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Reduced vitamin D levels in painful diabetic peripheral neuropathy

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Abstract

Aim Recent studies have reported an association between low vitamin D levels and diabetic peripheral neuropathy. However, many of these did not differentiate between people with painful diabetic peripheral neuropathy and those with painless diabetic peripheral neuropathy, or assess major confounding factors including sunlight exposure and daily activity. Our study addressed these limitations and evaluated vitamin D levels in people with carefully phenotyped diabetic peripheral neuropathy and controls.

Methods Forty-five white Europeans with Type 2 diabetes and 14 healthy volunteers underwent clinical and neurophysiological assessments. People with Type 2 diabetes were then divided into three groups (17 with painful diabetic peripheral neuropathy, 14 with painless diabetic peripheral neuropathy and 14 with no diabetic peripheral neuropathy). All had seasonal sunlight exposure and daily activity measured, underwent a lower limb skin biopsy and had 25-hydroxyvitamin D measured during the summer months, July to September.

Results After adjusting for age, BMI, activity score and sunlight exposure, 25-hydroxyvitamin D levels (nmol/l) (SE) were significantly lower in people with painful diabetic peripheral neuropathy [painful diabetic peripheral neuropathy 34.9 (5.8), healthy volunteers 62.05 (6.7), no diabetic peripheral neuropathy 49.6 (6.1), painless diabetic peripheral neuropathy 53.1 (6.2); ANCOVA $P = 0.03$]. Direct logistic regression was used to assess the impact of seven independent variables on painful diabetic peripheral neuropathy. Vitamin D was the only independent variable to make a statistically significant contribution to the model with an inverted odds ratio of 1.11. Lower 25-hydroxyvitamin D levels also correlated with lower cold detection thresholds ($r = 0.39$, $P = 0.02$) and subepidermal nerve fibre densities ($r = 0.42$, $P = 0.01$).

Conclusions We have demonstrated a significant difference in 25-hydroxyvitamin D levels in well-characterized people with painful diabetic peripheral neuropathy, while accounting for the main confounding factors. This suggests a possible role for vitamin D in the pathogenesis of painful diabetic peripheral neuropathy. Further prospective and intervention trials are required to prove causality between low vitamin D levels and painful diabetic peripheral neuropathy.

Introduction

Painful diabetic peripheral neuropathy (painful-DPN) affects up to a quarter of all people with diabetes and can lead to significant curtailment of quality of life [1]. People with painful-DPN can present with various degrees of unremitting

burning, aching and 'electric shock' pains in their feet and legs [2]. Night-time exacerbations and contact hypersensitivity to bed clothes result in loss of sleep, and painful-DPN can be disabling. The pathophysiology of painful-DPN is poorly understood, and there are no universally accepted disease modifying treatments for DPN [3]. The mainstay of treatment is symptom control with pharmacotherapy, which has limited efficacy and often dose-limiting side-effects [4].

A recent meta-analysis of six observational studies found an association between serum vitamin D levels and DPN in people with Type 2 diabetes [5]. A number of clinical and

What's new?

- Although there have been consistent associations between vitamin D levels and diabetic peripheral neuropathy, many studies did not differentiate between people with painful and painless diabetic peripheral neuropathy, assess major confounding factors including seasonal sunlight exposure and daily activity, and lacked detailed assessment of peripheral neuropathy including measurement of skin intra-epidermal nerve density.

- Our study addressed these limitations and found a significant reduction in 25-hydroxyvitamin D levels in well-characterized people with painful diabetic peripheral neuropathy.

