

Title Page

Small Fibre Pathology in Chronic Whiplash-Associated Disorder: A Cross-Sectional Study

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Significance: Our study found decreased intraepidermal nerve fibre density, reduced dermal innervation, thermal hypoaesthesia and hypersensitivity in people with chronic WAD, suggestive of small fibre pathology. This observation of peripheral nervous system pathology in chronic whiplash provides novel insights on mechanisms underpinning symptoms and challenges commonly held beliefs regarding this condition.

Abstract

Background: Mechanisms underpinning ongoing symptoms in chronic whiplash associated-disorder (WAD) are not well understood. People with chronic WAD can exhibit sensory dysfunction consistent with small nerve fibre pathology, including thermal hypoaesthesia and hyperalgesia. This study investigated small fibre structure and function in chronic WAD.

Methods: Twenty-four people with chronic WAD (median [IQR] age 49 [15] years, 16 females) and 24 pain-free controls (50 [17] years, 16 females) were recruited. Intraepidermal nerve fibre density (IENFD) and dermal innervation were assessed by skin biopsy. This was performed at i) the lateral index finger on the primary side of pain and ii) superior to the lateral malleolus on the contralateral side. Quantitative sensory testing was performed over the hand.

Results: The WAD group exhibited lower IENFD at the finger (WAD: median [IQR] 4.5 [4.9] fibres/mm; control 7.3 [3.9]; $p = 0.010$), but not the ankle (WAD: mean [SD] 7.3 [3.7] fibres/mm; control 9.3 [3.8]; $p = 0.09$). Dermal innervation was lower in the WAD group at the finger (WAD: median [IQR] 3.7 [2.8] nerve bundles/mm²; controls: 4.9 [2.1]; $p = 0.017$) but not the ankle (WAD: median [IQR] 2.1 [1.9] nerve bundles/mm²; controls: 1.8 [1.8]; $p = 0.70$). In the WAD group, hand thermal and light touch detection were impaired, and heat pain thresholds were lowered ($p \leq 0.037$).

Conclusions: Findings suggest small fibre structural and functional deficits in chronic WAD, implicating potential involvement of small fibre pathology.

Key words: whiplash injuries; neck pain; small fibre pathology; quantitative sensory testing; skin biopsy

Abbreviations: DASS = Depression, Anxiety and Stress Scale (-D, -A, -S refer to depression, anxiety and stress subscales); IENFD = intraepidermal nerve fibre density; IES-R = Impact of Events Scale Revised; NDI = Neck Disability Index; NPSI = Neuropathic Pain Symptom Inventory; NRS = Numerical rating scale; PCS = Pain Catastrophizing Scale; QST = quantitative sensory testing; S-LANSS = Self-complete Leeds Assessment of Neuropathic Symptoms and Signs; WAD = whiplash-associated disorder

Main Text

Introduction

Whiplash-associated disorder (WAD), as a consequence of a motor vehicle crash, incurs significant social and economic costs (Connelly & Supangan, 2006; Naumann, Dellinger, Zaloshnja, Lawrence, & Miller, 2010), with around half of injured individuals not fully recovering, and approximately one quarter reporting ongoing moderate to severe pain and disability (Sterling, Hendrikz, & Kenardy, 2010). Current conservative treatments for WAD, such as reassurance, education, exercise and simple analgesics are not very effective, typically showing only small effect sizes (Rushton et al., 2011; Teasell et al., 2010; Wong et al., 2016).

One reason for the modest effect of current treatments for WAD may be due to a lack of understanding of the mechanisms underlying this condition. Chronic WAD is thought to represent a complex interplay of musculoskeletal, psychosocial and neurological factors (Banic et al., 2004; Bogduk, 2011; Kamper et al., 2012; Sterling, 2010; Sterling, Jull, Vicenzino, & Kenardy, 2003). While it is plausible that some form of tissue lesion may occur in the initial injury (Uhrenholt, Grunnet-Nilsson, & Hartvigsen, 2002), routine clinical imaging or electrodiagnostic testing typically does not identify a pathology that explains patients' symptoms (Curatolo et al., 2011; Farrell, Smith, Hancock, Webb, & Sterling, 2019). Nevertheless, sensory impairments such as elevated thermal detection thresholds (Chien, Eliav, & Sterling, 2008b, 2009, 2010; Chien & Sterling, 2010; Raak & Wallin, 2006) and widespread hyperalgesia (Chien et al., 2008b, 2009; Schneider et al., 2010) have been reported in patients with chronic WAD (van Oosterwijck, Nijs, Meeus, & Paul, 2013). The

impairment in thermal detection is consistent with sensory features found in small fibre pathology, which refers to dysfunction and/or degeneration of small sensory nerve fibres (A δ and C fibres) (Schmid, Bland, Bhat, & Bennett, 2014; Üçeyler et al., 2013). Small fibre pathology has been observed in a range of neuropathic pain conditions including carpal tunnel syndrome (Schmid et al., 2014), lumbar radicular pain (Andrasinova et al., 2019) and diabetic neuropathy (Chao et al., 2010). Recent data suggest that small fibre pathology is also a feature of chronic pain conditions, that are not traditionally classified as neuropathic (e.g., fibromyalgia (Grayston et al., 2019)), but its presence in WAD is yet to be investigated.

