [31]. Therefore, across a group of patients with pain due to multiple and different dominant mechanisms the lack of correlation is not that surprising. Indeed, in previous mechanistic studies addressing the role of deficient descending pain modulation in painful diabetic neuropathy, although abnormalities in conditioned pain modulation (CPM) predicted the response to the selective serotonin-noradrenaline reuptake inhibitor Duloxetine, they did not correlate with pain severity [6].

Therefore, this novel translational observation raises the intriguing possibility that HRDD could be used, either in the clinic or the setting of a clinical trial, to identify or stratify patients with painful neuropathy driven predominantly by impaired spinal inhibition who may respond preferentially to medications, such as duloxetine, which target spinal inhibition. Indeed, the absence of mechanism specific stratification may well explain the relatively modest outcomes in most clinical trials of drugs for painful diabetic neuropathy [32].

Testing of HRDD is unlikely to be applicable to all patients, particularly those with severe diabetic neuropathy because of the increased likelihood of a severely attenuated or absent H-reflex. However, HRDD assessment is applicable for 75-80% of patients with diabetes. Painful symptoms are reported in up to a third of patients with mild and moderate diabetic neuropathy as well as in approximately one quarter of patients with diabetes without confirmed neuropathy[1].

Enhanced HRDD was observed in a sub-group of the patients with diabetes without pain, such that mean HRDD for the entire group was significantly higher than controls. This sub-group shared similar indices of peripheral neuropathy with the sub-group of diabetic patients with pain and most dramatic loss of HRDD and was also indistinguishable from subjects with diabetes, no pain and normal HRDD. It is well documented that spinal nociceptive transmission can be reduced by engagement of inhibitory pathways in the descending pain modulatory system (DPMS) [33]. Conversely, loss of endogenous pain suppression due to a reduced capacity for inhibition and/or enhanced facilitation in DPMS pathways has been implicated in diverse pain states, including painful diabetic neuropathy [6, 34-36]. Whether the dynamic alterations in the DPMS that either enhance or suppress inhibition in the spinal cord are linked to the mechanisms underlying painful diabetic neuropathy related spinal inhibitory dysfunction and HRDD requires further study.

Our novel observation of enhanced HRDD in some subjects with painless diabetic neuropathy raises the possibility that spinal inhibitory systems, as reflected by HRDD, can also be augmented to suppress peripheral nociceptive inputs that may otherwise cause pain. It will be of interest to assess whether the patients with no pain but exaggerated HRDD show peripheral hyperexcitability or spontaneous activity, electrophysiological indices that have been linked to neuropathic pain in some patients [37].

A potential limitation of the study is that the control group were significantly younger than the patient groups with diabetes. Whilst this has potential to impact the significance of neuropathy parameters between patients with diabetes and control subjects, the patient cohorts with and without pain were well matched for age. Furthermore, there was no significant correlation between age and HRDD and the findings for HRDD between patients with and without pain and controls were highly significant following adjustment for age using analysis of covariance (ANCOVA). We also acknowledge this is a cross sectional study comprised of relatively small cohorts of patients. A small proportion of patients were taking anti-neuropathic pain medication and we did not evaluate the effect of drugs on either HRDD or VAS pain scores.

In conclusion we show reduced and enhanced HRDD in patients with and without painful diabetic neuropathy, respectively which was not associated with the presence or severity of diabetic neuropathy. Prospective and pharmacological intervention studies are required to systematically address the utility of HRDD to target therapies in the clinic and for trial enrichment in clinical trial of new therapies for painful diabetic neuropathy.

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Conflict of Interest.

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**References**

1. Abbott, C.A., et al., *Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K.* Diabetes Care, 2011. **34**(10): p. 2220-4.

2. Zelman, D.C., N.A. Brandenburg, and M. Gore, *Sleep impairment in patients with painful diabetic peripheral neuropathy.* Clin J Pain, 2006. **22**(8): p. 681-5.