- Further studies including prospective and intervention trials are required to prove the causality between low vitamin D levels and painful diabetic peripheral neuropathy. If causality is confirmed, this will have a significant impact on clinical practice as there would be a clear rationale for early screening and treatment for low vitamin D in people with painful diabetic neuropathy.

animal studies have also examined the role of vitamin D in the pathogenesis of DPN. Although the findings have been inconsistent and occasionally contradictory, they have linked serum vitamin D levels with DPN [6,7]. The mechanisms that underpin the associations between vitamin D levels and DPN are not fully understood [6,7]. Pain sensitivity associated with low vitamin D levels may be directly mediated by its action on the dorsal root ganglia, specifically small nerve fibres [8]. Vitamin D deficiency has also been linked to hypertension [9], dysglycaemia [10] and dyslipidaemia [11] that have been implicated as independent risk factors for DPN [12].

Many studies reporting an association between low vitamin D levels and DPN did not differentiate between people with painful-DPN and painless-DPN or assess small fibre function and morphology on skin biopsy. Moreover, studies did not evaluate major confounding factors including seasonal sunlight exposure and daily activity. Vitamin D may have differing aetiological roles in painful- and painless-DPN subtypes and there is thus a clear rational for measuring vitamin D levels in carefully phenotyped people with DPN, while accounting for potential confounding factors.

Methods

Study design and participants

Fifty-nine white Europeans [without diabetes, *n*=14; Type 2 diabetes with no neuropathy (no-DPN), *n*=14; painless-DPN, *n*=14; and painful-DPN, *n*=17] participated in this study. People with diabetes were recruited from the Sheffield Teaching Hospitals NHS Foundation Trust diabetes database and outpatient clinics between August 2013 and September 2014. All had Type 2 diabetes diagnosed according to World Health Organization (WHO) criteria. Exclusion criteria included: non-diabetic neuropathies, history of alcohol consumption of > 24 units a week, diabetic neuropathies other than DPN, neurological or other systemic disorders. People on either maintenance or high-dose vitamin D supplementation were excluded, as were those using sunbeds. All participants gave written, informed consent before participation in the study, which had prior Ethics Approval by the Sheffield Research Ethics Committee (Study Number STH15701).

Neuropathy assessment

All those taking part in the study underwent: (1) evaluation of the Douleur Neuropathique-4 (DN4) questionnaire [13], the painful-DPN group only also completed the Neuropathic Pain Symptom Inventory; (2) Neuropathy Impairment Score Lower Limb [NIS(LL)] to assess clinical peripheral neurological status [14]; (3) cardiac autonomic function tests using the O'Brien protocol [15], which included a total of five measurements (heart rate variability during supine rest, standing, deep breathing, Valsalva manoeuvre and postural drop in blood pressure); (4) nerve conduction studies of the sural, common peroneal and tibial nerves at a stable skin temperature of 31 °C and a room temperature of 24 °C, using a Medelec electrophysiological system (Synergy Oxford Instruments, Oxford, UK); and (5) detailed quantitative sensory testing to assess large and small fibre function according to the German Research Network on Neuropathic Pain (DFNS) protocol [16] utilizing the Medoc TSA 2 Neurosensory analyser (Medoc Ltd., Ramat Yishai, Israel). The DFNS protocol includes seven tests measuring 13 parameters. This extended quantitative sensory testing protocol assesses the function of various somatosensory modalities corresponding to specific receptors, peripheral nerve fibres and their central pathways [17].

An overall neuropathy composite score of NIS(LL)+7 was obtained by combining the NIS(LL) plus seven tests of nerve function [18]. This is a validated, composite measure of neuropathy severity that has been used in epidemiological and population-based studies. Based on these assessments and the DN4 questionnaire score, people with Type 2 diabetes were divided into three groups: (1) no-DPN, people with Type 2 diabetes who were asymptomatic with normal clinical and neurophysiological assessments; (2) painless-DPN; comprising people with distal symmetrical polyneuropathy who were pain-free [18]; and (3) painful-DPN, people with painful

neuropathic symptoms involving the feet and or legs in a distal symmetrical fashion together with evidence of peripheral neuropathy[18].