Small fibre pathology can be assessed using skin biopsies, which allows quantification of intraepidermal nerve fibre density (IENFD) to evaluate structural integrity of small nerve fibres, and by using quantitative sensory testing (QST), which evaluates functional integrity of small nerve fibres (Hoeijmakers, Faber, Lauria, Merkies, & Waxman, 2012; Terkelsen et al., 2017). In chronic WAD, whilst functional changes of small nerve fibres have been reported (Chien et al., 2008b, 2009, 2010; Chien & Sterling, 2010), no study to date has investigated their structural integrity using skin biopsies. If small fibre pathology is present in people with chronic WAD, it could provide an explanation for the thermal hypoaesthesia and hyperalgesia observed in this condition. The primary aim of the present study was to investigate structure and function of small nerve fibres in people with chronic WAD. An exploratory secondary aim was to examine associations between small fibre structure and patients' clinical characteristics.

Methods

Ethics Statement

This study received approval from Griffith University Human Research Ethics Committee and reciprocal approval from The University of Queensland Human Research Ethics Committee. All participants provided written informed consent and the study was conducted in accordance with ethics standards laid down in the Declaration of the Helsinki.

Participants

Participants were recruited through electronic and poster advertising at a university campus, as well as a database of research volunteers of RECOVER Injury Research Centre (Brisbane, Australia), between October 2016 and September 2019.

Individuals interested in participating in the study were screened for eligibility by telephone. Two groups of participants were recruited, a WAD group and a control group. The WAD group comprised individuals with WAD Grade II (neck pain without fracture or explicit neurological deficit apparent on bedside clinical examination [upper limb muscle power, deep tendon reflexes, light touch sensation (Spitzer et al., 1995)]) lasting more than three months. The control group was age- and sex-matched 1:1 to participants in the WAD group (\pm 5 years) and comprised healthy volunteers without a history of neck injury or significant neck pain (lasting more than one week).

For both groups, exclusion criteria comprised known diagnoses of sensory neuropathy, other conditions affecting the upper or lower extremities (e.g. carpal tunnel syndrome, complex regional pain syndrome, osteoarthritis), specific spinal pathology (e.g. malignancy or radiculopathy), or other significant medical conditions (e.g. fibromyalgia, rheumatoid arthritis, diabetes mellitus, systemic lupus erythematosus, HIV/AIDS, multiple sclerosis, cancer). Participants were also excluded if they had a history of cervical spine surgery, were taking anti-coagulant medication (e.g. warfarin), were allergic to lidocaine, or were pregnant.

Participation involved attending a single appointment at a university research laboratory, which included completion of a neurological examination, questionnaires, QST undertaken by a trained researcher, and skin biopsies performed by a medical practitioner.

Clinical Questionnaires

Demographic details including age and sex were recorded for all participants. Duration of symptoms was recorded for participants with WAD, as was neck pain intensity during the preceding week on a 0-10 numerical pain rating scale and the location of pain and paraesthesia using a body chart. All participants completed the following questionnaires: Neck Disability Index (NDI), which assesses neck pain related disability quantified as 0-100%, with higher scores reflecting greater disability, and scores > 28% considered indicative of moderate to severe pain related disability (Sterling et al., 2010; Vernon & Mior, 1991); Pain Catastrophising Scale (PCS) which measures catastrophic thinking regarding pain with 13 Likert scale

questions scored from 0-52, with higher scores representing greater catastrophising (Sullivan, Bishop, & Pivik, 1995) and scores ≥ 30 suggesting clinically relevant catastrophising (Sullivan, 2009); Depression, Anxiety and Stress Scale (DASS 21), which assesses symptoms of depression, anxiety and stress using 21 Likert scale questions comprising three subscales (0-28 each subscale), with higher scores representing greater symptom severity (Antony, Bieling, Cox, Enns, & Swinson, 1998). Moderate, severe or extremely severe symptoms are reflected by scores of ≥ 14 , ≥ 10 and ≥ 19 for depression, anxiety and stress subscales, respectively (Lovibond & Lovibond, 1995). In addition, participants in the WAD group completed the following questionnaires: Self-complete Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS), which identifies neuropathic pain based on self-reported symptoms and signs (scored 0-24), with scores ≥ 12 suggestive of pain of predominately neuropathic origin (Bennett, Smith, Torrence, & Potter, 2005); Neuropathic Pain Symptom Inventory (NPSI), which evaluates severity of different neuropathic pain symptoms, giving a total score 0-100, with higher scores reflecting more severe symptoms (Bouhassira et al., 2004); and Impact of Events Scale Revised (IES-R), which evaluates post-traumatic stress symptoms using 22 Likert scale questions (scored 0-88), with scores ≥ 33 consistent with post-traumatic stress disorder (Creamer, Bell, & Failla, 2003; Weiss, 2007).