3. Gore, M., et al., *Pain severity in diabetic peripheral neuropathy is associated with patient functioning, symptom levels of anxiety and depression, and sleep.* J Pain Symptom Manage, 2005. **30**(4): p. 374-85.

4. Finnerup, N.B., et al., *Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis.* Lancet Neurol, 2015. **14**(2): p. 162-73.

5. Smith, S.M., et al., *The Potential Role of Sensory Testing, Skin Biopsy, and Functional Brain Imaging as Biomarkers in Chronic Pain Clinical Trials: IMMPACT Considerations.* J Pain, 2017. **18**(7): p. 757-777.

6. Yarnitsky, D., et al., *Conditioned pain modulation predicts duloxetine efficacy in painful diabetic neuropathy.* Pain, 2012. **153**(6): p. 1193-8.

7. Demant, D.T., et al., *The effect of oxcarbazepine in peripheral neuropathic pain depends on pain phenotype: a randomised, double-blind, placebo-controlled phenotype-stratified study.* Pain, 2014. **155**(11): p. 2263-73.

8. Basbaum, A.I., et al., *Cellular and molecular mechanisms of pain.* Cell, 2009. **139**(2): p. 267-84.

9. Jolivalt, C.G., et al., *Allodynia and hyperalgesia in diabetic rats are mediated by GABA and depletion of spinal potassium-chloride co-transporters.* Pain, 2008. **140**(1): p. 48-57.

10. Lee-Kubli, C.A. and N.A. Calcutt, *Altered rate-dependent depression of the spinal H-reflex as an indicator of spinal disinhibition in models of neuropathic pain.* Pain, 2014. **155**(2): p. 250-60.

11. Malmberg, A.B., et al., *Impaired formalin-evoked changes of spinal amino acid levels in diabetic rats.* Brain Res, 2006. **1115**(1): p. 48-53.

12. Marshall, A.G., et al., *Spinal Disinhibition in Experimental and Clinical Painful Diabetic Neuropathy.* Diabetes, 2017.

13. Lee-Kubli, C., et al., *The H-Reflex as a Biomarker for Spinal Disinhibition in Painful Diabetic Neuropathy.* Curr Diab Rep, 2018. **18**(1): p. 1.

14. Burke, D., S.C. Gandevia, and B. McKeon, *The afferent volleys responsible for spinal proprioceptive reflexes in man.* J Physiol, 1983. **339**: p. 535-52.

15. Burke, D., S.C. Gandevia, and B. McKeon, *Monosynaptic and oligosynaptic contributions to human ankle jerk and H-reflex.* J Neurophysiol, 1984. **52**(3): p. 435-48.

16. Boulenguez, P., et al., *Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury.* Nat Med, 2010. **16**(3): p. 302-7.

17. Ishikawa, K., et al., *Low frequency depression of the H wave in normal and spinal man.* Exp Neurol, 1966. **15**(1): p. 140-56.

18. Matsushita, A. and C.M. Smith, *Spinal cord function in postischemic rigidity in the rat.* Brain Res, 1970. **19**(3): p. 395-410.

19. Bos, R., et al., *Activation of 5-HT2A receptors upregulates the function of the neuronal K-Cl cotransporter KCC2.* Proc Natl Acad Sci U S A, 2013. **110**(1): p. 348-53.

20. Laakso, M., *Biomarkers for type 2 diabetes.* Mol Metab, 2019. **27s**: p. S139-s146.

21. Tesfaye, S., et al., *Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments.* Diabetes Care, 2010. **33**(10): p. 2285-93.

22. Malik, R.A., et al., *Small fibre neuropathy: role in the diagnosis of diabetic sensorimotor polyneuropathy.* Diabetes Metab Res Rev, 2011. **27**(7): p. 678-84.

23. Treede, R.D., et al., *Chronic pain as a symptom or a disease: the IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11).* Pain, 2019. **160**(1): p. 19-27.

24. Dworkin, R.H., et al., *Considerations for improving assay sensitivity in chronic pain clinical trials: IMMPACT recommendations.* Pain, 2012. **153**(6): p. 1148-58.