Sunlight exposure and activity measurement

All those taking part in the study completed a validated sunlight exposure and activity questionnaire [19]. This had been measured for each month over the preceding 12 months (Appendix S1) [19]. The final sunlight exposure score was calculated based on: which parts of the body were exposed as a percentage of total body surface area using the 'rule of nine' (face 9%, plus hands 4%, plus arms 18%, plus legs 36%, plus some or all trunk 36% (Appendix S2); and duration of outdoor exposure ('seldom', 10 min; 'occasionally', 20 min; 'often', 80 min).

Vitamin D assay

Vitamin D is a fat-soluble steroid precursor that is mainly produced in skin by direct exposure to sunlight. The two most important forms of vitamin D are cholecalciferol (D3) and ergocalciferol (D2). The human body cannot produce D2 but is ingested in fortified foods or supplements. Vitamin D is not biologically active and undergoes successive hydroxylation in liver and kidney to form active 1,25-dihydroxyvitamin D. The major storage form is 25-hydroxyvitamin D [25 (OH)D] with levels ~ 1000-fold greater than the active 1,25 dihydroxyvitamin D. Hence 25(OH)D measurement is considered to be adequate to calculate overall vitamin D status.

We used an electrochemiluminescence binding assay for the quantitative determination of total serum 25(OH)D (Roche Diagnostics, USA). Blood samples were collected only during the summer months (July, August and September) to avoid seasonal variation in sunlight exposure. This assay technique has 100% cross reactivity for 25-hydroxyvitamin D3 and 92% cross reactivity for 25-hydroxyvitamin D2. Interassay variability was 7.1% and intra-assay variability was 5.2% at 50 nmol/l and 3.4% at 120 nmol/l.

Skin biopsy procedure

Skin biopsy specimens were obtained from the distal calf (10 cm above the lateral malleolus), in accordance with the guidelines published by the European Federation of Neurological Societies on the use of skin biopsy in the diagnosis of peripheral neuropathy [20]. The biopsy was fixed in fresh periodate–lysine–paraformaldehyde (2%) for 12–24 h. Tissue was then washed in 0.1 M phosphate buffer and stored for 2–3 days in 15% sucrose in 0.1 M phosphate buffer. After embedding in OCT (Fisher Scientific UK Ltd), the tissue was snap-frozen and stored at −80 °C. Section (15 μm) sections were cut with a freezing microtome for assessment of intra-epidermal and sub-epidermal nerve fibre density. Sections were incubated overnight with primary antibodies

to the structural nerve marker PGP9.5 (1 : 40 000; Ultraclone, Wellow, UK), detected using avidin–biotin peroxidase methods (Vector Laboratories, Peterborough, UK) giving black, positive immunostaining as described previously [21]. Tissue sections were counterstained for nuclei in 0.1% w/v aqueous neutral red. Omission of primary antibodies and sequential dilution of antibodies gave appropriate results for specificity.

Intra-epidermal nerve fibres were counted along the length of four non-consecutive sections as described and validated previously [22]. The length of epithelium in each counted section was measured using computerized microscopy software (Olympus ANALYSIS 5.0 Soft, Olympus UK, Southend, UK) and results expressed as fibres/mm length of section. Sub-epidermal nerve fibres were measured by image analysis where digital photomicrographs were captured via video link to an Olympus BX50 microscope with a depth of 200 μm below the basal epidermis [22]. The grey-shade detection threshold was set at a constant level to allow detection of positive immunostaining and the area of highlighted immunoreactivity was obtained as a percentage (% area) of the field scanned. Images were captured (×40 objective magnification) along the entire length and the mean values were used for statistical analysis. Quantification was performed by two independent blinded observers and there was no significant difference between observers.