Quantitative Sensory Testing

Quantitative sensory testing was performed using the protocol described by the German Research Network on Neuropathic Pain (Rolke, Baron, et al., 2006) by a single assessor (SFF). Testing was undertaken over the palmar side of the hand and

index finger in a cervical (C7) dermatome (Lee, McPhee, & Stringer, 2008). Previous studies have observed sensory changes in this region in chronic WAD (Chien et al., 2009; Chien & Sterling, 2010). For participants in the WAD group, QST was performed on the primary side of their neck pain symptoms, and for control group participants, testing was undertaken on the non-dominant hand as QST parameters do not vary between sides (Rolke, Magerl, et al., 2006). Cold and warm detection thresholds, thermal sensory limen, and cold and heat pain thresholds were performed using a SENSELab Thermotest (Somedic, Horby, Sweden) on the palmar aspect of the hand over the second metacarpophalangeal joint using a 25 × 50 mm thermode. Paradoxical heat sensations were recorded during thermal sensory limen testing. Mechanical detection threshold, mechanical pain threshold, mechanical pain sensitivity, dynamic mechanical allodynia, and wind-up ratio were tested over the palmar aspect of the proximal phalanx of the index finger. Mechanical detection threshold was assessed using SENSELab von Frey hairs (Somedic, Horby, Sweden) and mechanical pain threshold was assessed using weighted pinpricks (MRC Systems GmbH, Heidelberg, Germany). Mechanical pain sensitivity was assessed with a 0-100 numerical pain rating scale using the same weighted pinpricks, and intermixed between these stimulations were light touch stimulations using a cotton wisp, a cotton tip, and a standardised brush (Somedic, Horby, Sweden) to assess dynamic mechanical allodynia. Wind-up ratio was reported as the mean numerical pain rating of three series of ten pinprick stimulations (256 mN) divided by the mean pain rating of three single stimulations. Vibration detection threshold was assessed using a Rydel-Seiffer tuning fork (Armo Bydand, Castle Hill, Australia) at the palmar aspect of the head of the second metacarpal (Schmid et al., 2014). Pressure pain

threshold was tested at the thenar eminence using a SENSELab AB algometer (Somedic, Horby, Sweden) (Baselgia, Bennett, Silbiger, & Schmid, 2017).

QST data were log-transformed in order to produce a normal distribution (with the exceptions of cold pain threshold, heat pain threshold, vibration detection threshold, paradoxical heat sensation and dynamic mechanical allodynia) (Magerl et al., 2010). A small constant of 0.1 was added to mechanical pain sensitivity to avoid loss of data when pain was rated at zero (Rolke, Magerl, et al., 2006). In accordance with Rolke et al. (2006), Z-scores were calculated as $Z = (\text{value}_{\text{patient}} - \text{mean value}_{\text{control group}}) \div \text{standard deviation}_{\text{control group}}$. Positive Z-scores indicate gain of function, and negative Z-scores represent loss of function (Rolke, Magerl, et al., 2006).

Skin Biopsy

Skin punch biopsies (3 mm) were taken from the ventro-lateral aspect of the proximal phalanx of the index finger, as well as from the lateral distal leg approximately 10 cm above the lateral malleolus. These two locations were selected as i) thermal hypoaesthesia and hyperalgesia has been observed in the hand in chronic WAD (Chien et al., 2009; Chien & Sterling, 2010) and the index finger is an established site for assessment of small fibre pathology in the upper extremity (Schmid et al., 2014), and as ii) including the leg location as distal site allowed investigation of a systemic difference in IENFD. Biopsies were performed under sterile conditions by a medical practitioner. Local anaesthesia was achieved by subcutaneous injection of lidocaine (1%, 0.2-0.4 mL) at both finger and leg sites. Each biopsy was fixed in fresh periodate-lysine-paraformaldehyde (2%) for 30

minutes, then washed in 0.1 M phosphate buffer and immersed in 15% sucrose in 0.1 M phosphate buffer for two days at 4°C. Biopsies were then embedded in OCT Compound (Tissue-Tek, Sakura Finetek, Torrance, CA) and frozen at -80°C.

The tissue was sectioned into 50 µm sections using a cryostat and immunohistochemistry was performed using an established free-floating protocol (Doppler, Werner, & Sommer, 2013; Schmid et al., 2014). Sections underwent one hour immersion in 5% fish gelatin and 0.5% Triton® X-100 in phosphate buffered saline (PBS) prior to incubation overnight at 4°C with primary antibody to protein gene product (PGP) 9.5 as a pan-axonal marker (1:200, rabbit polyclonal, cat # 516-3344, Zytomed Systems, Berlin, Germany). The antibody to PGP 9.5 is the recommended primary antibody for quantification of human IENFD (Lauria et al., 2010). Sections were double stained with primary antibody to myelin basic protein (MBP) (1:500, rat monoclonal, cat # ab7349, Abcam, Cambridge, United Kingdom) to denote myelinated nerve fibres. On the second day, the sections were washed in 0.1% Triton® X-100 in PBS, before incubation overnight at 4°C with secondary antibodies (CY™3, 1:1000, anti-rabbit, cat # JI711165152, Jackson Immuno Research, West Grove, PA; Alexa Fluor™ 488, 1:500, anti-rat, cat # A11006, Life Technologies Corporation, Eugene, OR). On the third day, the sections were washed three times in 0.1% Triton® X-100 in PBS, then in PBS alone, followed by mounting on slides in Vectashield H-1200 mounting medium (Vector Laboratories, Burlingame, CA). A negative control run (omission of primary antibodies) was performed on eight sections from two biopsies to confirm the specificity of the primary antibodies for visualisation of nerve fibres.