25. Tavakoli, M. and R.A. Malik, *Corneal confocal microscopy: a novel non-invasive technique to quantify small fibre pathology in peripheral neuropathies.* J Vis Exp, 2011(47).

26. Sierra-Silvestre, E., et al., *Altered pain processing in patients with type 1 and 2 diabetes: systematic review and meta-analysis of pain detection thresholds and pain modulation mechanisms.* BMJ Open Diabetes Res Care, 2020. **8**(1).

27. Kalteniece, A., et al., *Corneal confocal microscopy detects small nerve fibre damage in patients with painful diabetic neuropathy.* Sci Rep, 2020. **10**(1): p. 3371.

28. Trujillo-Hernandez, B., et al., *F-wave and H-reflex alterations in recently diagnosed diabetic patients.* J Clin Neurosci, 2005. **12**(7): p. 763-6.

29. Millan-Guerrero, R., et al., *H-reflex and clinical examination in the diagnosis of diabetic polyneuropathy.* J Int Med Res, 2012. **40**(2): p. 694-700.

30. Raja, S.N., et al., *The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises.* Pain, 2020. **161**(9): p. 1976-1982.

31. Calcutt, N.A., *Diabetic neuropathy and neuropathic pain: a (con)fusion of pathogenic mechanisms?* Pain, 2020. **161**(Suppl 1): p. S65-S86.

32. Alam, U., G. Sloan, and S. Tesfaye, *Treating Pain in Diabetic Neuropathy: Current and Developmental Drugs.* Drugs, 2020. **80**(4): p. 363-384.

33. Lockwood, S. and A.H. Dickenson, *What goes up must come down: insights from studies on descending controls acting on spinal pain processing.* J Neural Transm (Vienna), 2020. **127**(4): p. 541-549.

34. Yarnitsky, D., *Conditioned pain modulation (the diffuse noxious inhibitory control-like effect): its relevance for acute and chronic pain states.* Curr Opin Anaesthesiol, 2010. **23**(5): p. 611-5.

35. Segerdahl, A.R., et al., *A brain-based pain facilitation mechanism contributes to painful diabetic polyneuropathy.* Brain, 2018. **141**(2): p. 357-364.

36. Niesters, M., et al., *Tapentadol potentiates descending pain inhibition in chronic pain patients with diabetic polyneuropathy.* Br J Anaesth, 2014. **113**(1): p. 148-56.

37. Serra, J., et al., *Effects of a T-type calcium channel blocker, ABT-639, on spontaneous activity in C-nociceptors in patients with painful diabetic neuropathy: a randomized controlled trial.* Pain, 2015. **156**(11): p. 2175-83.