Statistical analysis

All analyses were performed using the statistical package SPSS Version 24.0 (SPSS, IBM Corp, Armonk, NY, USA). Baseline characteristics were described as mean and standard deviations (SD) and percentages for categorical variables. One-way analysis of variance (ANOVA) was used to compare mean of baseline characteristics and post hoc analysis to make comparisons between the group means. Analysis of covariance (ANCOVA) was performed to examine differences in mean serum vitamin D levels between the study groups using sunlight exposure score, activity score, age and BMI as covariates. Direct logistic regression was performed and odds ratio calculated to assess the impact of various independent factors on painful-DPN. We also examined the relationship between serum vitamin D levels and demographic variables, markers of DPN severity, pain severity, intra-epidermal nerve fibre density and autonomic function scores using Pearson's correlation for parametric variables, and Spearman's rank correlation for non-parametric variables.

Results

Table 1 summarizes demographic details and study assessments performed for each group. DPN groups had higher BMI (ANOVA *P*=0.02) and were older (*P*=0.009) compared with healthy volunteers and the no-DPN group. There were

Table 1 Demographic characteristics, sunlight exposure and activity scores

	Healthy volunteer	No DPN	Painless DPN	Painful DPN	Post hoc P-value comparing painful and painless DPN
n	14	14	14	17	
Age (years)	52·1 (11·8)	55·4 (8·2)	63·2 (8·4)	61·8 (9·7)	0·70
BMI (kg/m^2)	26·1 (4·6)	30·1 (6·7)	31·1 (5·5)	32·8 (5·6)	0·41
Diabetes duration (years)*	–	6.5 (6.0)	14·5 (7·0)	15·0 (22·0)	0·70
HbA1c (mmol/mol)	–	62 (21)	62 (6)	67 (17)	0·43
HbA1c (%)		7.9 (1.9)	7.9 (0.6)	8.3 (1.6)	
Total cholesterol (mmol/l)*	–	4·1 (1·4)	3·2 (0·8)	4·0 (1·6)	0·01
Urine ACR (mg/mmol)*	–	0·6 (0·9)	4·1 (7·2)	3·3 (30·1)	0·14
eGFR (ml/min/1.73m^2)*	-	82 (17.5)	77.0 (23·5)	69.5 (22·5)	0·38
MAP (mmHg)	91·1 (12·8)	101·49 (7·4)	95 (11·0)	102 (10·7)	0·09
Sunlight exposure score	347 (176)	305 (125)	384 (149)	389 (296)	0·95
Activity score*	11.0 (4.0)	11.0 (3.0)	10·0 (4.0)	10 (4.0)	0·31

Scores are given as mean (SD), except *median (i.q.r.).
DPN, diabetic peripheral neuropathy; ACR, albumin creatinine ratio; eGFR, estimated glomerular filtration rate; MAP, mean arterial pressure

Table 2 Neuropathy parameters

	Healthy volunteers	No DPN	Painless DPN	Painful DPN	Post hoc P-value comparing painful and painless DPN
NISLL+7 score	0·9 (1·3)	1·5 (1·1)	19 (18·6)	25·5 (13·0)	0·03
DN4 score	0 (0)	0·21 (0·5)	1·9 (0·8)	5·8 (1·7)	<0·01
CDT (°C)	28·3 (1·7)	25·7 (2·8)	20·6 (11·3)	11·2 (10·3)	<0·01
WDT (°C)	38·2 (3·4)	39·3 (3·6)	46·2 (3·3)	47·0 (2·8)	0.50
TSL (°C)	10·6 (5·7)	15·7 (8·5)	30·8 (12·1)	38·5 (11·4)	0.03
IENFD (fibres/mm)	5·7 (1·4)	6·2 (3·1)	0.85 (1.6)	0.77 (1.7)	0.91
SENFD (% area)	1·1 (0·5)	1·9 (1·0)	0·43 (0·6)	0·16 (0·2)	0.28
O'Brien autonomic function score	0 (0)	0.15 (0.55)	1·3 (1.3)	1·6 (2.2)	0.50
HRV with deep breath	1.4 (0.09)	1.4 (0.17)	1.19 (0.07)	1.19 (0.09)	0.97