Measurement of IENFD was undertaken by a single, blinded assessor (SFF) using a Nikon Ti Eclipse wide-field microscope (Nikon Instruments Inc., Melville, NY) and NIS Elements AR 4.30 imaging software (Nikon Instruments Inc., Melville, NY). Counting was performed at 40x magnification down the microscope on three PGP 9.5 stained sections per participant and quantified as fibres/mm, in accordance with the guidelines described by Lauria et al. (2010). Dermal innervation was assessed as described before (Doppler, Werner, Henneges, & Sommer, 2012) by quantifying nerve fibre bundles comprising at least five PGP 9.5⁺ axons, excluding the subepidermal plexus. The percentage of dermal PGP 9.5⁺ axon bundles containing one or more MBP⁺ fibres was also quantified, as per previous studies (Schmid et al., 2014; Üçeyler et al., 2013). Number of Meissner corpuscles per millimetre of epidermis was counted only at the finger (Schmid et al., 2014) as they are not present in hairy skin in the leg.

Statistical Analysis

Data were analysed using Stata 13.1 (StataCorp, College Station, TX) statistical analysis package. The distribution of continuous variables (i.e., questionnaires, QST parameters, IENFD, dermal fibres and Meissner corpuscles) was examined by visual inspection and using Shapiro-Wilk testing. Two-tailed independent t-tests and Wilcoxon rank-sum (Mann-Whitney) testing was used to compare group data (i.e., age, duration of symptoms, questionnaires, QST parameters, IENFD, dermal fibres and Meissner corpuscles), as appropriate to distribution. Effect size was estimated for significant differences using Cohen's d (or r for non-parametric data), interpreted as small ($d = 0.2$, $r = 0.1$), medium ($d = 0.5$, $r = 0.3$) or large ($d = 0.8$, $r = 0.5$) (Fritz &

Morris, 2011). Spearman rho correlations were used to investigate associations between IENFD, dermal innervation and patient clinical characteristics, with Benjamini-Hochberg corrections at 5% false discovery rate. Significance was set at $\alpha \leq 0.05$.

A priori sample size calculation revealed that 21 participants were required in each group to detect a between group difference in IENFD of 3.0 fibres/mm at 90% power, $\alpha \leq 0.05$ and with an estimated standard deviation of 2.9. This was based on a previous study (Schmid et al., 2014) which found a between group difference of 4.2 fibres/mm between people with a peripheral nerve injury (carpal tunnel syndrome) and healthy controls. As a frank nerve lesion is not apparent in WAD Grade II, the present study was conservatively powered to detect a 30% smaller between group difference.

Data Availability

The data that support the findings of this study are available in a de-identified format from the corresponding author with the approval of all contributing authors, upon reasonable request from a qualified investigator.

Results

Demographics, Clinical Information and Questionnaires

Twenty-four people with chronic WAD and 24 pain-free control participants were recruited into the study. Demographic, clinical and questionnaire data for the study groups are presented in Table 1. The duration of symptoms in the WAD group ranged from three months to 44 years (median [IQR] 5 [20] years). While no individuals in the WAD group had explicit neurological deficits based on clinical neurological examination, 10/24 (42%) reported occasional upper limb paraesthesia and 11/24 (46%) reported pain radiating into the upper limb (shoulder, arm, forearm and/or hand) in addition to neck symptoms. WAD group participants reported the following concurrent medical conditions: depression/anxiety (n = 4), atrial fibrillation (n = 2), hypertension (n = 2), hypercholesterolaemia (n = 1), and low back pain (n = 5). Medication use in the WAD group comprised: selective serotonin reuptake inhibitor (n = 3), diazepam (n = 1), ibuprofen (n = 3), diclofenac (n = 1), paracetamol (n = 1), aspirin (n = 4), antihypertensive (n = 2), cholesterol lowering medication (n = 1), and fish oil tablets (n = 2).

Participants in the WAD group scored higher than controls on the NDI, PCS and DASS questionnaires. Based on their NDI scores, 15/24 (63%) participants with WAD were classified as reporting moderate to severe pain related disability, and 6/24 (25%) had S-LANSS scores suggestive of pain predominately of neuropathic origin. Two of 24 (8%) had PCS scores suggesting clinically relevant catastrophising and 5/23 (22%, one non-responder) had IES-R scores consistent with post-traumatic stress disorder. Moderate, severe or extremely severe scores for the DASS 21 were observed in 9/24 (38%), 8/24 (33%) and 8/24 (33%) of WAD group participants for the depression, anxiety and stress subscales, respectively.

Quantitative Sensory Testing

Results of QST for the WAD and control groups can be seen in Figure 1. For three participants (two in the WAD group, one in the control group), wind up ratio could not be calculated due to zero ratings in single stimulations and these data were not included in the analysis (Rolke, Baron, et al., 2006). A technical issue lead to missing thermal testing data in four participants (WAD: n = 2 CPT missing only, n = 1 CDT, WDT, TSL, HPT, CPT missing; controls: n = 1 WDT, TSL, CPT, HPT missing). In two control group participants, WUR, VDT and PPT data were not collected due to time constraints.