**Table 1.** Demographic and neuropathy parameters for patients with type 1 and type 2 diabetes and control subjects.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Patients with Type 1 diabetes (n=37) | Patients with Type 2 diabetes (n=67) | Controls (n=34) |
| Gender (Female/Male) | 12/25 | 24/43 | 20/14 |
| Ethnicity (White/Asian/Black) | 34/2/1 | 44/19/4 | 25/7/2 |
|  | Median ± Interquartile Range |  |  |
| Age (years) | 51.0 (42.5-65) ++++ | 66.0 (60-73.75) \*\*\*\* | 46.5 (31-55) |
| Duration (years) | 17.0 (7.5-34.5) + | 15.0 (7.25-19) |  |
| HbA1c % | 7.2 (6.7-8.4) | 7.3 (6.6-8.1) | 5.4 (4.9-5.7) |
| HbA1c (mmol/mol) | 55.5 (49.25-68) \*\*\*\* | 56.0 (49-64.5) \*\*\*\* | 35.0 (30.5-38.75) |
| BMI (kg/m²) | 25 (23-31.66) ++ | 29.2 (26.5-34.08) \*\*\*\* | 23.8 (22.5-25.4) |
| VAS Current | 20 (8-46.5) |  |  |
| VAS Average 24hrs | 35.5 (15.8-64.5) |  |  |
| VAS Worst 24hrs | 56 (30-80) |  |  |
| SNAP (µV) | 9.1 (4.9-16) \*\* | 6.5 (3.6-12) \*\*\*\* | 17.0 (15-22) |
| SNCV (m/s) | 41.2 (40-46.7) \*\*\*\* | 43.8 (40-46.7) \*\*\* | 48.3 (45.2-51.9) |
| PMNAP (mV) | 4.2 (2.25-6.3) | 3.3 (2.25-4.9) \*\* | 4.9 (3.4-7.5) |
| PMNCV (m/s) | 41.3 (38-44.5) \*\*\*\* | 41.4 (38.4-43.7) \*\*\*\* | 47.5 (43.4-50) |
| CDT (°C) | 27.8 (23.3-29.6) \*\*\* | 28.0 (24.1-29.9) \*\* | 29.8 (28.5-30.5) |
| WDT (°C) | 40.3 (36.4-46.3) \*\* | 40.0 (37.7-43.6) \*\*\* | 36.3 (34.8-39.3) |
| VDT (0-8) | 7 (4-8) \*\* | 7 (5.3-8) \* | 8 (6.6-8) |
|  | Mean ± SE |  |  |
| CNFD (no.mm²) ^ | 25.73 ± 1.44 \*\* | 24.23 ± 1.05 \*\*\* | 32.36 ± 1.59 |
| CNFL (mm/mm²) ^ | 18.62 ± 1.24 \*\* | 18.72 ± 0.91 \*\* | 25.11 ± 1.35 |
| CNBD (no.mm²) ^ | 51.02 ± 5.45 | 47.58 ± 4.14 | 59.74 ± 6.85 |
| RDD meanH2-10 @ 1 Hz ^ | 40.51 ± 3.46 | 48.79 ± 2.78 | 41.66 ± 3.92 |

*Nonparametric data are median ± interquartile range: Mann Whitney or Kruskal-Wallis with Dunn post hoc test. Parametric data are mean ± SE: ^ANCOVA values adjusted for age (post hoc LSD).\*P < 0.05 compared to controls \*\*P < 0.01 compared to controls \*\*\*P < 0.001 compared to controls \*\*\*\*P < 0.0001 compared to controls. +P<0.05 compared to patients with type 2 diabetes ++P<0.01 compared to patients with type 2 diabetes ++++P<0.0001 compared to patients with type 2 diabetes.*