Values are given as mean (SD).
DPN, diabetic peripheral neuropathy; NISLL+7, Neuropathy Impairment Score of the Lower Limb plus 7 tests of nerve function; DN4, Douleur Neuropathique-4 painful neuropathy score; CDT, cooling detection threshold; WDT, warm detection threshold; TSL, thermal sensory limen; IENFD, intra-epidermal nerve fibre density; SENFD, sub-epidermal nerve fibre density; HRV, heart rate variability.
*ANOVA $P < 0.01$; post-hoc analysis revealed no significant differences in mean neurophysiological measures between healthy volunteers and no-DPN, ($P > 0.05$), except for SENFD, ($P = <0.01$). Significant differences in post-hoc analysis were seen between healthy volunteers and the no-DPN with neuropathy groups (painless- and painful-DPN; $P < 0.01$).

no significant differences in estimates of sunlight exposure ($P=0.65$) or outdoor activity between study groups ($P=0.46$; Table 1). The DPN groups had longer duration of diabetes but there was no significant difference in HbA$_{1c}$ between them ($P=0.63$). Table 2 shows clinical, neurophysiological and skin biopsy data for the different groups. Neuropathy groups had significantly high NIS(LL)+7 scores and very low intra-epidermal nerve fibre density ($P<0.01$) indicating well established DPN. Quantitative sensory testing parameters were abnormal in the neuropathy groups; however, the cold detection threshold was significantly lower in the painful-DPN compared with the painless-DPN group.

After adjusting for age, BMI, activity score and sunlight exposure, 25(OH)D levels (nmol/l) (SE) were significantly lower in painful-DPN [34.9 (5.8), healthy volunteers 62.05 (6.7), no-DPN 49.6 (6.1), painless-DPN 53.1 (6.2); ANCOVA $P=0.03$; Fig. 1]. Pairwise comparisons revealed main group difference between painful-DPN vs. painless-DPN ($P = 0.02$) and painful-DPN vs. healthy volunteers ($P = 0.002$). Direct logistic regression was performed to assess the impact of seven independent variables (age, BMI, sunlight exposure score, activity score, diabetes duration, mean arterial blood pressure and vitamin D level) on painful-DPN. The full model containing all predictors was statistically significant [x^2 (7, $N = 40$) = 17.3, $P = 0.01$]. As shown in Table 3, vitamin D was the only independent variable that made a statistically significant contribution to the model with an inverted odds ratio of 1.11. This suggests that for each unit reduction in vitamin D the odds of painful-DPN increased by a factor of 1.11.

There was also a significant negative correlation between serum 25(OH)D levels and pain scores (DN4; $r = -0.30$, $P = 0.02$; Fig. 2). However, there was no significant correlation between 25(OH)D and nerve conduction studies, markers of large fibre neuropathy. There were significant correlations between 25(OH)D and either cold detection threshold ($r = 0.39$, $P = 0.02$) and thermal sensory limen ($r = -0.34$, $P = 0.04$). There was a significant negative correlation between HbA$_{1c}$ and serum 25(OH)D levels ($r = -0.36$, $P = 0.01$). Autonomic function tests showed significant reduction in total O'Brien score and heart rate variability during deep breath in the neuropathy groups but was reduced equally in both groups (Table 2). Hence pairwise comparison showed, there was no difference between painful- and painless-DPN (total O'Brien autonomic score; post hoc $P = 0.5$ and heart rate variability; $P = 0.97$). There was a positive correlation between heart rate variability during deep breath and vitamin D ($r = 0.29$, $P = 0.03$)
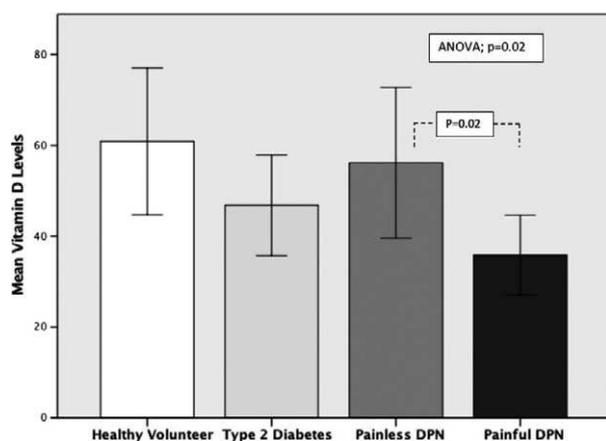
but this was not prominent with a total O'Brien score that includes five autonomic measurements ($r = -0.26$, $P = 0.052$).