Participants in the WAD group had elevated cold (t-test $t = 2.54$, $p = 0.015$, $d = 0.74$) and warm (t-test $t = 3.65$, $p < 0.001$, $d = 1.08$) detection thresholds as well as greater thermal sensory limens (t-test $t = 2.87$, $p = 0.006$, $d = 0.85$), compared with the control group. These represent medium, large and large effect sizes, respectively. Heat pain thresholds were reduced in the WAD group (t-test $t = -2.15$, $p = 0.037$, $d = 0.63$), reflecting a medium effect size. Mechanical detection threshold was also raised in the WAD group compared with the control group (t-test $t = 3.65$, $p < 0.001$, $d = 1.05$), representing a large effect size. Paradoxical heat sensation was reported by one participant with WAD, and no participant reported dynamic mechanical allodynia.

Intraepidermal Nerve Fibre Density

Intraepidermal nerve fibre density was reduced in the WAD group when compared to the control group at the finger but not the ankle (Figure 2). At the index finger, median (IQR) IENFD for the WAD group was 4.5 (4.9) fibres/mm compared with 7.3 (3.9) fibres/mm for the control group (rank-sum $z = 2.58$, $p = 0.010$, $r = 0.37$ [medium effect size]). At the ankle, there was a trend for reduced IENFD in patients with WAD, however this was not statistically significant (WAD group: mean [SD] IENFD 7.3 [3.7] fibres/mm; control group: 9.3 [3.8] fibres/mm; t-test $t = 1.75$, $p = 0.09$). For one male in the control group, ankle IENFD was not assessed due to unsuccessful fixation of the biopsy tissue. No specific immunofluorescent staining was observed in the negative control sections.

Dermal Innervation

At the finger, the density of PGP 9.5⁺ nerve fibre bundles in the dermis was reduced in the WAD group when compared to the control group (WAD: median [IQR] 3.7 [2.8] bundles/mm²; controls: 4.9 [2.1]; rank-sum $z = 2.39$, $p = 0.017$, $r = 0.3537$ [medium effect size]) (Figure 3). The percentage of bundles containing at least one MBP⁺ fibre did not differ between groups (WAD: median [IQR] 90% [23]; controls: 81% [12]; rank-sum $z = -1.88$, $p = 0.06$). Density of Meissner corpuscles did not differ between groups (WAD: median [IQR] 0.41 [0.51] Meissner corpuscles/mm; controls: 0.61 [0.52]; rank-sum $z = 1.39$, $p = 0.16$) (Figure 3).

At the ankle, there were no between group differences in density of PGP 9.5⁺ nerve fibre bundles in the dermis (WAD: median [IQR] 2.1 [1.9] bundles/mm²; controls: 1.8 [1.8]; rank-sum $z = -0.38$, $p = 0.70$) (Figure 3). There were also no between group

differences in the percentage of bundles containing at least one MBP⁺ fibre (WAD: mean [SD] 49% [30]; controls: 54% [22]; t-test $t = 0.65$, $p = 0.52$) (Figure 3).

Correlation of IENFD with Clinical Characteristics

Spearman rho correlations of IENFD and dermal nerve fibre bundle density with patient characteristics can be seen in Table 2. None of the significant correlations survived correction for multiple testing. These data are presented graphically as supplementary material (Figures S1-S4).

Discussion

Our findings provide structural and functional evidence of small fibre pathology in people with chronic WAD when compared to age and sex-matched pain-free controls. IEFND was lower in the WAD group than controls at the index finger on the primary side of neck pain, with a similar trend not reaching significance at the contralateral ankle. Consistent with these structural findings, thermal detection thresholds were impaired in the WAD group, implicating loss of small fibre function. Heat pain thresholds were lower in the WAD group, potentially indicative of concurrent small fibre hyperexcitability. Dermal innervation was largely preserved with sparing of the receptor organs (Meissner corpuscles) and percentage of myelinated axon bundles. However, there was a reduction in PGP 9.5⁺ axon bundles in the finger, which may reflect the identified impairment in mechanical detection thresholds. While there were no significant correlations between clinical

characteristics and IENFD, the identified structural small fibre degeneration combined with the observed changes of small fibre function provide novel insights into neurophysiologic mechanisms involved in WAD. Our findings are of interest especially since routine clinical tests (e.g. magnetic resonance imaging [MRI], electrodiagnostic testing) cannot identify a pathology in these patients (Curatolo et al., 2011).