**Table 2.**  Demographic and neuropathy parameters for patients with diabetes, with and without pain, and control subjects.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Diabetes with Pain (n=42) | Diabetes without pain (n=62) | Controls (n=34) |
| Type of Diabetes (1/2) | 11/31 | 26/36 |  |
| Gender (Female/Male) | 18/24 | 18/44 | 20/14 |
| Ethnicity (White/Asian/Black) | 32/8/2 | 46/13/3 | 25/7/2 |
|  | Median ± Interquartile Range |  |  |
| Age (years) | 61.5 (49.8-69.5) \*\*\* | 65.0 (51.5-71) \*\*\*\* | 46.5 (31-55) |
| Duration (years) | 12.5 (4.8-20.3) | 16.0 (10.0-23.3) |  |
| HbA1c % | 7.0 (6.2-7.5) | 7.5 (6.8-8.4) | 5.4 (4.9-5.7) |
| HbA1c (mmol/mol) | 53.0 (44.5-58) \*\*\* | 58.5 (51-68.25) \*\*\*\* | 35.0 (30.5-38.75) |
| BMI (kg/m²) | 29.2 (25.5-34.9) \*\*\*\* | 27.3 (24.3-31.9) \*\* | 23.8 (22.5-25.4) |
| SNAP (µV) | 7.7 (3.7-15) \*\*\* | 6.9 (4.3-11.5) \*\*\*\* | 17.0 (15-22) |
| SNCV (m/s) | 43.1 (40-46.7) \*\*\* | 42.4 (40-46.7) \*\*\*\* | 48.3 (45.2-51.9) |
| PMNAP (mV) | 3.8 (2.4-5.7) | 3.5 (2.4-5.9) \* | 4.9 (3.4-7.5) |
| PMNCV (m/s) | 41.4 (38.1-43.7) \*\*\*\* | 41.2 (38.6-44) \*\*\*\* | 47.5 (43.4-50) |
| CDT (°C) | 27.8 (23.3-29.6) \*\*\* | 28.0 (24.1-29.9) \*\* | 29.8 (28.5-30.5) |
| WDT (°C) | 40.3 (36.4-46.3) \*\* | 40.0 (37.7-43.6) \*\*\* | 36.3 (34.8-39.3) |
| VDT | 7 (4-8) \*\* | 7 (5.3-8) \* | 8 (6.6-8) |
| VAS Current | 20 (8-46.5) |  |  |
| VAS Average 24hrs | 35.5 (15.8-64.5) |  |  |
| VAS Worst 24hrs | 56 (30-80) |  |  |
|  | Mean ± SE |  |  |
| CNFD (no.mm²) ^ | 23.84 ± 1.84\*\*\* | 25.30 ± 1.31\*\*\* | 32.36 ± 1.59 |
| CNFL (mm/mm²) ^ | 18.16 ± 1.563\*\*\* | 18.43 ± 1.11\*\*\* | 25.11 ± 1.35 |
| CNBD (no.mm²) ^ | 46.64 ± 5.18 | 47.61 ± 4.20 | 59.74 ± 6.85 |
| HRDD meanH2-10 @ 1 Hz ^ | 60.40 ± 2.74 \*\*\* | 34.92 ± 2.33 \* +++ | 43.46 ± 3.31 |

*Non-parametric data are expressed as median ± interquartile range: Mann Whitney or Kruskal-Wallis with Dunn post hoc test. Parametric data are expressed as mean ± SD: ^ANCOVA values adjusted for age (post hoc LSD). \*P < 0.05 compared to controls \*\*P < 0.01 compared to controls \*\*\*P < 0.001 compared to controls \*\*\*\*P < 0.0001 compared to controls. +++P<0.001 compared to painful diabetic neuropathy.*

**Figure 1.** Study flow diagram

Graphical user interface

Description automatically generated with medium confidence

Chart, diagram, schematic

Description automatically generated**Figure 2. A:** Individual HRDD (mean of responses H2-10 as % of H1) at 1Hz in patients with type 1 or type 2 diabetes and control subjects. Patients reporting painful symptoms are shown in red. The dotted line represents ± 2 standard deviations of the mean value of control subjects. **B:** Individual HRDD (mean of responses H2-10 as % of H1) at 1Hz in control subjects (black circle), patients with diabetic neuropathy with pain (red circle/black outline), patients without diabetic neuropathy with pain (black circle/red outline), patients with diabetic neuropathy without pain (green circle/ black outline) and patients without diabetic neuropathy without pain (black circles/green outline). Statistically significant differences were identified with one-way ANOVA followed by Tukey’s multiple comparisons test; \*\*p<0.01, \*\*\*p<0.001 and \*\*\*\*p<0.0001. **C:** Group mean H wave amplitude responses to stimulus train at 1Hz in control subjects (black circle/black line), patients with painful diabetic neuropathy (red circle/red line) and patients with painless diabetic neuropathy (green circle/green line). **D:** Individual HRDD (mean of responses H2-10 as % of H1) at 1Hz in control subjects, patients with painful diabetic neuropathy and patients with painless diabetic neuropathy. Statistically significant differences were identified with one-way ANOVA followed by Tukey’s multiple comparisons test; \*p<0.05, \*\*\*p<0.001 and \*\*\*\*p<0.0001. Neuropathic pain medication: Duloxetine (cyan), Gabapentin/Pregabalin (yellow), SSRI (blue), Amitriptyline (orange). Tricyclic (purple).