Both intra- and sub-epidermal nerve fibres were significantly lower in the painful- and painless-DPN groups compared with no-DPN and healthy volunteers (ANCOVA $P < 0.01$; Table 2; Fig. 3). Because of the well-established DPN and hence severely reduced intra-epidermal nerve fibre counts, we were unable to perform correlation analyses for intra-epidermal nerve fibres, but correlations with sub-epidermal nerve fibres showed a significant positive correlation between sub-epidermal nerve fibre density and 25(OH)D levels ($r = 0.42$, $P = 0.01$).

Discussion

A number of recent studies have reported reduced vitamin D levels in DPN [5] although many of these did not assess major

FIGURE 1 Mean 25(OH)D (nmol/l) levels of groups with 95% confidence interval, showing significant reduction in 25(OH)D in the Painful-DPN group. ANOVA, $P < 0.01$; post-hoc pairwise comparisons showed group differences between Painful-DPN vs Painless-DPN ($P = 0.02$) and Painful-DPN vs HV ($P = 0.002$). ANOVA, analysis of variance; DPN, diabetic peripheral neuropathy.
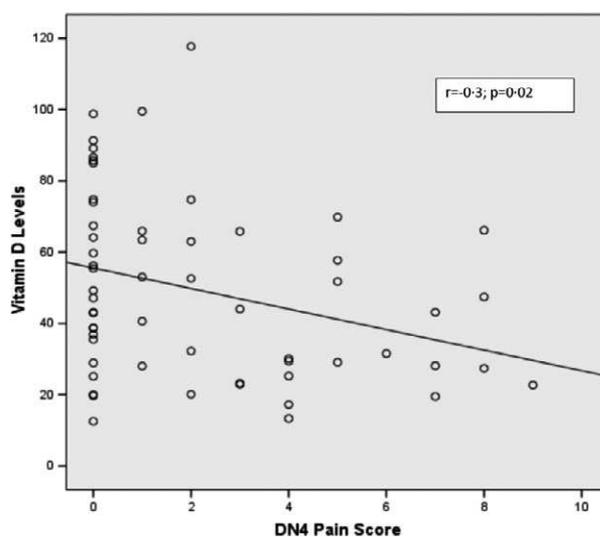

FIGURE 2 Nonparametric correlation between pain score DN4 and vitamin D [25(OH)D] levels (nmol/l) showing significant negative correlation. DPN, diabetic peripheral neuropathy.

Table 3 Logistic regression predicting likelihood of painful-DPN

	B	SE	Wald	P	Odds ratio	95% CI for odds ratio	
						Lower	Upper
Age	0.10	0.06	2.8	0.09	1.11	0.98	1.25
BMI	0.02	0.06	0.13	0.70	1.02	0.89	1.17
Sunlight exposure	0.002	0.002	0.66	0.41	1.00	0.99	1.0
Activity score*	−0.34	0.20	2.7	0.10	1.40	0.94	2.12
Vitamin D*	−0.05	0.02	4.9	0.02	1.11	1.0	1.11
Diabetes duration	0.08	0.05	2.5	0.11	1.08	0.98	1.19
MAP	0.07	0.04	2.9	0.08	1.07	0.98	1.17
Constant	−11.18	6.9	2.6	0.10	0.00		