Reduced IENFD has been observed in several neuropathic pain conditions, such as carpal tunnel syndrome, lumbar radicular pain and diabetic neuropathy (Andrasinova et al., 2019; Chao et al., 2010; Schmid et al., 2014). In contrast, the present study demonstrates reduced IENFD in a common musculoskeletal pain condition without clear neuropathic involvement. There is some indication for elements of nervous system dysfunction in some patients with WAD Grade II (e.g. hypoaesthesia (Chien et al., 2008b, 2009), widespread hyperalgesia (Sterling et al., 2003), hyperexcitable spinal cord reflexes (Sterling, 2010)), and S-LANSS scores inferring a neuropathic component to their pain (Smith et al., 2013). In a recent small study in patients with chronic WAD Grade II, Greening et al. (2018) noted possible peripheral nervous system compromise resulting in increased T2-weighted signal intensity of spinal nerve roots, brachial plexus and distal median nerve apparent on MRI. This was interpreted as presence of intraneural inflammation. Together with our data, this suggests that compromise of the peripheral nervous system may form part of the pathology in some patients with WAD Grade II despite the absence of an explicit neurological lesion on routine clinical tests.

The here identified small fibre deficit in patients with WAD may develop after the trauma or could be pre-existing and therefore predisposes patients to developing chronic pain. Small fibre pathology may arise from metabolic, inflammatory, neurotoxic, infectious, mechanical and genetic factors, or alternately may be insidious in onset (Terkelsen et al., 2017). We carefully excluded patients if they reported relevant concurrent medical conditions making these unlikely to explain reduced IENFD and dermal innervation in our WAD participants. One possible explanation for the observed small fibre pathology may be that whiplash trauma leads to mild injury to peripheral nerve tissue in some patients, sufficient to cause intraneural inflammation and small fibre degeneration, but not severe enough for explicit neuropathy (Greening et al., 2018). Animal models demonstrate that intraneural inflammation can arise through mild neural stretch (Kitamura, Takagi, Yamaga, & Morisawa, 1995) and compression injuries, which are predominantly associated with small rather than large fibre axonal compromise (Schmid, Coppieters, Ruitenbergh, & McLachlan, 2013). This would be consistent with the reported increase in T2 signal in nerve tissue at both proximal (neck, brachial plexus) and distal (median nerve at wrist) sites in patients with chronic WAD Grade II (Greening et al., 2018) and our finding of small fibre pathology at the index finger.

Another consideration may be that small fibre pathology occurs systemically in some people with chronic WAD. Reduced IENFD has been observed across a range of chronic pain conditions, including fibromyalgia (Grayston et al., 2019), central post-stroke pain (Cavalier, Albrecht, Amory, Bernardini, & Argoff, 2016) and Gulf War Illness (Klein, Zirpoli, Downs, & Oaklander, 2018). Further, bilateral reductions in IENFD occur in people with unilateral complex regional pain syndrome (Rasmussen

et al., 2018). These observations suggest that non-specific or systemic reduction in IENFD may be a feature common to some chronic pain conditions. Such a reduction could plausibly arise secondary to an immune-mediated process, possibly related to chronic stress: The association between stress and chronic pain is well-established (Abdallah & Geha, 2017), including in WAD (Walton et al., 2013). Chronic stress is associated with raised systemic inflammatory markers (Hansel, Hong, Camara, & von Kanel, 2010) as well as altered skin immune function (Hunter, Momen, & Kleyn, 2015). Small fibre pathology can be associated with inflammation in the skin, evidenced by increased pro-inflammatory cytokine expression in affected skin in idiopathic small fibre neuropathy (Üçeyler et al., 2010). In our study, while the chronic WAD group reported greater stress than the control group, the observed group difference in IENFD at the ankle showed a trend, but did not reach significance ($p = 0.09$), and the correlation between stress and ankle IENFD ($\rho = -0.43$, $p = 0.03$) did not survive multiple comparisons correction. These data imply that a systemic reduction in IENFD, possibly associated with stress and inflammation, could be feasible however requires further investigation in a larger sample, appropriately powered for correlations and a more subtle group difference in IENFD at the ankle.

Sensory dysfunction has clinical significance in chronic WAD, as thermal hyperalgesia and hypoaesthesia are associated with greater pain and disability in this population (Chien et al., 2010; Sterling et al., 2003). In the present study, consistent with small fibre pathology, thermal detection was impaired in the WAD group. This is in accordance with prior studies of chronic WAD (Chien et al., 2008b, 2009, 2010; Chien & Sterling, 2010) and other conditions associated with small fibre

pathology (Andrasinova et al., 2019; Løseth, Stålberg, Jorde, & Mellgren, 2008; Schley et al., 2012; Schmid et al., 2014; Üçeyler et al., 2013). We also observed lowered heat pain thresholds in the WAD group, consistent with prior findings in chronic WAD (Raak & Wallin, 2006; Scott, Jull, & Sterling, 2005; Sterling et al., 2003). This is seemingly counter-intuitive, but has previously been reported in patients with polyneuropathies, where the majority show a loss of function phenotype, but approximately 30% report thermal hyperalgesia (Baron et al., 2017). Thermal hyperalgesia can also be seen in fibromyalgia (Brietzke et al., 2019; Hurtig, Raak, Kendall, Gerdle, & Wahren, 2001), a condition arguably more akin to chronic WAD than polyneuropathies, which also features reduced IENFD (Grayston et al., 2019). Thermal hyperalgesia in chronic WAD has been attributed to central sensitisation, due to the widespread distribution of hyperalgesia at local (neck) and remote (upper and lower limbs) sites, and presence of hyperalgesia to a variety of noxious stimuli (thermal, mechanical, electrical) (Chien et al., 2008b, 2009; Curatolo et al., 2001; Scott et al., 2005). Our findings offer an additional explanation, as reduced thermal pain thresholds are thought to reflect hyperexcitability of surviving nociceptors in the context of small fibre pathology (Serra et al., 2014), which may represent a mechanism contributing to thermal hyperalgesia in chronic WAD.

Mechanical detection thresholds were impaired in the WAD group, indicating a compromise in low threshold mechanoreceptor function (Rolke, Baron, et al., 2006). This might be reflected in our finding of reduced PGP⁺ axon bundles in the dermis, which contain both unmyelinated C fibres as well as A β and A δ fibres that may lose their myelin sheaths before entering the dermis (Doppler et al., 2012; Provitera et al., 2007). The comparable density of tactile receptor organs (Meissner corpuscles)

between WAD and control groups suggests however that the dermal findings may be driven by small rather than large fibres. It has been speculated that impaired mechanical detection in the absence of large fibre degeneration could reflect loss of C fibre tactile afferents, a mechanosensitive subgroup of C fibres responsible for conduction of pleasant light touch (Morrison et al., 2011). However, this fibre population has been observed in human hairy skin (Liljencrantz & Olausson, 2014), rather than glabrous skin where we took our measurements, although there is emerging evidence of their presence in rodent glabrous skin (Djoughri, 2016). Most likely, the challenge of sensitively quantifying large fibres in human skin warrants caution in interpreting these findings.

Following strict correction for multiple comparisons (5% FDR), no clinical variables were significantly correlated with IENFD or dermal innervation. Interestingly however, some negative correlations were observed between innervation densities and psychological variables (ankle IENFD and stress symptoms [DASS], finger PGP⁺ bundle density and anxiety symptoms [DASS], finger PGP⁺ bundle density and posttraumatic stress symptoms [IES-R]), inferring reduced innervation to be associated with greater stress/anxiety symptoms, **in line with the proposition of a stress-related immune-mediated process contributing to small fibre pathology proposed above.** **Assessment of correlations between structural and clinical outcomes was a secondary, exploratory aim of this study.** While these correlations did not survive correction for multiple comparisons, these trends warrant investigation in a larger sample adequately powered for such correlations.

In our data, IENFD was not associated with any measure of pain or disability, consistent with findings from other patient populations (Caro & Winter, 2014; Schley et al., 2012; Schmid et al., 2014; Üçeyler et al., 2013). This implies that pain experienced by patients does not directly arise from reduced IENFD, but may either be unrelated or secondary to downstream effects such as hyperexcitability of surviving nociceptors (Serra et al., 2014). It should also be noted that while there were group differences in finger IENFD and dermal innervation between WAD and controls, not all WAD patients had reduced IENFD or dermal innervation. Chronic WAD Grade II is a heterogeneous condition and it is likely that variations in nature, location and intensity of signs and symptoms reflect similarly varied underpinning neurophysiologic mechanisms. Our findings suggest that small fibre pathology may be one such mechanism present in a subset of people with chronic WAD.

Some limitations should be considered when interpreting the present study. While QST was performed in accordance with the standardised protocol (Rolke, Baron, et al., 2006) by a single researcher, this assessor was not blinded to the WAD/control status of participants. Standardised instructions (Rolke, Baron, et al., 2006) were used to address this potential source of bias. Further, the study's cross-sectional design does not allow inferences of causation regarding the observed between group differences in IENFD, dermal innervation and sensory function. It should also be noted that while prior authors have investigated IENFD at the proximal index finger (Doppler, Rittner, Deckart, & Sommer, 2015; Schmid et al., 2014), large datasets of age- and sex-stratified normative values do not yet exist for this location. We have therefore collected our own carefully age- and sex-matched control data.

Future research should employ a longitudinal approach to examine the temporal development of small fibre pathology following a whiplash injury and its potential clinical significance for prognosis or management. It has been observed that cold hypoaesthesia and hypersensitivity occur soon after whiplash injury (Chien, Eliav, & Sterling, 2008a), however it is not clear if this difference in small fibre function is accompanied by reduced IENFD in the acute stage. Further investigation of small fibre pathology in chronic WAD at the ankle is required in a larger sample, powered to detect a more subtle systemic difference in IENFD. Whereas we evaluated IENFD in a pre-determined area with established loss of small fibre function, it would also be valuable to assess small fibre pathology in symptomatic regions (e.g. area of maximal pain), to investigate the localised relationship between presence of pain, IENFD and sensory function in this population (Patel & Kamerman, 2019).

In conclusion, our findings provide evidence of differences in small nerve fibre structure and function between people with chronic WAD and controls. At the finger, IENFD and dermal innervation were lower in the chronic WAD group than controls. Thermal and light touch detection thresholds were impaired in the chronic WAD group, and heat pain thresholds were lowered. While IENFD was not associated with clinical characteristics, these findings provide novel insight into potential neurobiological mechanisms involved in chronic WAD.

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SFF, MS and ABS analysed data; SFF, MS, HIR and ABS discussed the results and commented on the manuscript; SFF, MS, HIR and ABS approved final manuscript for publication.