MAP, mean arterial blood pressure.
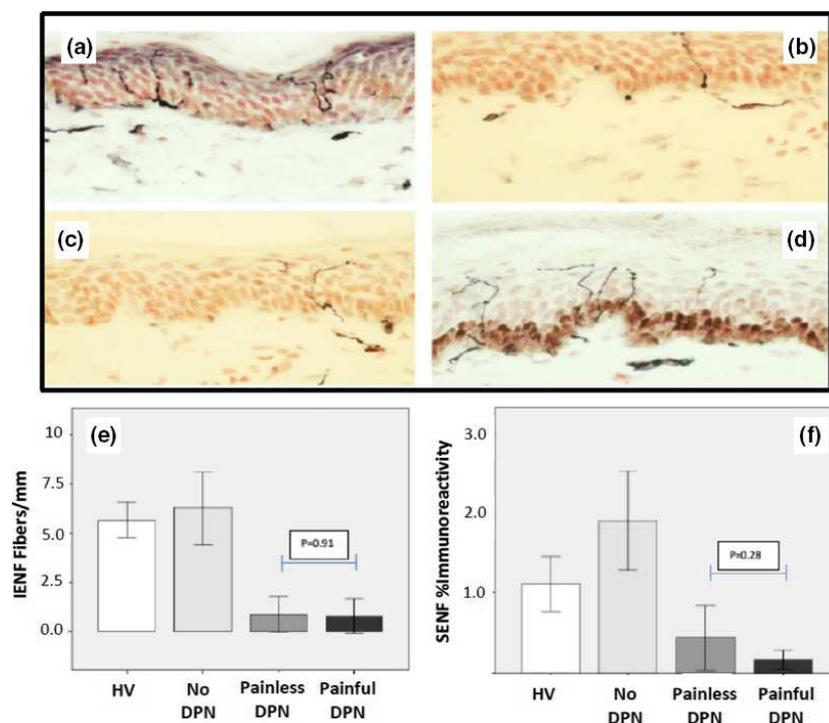*Inverted odds ratio.

FIGURE 3 Skin biopsy showing comparable nerve fibres in healthy volunteer controls (HV, A) and Type 2 diabetes without neuropathy (No-DPN, D). Painful-DPN (B) and Painless-DPN (C) groups had significant and comparable reduction in intra-epidermal (IENFD) and sub-epidermal nerve fibre density (SENFD). Box chart E shows mean IENFD and box chart F shows mean SENFD based on immunoreactivity for PGP9·5 antibody, with 95% confidence interval. Figure E (IENFD) showed ANOVA, $P < 0.01$; but post-hoc pairwise comparisons showed no group differences between Painful-DPN vs Painless-DPN ($P = 0.91$) but both neuropathy groups (Painful and Painless DPN) had significant reduction in IENFD compared to HV ($P = < 0.001$). Figure F (SENFD) ANOVA, $P < 0.01$ and post-hoc pairwise comparisons between Painful-DPN vs Painless-DPN ($P = 0.28$). DPN, diabetic peripheral neuropathy.

confounding factors including seasonal sunlight exposure and daily activity. In addition, most studies did not differentiate between painful-DPN and painless-DPN [23]. There is some evidence, although not consistent, that vitamin D supplementation improves painful neuropathic symptoms [24], suggesting that vitamin D may have a role in the pathogenesis of painful-DPN. Our study was designed to address these limitations by: (1) careful characterization and detailed phenotyping of DPN, using internationally recognized standards; (2) restricting the collection of vitamin D blood samples to during the summer months (July to September); (3) quantifying sunlight exposure and activity levels, using validated questionnaires to estimate exposure to UV radiation; and (4) examining the relationship of vitamin D with measures of small and large nerve fibre function using quantitative sensory testing, skin biopsy and nerve conduction studies.

After adjusting for age, BMI, activity score and sunlight exposure, we demonstrated significantly lower serum 25 (OH)D levels in people with painful-DPN compared with painless-DPN, no-DPN and healthy volunteers. In our study cohort with established DPN, a comprehensive cardiac autonomic function test showed equally reduced scores in both painful- and Painless-DPN, without any statistically significant difference between the groups. Although there was no significant correlation between vitamin D and total

O'Brien autonomic score, there was a trend towards this. Serum 25(OH)D levels were lowest in people with the highest reported pain scores. Because pain and thermal perception are mediated by small sensory fibres, our findings suggest that vitamin D deficiency may have a role in the pathogenesis of small-fibre neuropathy particularly affecting nociceptors, contributing to the development of painful-DPN. This view is supported by the marked reduction in the cold detection threshold, considered to be mediated by Aδ fibres, in the painful-DPN group, and the significant correlation between vitamin D levels and cold detection threshold. Further, there was a significant relationship between sub-epidermal nerve fibre density and vitamin D levels, but not nerve conduction studies. The nerve conduction studies included in the NIS(LL)+7 test are predominantly a measure of large nerve fibre function, whereas skin biopsy is considered to be the gold standard for diagnosing and assessing small fibre neuropathy.

Vitamin D has an important role in promoting nerve growth factor (NGF) secretion. NGF is a target-derived protein that regulates the phenotype and sensitivity of nociceptor fibres, and its deficiency may lead to the development of clinical diabetic small fibre neuropathy [25]. A vitamin D3 derivative, induced NGF and prevented neurotrophic deficits in streptozotocin-diabetic rats

[26]. A recent study has shown positive correlation between vitamin D and serum NGF in people with Type 1 diabetes and neuropathy [27]. A study in rodents reported deactivation of vitamin D by the CYP24A1 enzyme in the presence of glucotoxicity [28]. This may result in decreased vitamin D-mediated NGF secretion, which in turn could lead to a predominantly small nerve fibre neuropathy. We evaluated the relationship between vitamin D and HbA1c and found a significant negative correlation, suggesting higher glucose load was associated with lower vitamin D levels. This suggests a possible mechanistic link between diabetes, vitamin D and neuropathy. The relationship between degeneration of nociceptor fibres and neuropathic pain is complex and future studies will need to examine this further; however, the disproportion between surviving nociceptor fibres and exposure to 'relative excess' of NGF might contribute to neuropathic pain in DPN [25,29].

Limitations of the study include the relatively small cohort size and the cross-sectional design. To minimize the impact of sample size, we carefully characterized and matched people in the different cohorts. The only statistically significant difference between subgroups was higher BMI and age for people in the painful-DPN cohort. This was expected, as age, duration of diabetes and features of the metabolic syndrome are risk factors for DPN in Type 2 diabetes [30]. To address this, we included BMI and age as covariates in the ANCOVA, and still found mean serum 25(OH)D levels were significantly lower in people with painful-DPN. Another limitation is that our neuropathy cohort had advanced neuropathy with high NIS(LL)+7 scores, and a few people had absent intra- and sub-epidermal nerve fibres on distal leg skin biopsy; however, they were similar in number in the painful-DPN and painless-DPN groups.

In conclusion, we have conducted a carefully designed study which included detailed clinical, neurophysiological and skin biopsy assessment of all those taking part, in order to accurately stratify the presence of peripheral neuropathy and neuropathic pain. We also quantified sunlight exposure and levels of outdoor physical activity, potentially important confounding factors that have not been examined in previous studies. Our study included groups of appropriately matched disease controls (Type 2 diabetes with no DPN), and healthy volunteers. The findings suggest a role for vitamin D in painful-DPN compared with painless-DPN. Further long-term prospective cohort or interventional studies are required to examine causality, i.e. if low serum vitamin D levels cause painful-DPN, or if they are a risk factor/surrogate marker for the development of painful-DPN.

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Supporting Information

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

Appendix S1. Sunlight exposure and activity questionnaire.
Appendix S2. Rule of nine explaining percentage of surface area exposed to sunlight.