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Figure Legends:

Figure 1: Quantitative sensory testing results for chronic whiplash (orange) and control (blue) groups at the finger. Data are presented as mean Z-scores with standard deviations. The grey region denotes ± 2 standard deviations. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. CDT = cold detection threshold; WDT = warm detection threshold; TSL = thermal sensory limen; CPT = cold pain threshold; HPT = heat pain threshold; MDT = mechanical detection threshold; MPT = mechanical pain threshold; MPS = mechanical pain sensitivity; WUR = wind-up ratio; VDT = vibration detection threshold; PPT = pressure pain threshold

Figure 2: Intraepidermal nerve fibre density (IENFD) for whiplash and control groups at finger and ankle sites. (a-d) Representative skin biopsy sections from a patient in the whiplash group and an age- and sex-matched control participant. The dermal-epidermal border is indicated with a dashed line. Epidermal small nerve fibres (arrows), papillary nerve fibres (asterisks) and sub-epidermal nerve fibres (arrow heads) are visible in red (PGP 9.5). In the finger, there are numerous small nerve fibres projecting into the epidermis in the control participant (a), whereas there is a reduced density of small nerve fibres in the epidermis of the whiplash patient (b). At the ankle, the density of small nerve fibres projecting into the epidermis is similar in the control participant (c) compared with the whiplash patient (d). Confocal microscope (Olympus FV3000) 20x objective. (e) Median (IQR) IENFD for the whiplash and control groups at the index finger. IENFD is lower in the whiplash group (* $p = 0.010$). (f) Mean (SD) IENFD for the whiplash and control groups at the ankle. There is no significant group difference ($p = 0.09$).

Figure 3: Dermal innervation for whiplash and control groups at finger and ankle sites, with representative histology images. (a) Median (IQR) bundles of protein gene product 9.5 positive (PGP⁺) fibres in the dermis for the whiplash and control groups at the index finger and the ankle. The density of dermal nerve bundles is lower in the whiplash group compared with controls at the finger (*p = 0.017) but not the ankle. (b) The median (IQR) percentage of dermal PGP⁺ nerve bundles containing myelin basic protein positive (MBP⁺) fibres is comparable between groups at the finger. (c) Mean (SD) percentage of dermal PGP⁺ nerve bundles containing MBP⁺ fibres is comparable between groups at the ankle. (d) The median (IQR) density of Meissner corpuscles in the finger is not different between the whiplash and control groups. (e) A bundle of dermal PGP⁺ fibres in the finger, containing no myelinated fibres (red, PGP 9.5). Cell nuclei appear blue (DAPI). (f) A bundle of dermal PGP⁺ fibres (red, PGP 9.5) in the finger also containing myelinated fibres (green, MBP). (g) A Meissner corpuscle in a dermal papilla of the finger. The corpuscle is formed by axons visible in red (PGP 9.5) and is innervated by myelinated fibres visible in green (MBP). Dotted line in inset indicates dermal-epidermal border.

Supplementary Figure 1: Associations between finger intraepidermal nerve fibre density (IENFD) and clinical outcomes (a-j). DASS = Depression, Anxiety and Stress Scale (-D, -A, -S refer to depression, anxiety and stress subscales); IES-R = Impact of Events Scale Revised; NDI = Neck Disability Index; NPSI = Neuropathic Pain Symptom Inventory; NRS = Numerical rating scale (pain intensity); PCS = Pain Catastrophizing Scale; S-LANSS = Self-complete Leeds Assessment of Neuropathic Symptoms and Signs.

Supplementary Figure 2: Associations between ankle intraepidermal nerve fibre density (IENFD) and clinical outcomes (a-j). DASS = Depression, Anxiety and Stress Scale (-D, -A, -S refer to depression, anxiety and stress subscales); IES-R = Impact of Events Scale Revised; NDI = Neck Disability Index; NPSI = Neuropathic Pain Symptom Inventory; NRS = Numerical rating scale (pain intensity); PCS = Pain Catastrophizing Scale; S-LANSS = Self-complete Leeds Assessment of Neuropathic Symptoms and Signs.

Supplementary Figure 3: Associations between density of bundles of protein gene product 9.5 positive (PGP+) fibres in the finger and clinical outcomes (a-j). DASS = Depression, Anxiety and Stress Scale (-D, -A, -S refer to depression, anxiety and stress subscales); IES-R = Impact of Events Scale Revised; NDI = Neck Disability Index; NPSI = Neuropathic Pain Symptom Inventory; NRS = Numerical rating scale (pain intensity); PCS = Pain Catastrophizing Scale; S-LANSS = Self-complete Leeds Assessment of Neuropathic Symptoms and Signs.

Supplementary Figure 4: Associations between density of bundles of protein gene product 9.5 positive (PGP+) fibres in the finger and clinical outcomes (a-j). DASS = Depression, Anxiety and Stress Scale (-D, -A, -S refer to depression, anxiety and stress subscales); IES-R = Impact of Events Scale Revised; NDI = Neck Disability Index; NPSI = Neuropathic Pain Symptom Inventory; NRS = Numerical rating scale (pain intensity); PCS = Pain Catastrophizing Scale; S-LANSS = Self-complete Leeds Assessment of Neuropathic Symptoms and Signs.