The Role of Nerve Pathology in the Development of Persistent Pain in Whiplash Associated Disorders

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Abstract

The World Health Organization reported the global burden of non-fatal road traffic collisions affects between 20-50 million people each year. Whiplash Associated Disorders (WAD) commonly occur after motor vehicle collisions and often include symptoms of pain and psychological distress. The most common type is WAD grade 2 (WAD2), which includes neck symptoms and musculoskeletal signs (e.g., tenderness and impaired neck movement) in the absence of a frank nerve injury on routine diagnostic testing (electrodiagnostic tests, traditional neurological examination). Contrary to the previous definition, new evidence demonstrates signs of nerve injury in patients with WAD2.

My research builds upon this evidence to answer novel objectives in a longitudinal cohort study assessing the temporal development of nerve pathology and its predictive ability in WAD2. To begin, I completed two systematic reviews of the literature integrating current evidence to inform the design of my longitudinal cohort study. The first systematic review summarised our current understanding of nerve pathology and neuropathic pain in people with WAD. My second systematic review aggregated the findings of blood-based biomarkers of nerve pathology to identify the most promising marker to measure within my cohort.

The findings from the longitudinal cohort study in WAD2 identified approximately one-third of participants with acute signs of neuropathic pain. In addition, I identified nearly two-thirds of participants with acute functional nerve pathology using quantitative sensory testing and neurological assessments. These measures improved six-months after injury but did not fully recover. Serum neurofilament light chain was elevated acutely but was comparable to controls six-months after injury. Intraepidermal nerve fibre densities from skin biopsies remained preserved in WAD2 participants acutely and six-months after whiplash injury. Transcriptional blood profiling six-months after injury revealed significant gene dysregulation for moderate/severe persistent pain versus minimal persistent pain when stratified by sex.

These novel insights help improve our understanding of the heterogenous nature of WAD2 and reveal important implications to better manage such patients.

Acknowledgements

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I am immensely grateful to my supervisor, Associate Professor Giorgos Baskozos, for his continuous support, positivity, and eagerness to teach. His expertise and knowledge in pain and bioinformatics have provided me with invaluable learning opportunities. I also wish to thank my clinical mentor, Dr Melissa Kolski, for instilling in me the confidence to pursue this path.

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Declaration

All work completed in this thesis was performed and completed by me, aside from the following:

Chapter 2. I led all aspects of this systematic review and meta-analysis with my co-authors, which has been previously published.

> *Fundaun J, Kolski M, Baskozos G, Dilley A, Sterling M, Schmid AB. Nerve pathology and neuropathic pain after whiplash injury: a systematic review and meta-analysis. Pain. 2022 Jul 1;163(7):e789-e811.*

Chapter 3. I led all aspects of this systematic review and meta-analysis with my co-authors, which has been previously published.

> *Fundaun J, Kolski M, Molina-Álvarez M, Baskozos G, Schmid AB. Types and Concentrations of Blood-Based Biomarkers in Adults With Peripheral Neuropathies: A Systematic Review and Meta-analysis. JAMA Netw Open. 2022 Dec 1;5(12):e2248593.*

Chapters 5 and 6: As described in detail in Chapter 4, my thesis includes data from a multicentre longitudinal cohort study collected in Oxford and Brighton, England. I collected and assessed all participants included in this thesis, besides the following:

- 11 acute WAD2 participants assessed by Soraya Koushesh (Oxford).
- 31 acute and follow-up WAD2 participants and 14 healthy control participants assessed by Dr Colette Ridehalgh (Brighton).
- Additional normative healthy control data for quantitative sensory testing, skin biopsies, and serum samples were collected by Professor Annina Schmid. The previously collected healthy control participant data used in my cohort included 25 participants for QST, 18 participants for skin biopsies and 11 participants for serum NfL.

Joel Fundaun

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Contents

List of abbreviations

BMI	Body Mass Index
CDT	Cold Detection Threshold
CI	Confidence Intervals
CNS	Central Nervous System
CPT	Cold Pain Threshold
CSF	Cerebrospinal Fluid
DASS42	Depression, Anxiety and Stress Scale 42
DN4	Douleur Neuropathique 4
ELISA	Enzyme-Linked Immunosorbent Assay
EMG	Electromyography
HPT	Heat Pain Threshold
IASP	International Association for the Study of Pain
ICC	Intra-Class Correlation
ICD	International Classification of Diseases
IENFD	Intraepidermal Nerve Fibre Density
IES-R	Impact of Events Scale-Revised
IQR	Interquartile Range
MDT	Mechanical Detection Threshold
MPS	Mechanical Pain Sensitivity
MRI	Magnetic Resonance Imaging
NDI	Neck Disability Index
NeuPSIG	Neuropathic Pain Special Interest Group
NfL	Neurofilament Light Chain

NSAID	Non-Steroidal Anti-Inflammatory Drug
PCS	Pain Catastrophisation Scale
PET	Positron Emission Tomography
PNS	Peripheral Nervous System
PPT	Pressure Pain Thresholds
PTSD	Posttraumatic Stress Disorder
QST	Quantitative Sensory Testing
RNA	Ribonucleic Acid
SD	Standard Deviation
Simoa	Single Molecule Array
SMD	Standardised Mean Differences
TBI	Traumatic Brain Injury
TNF-α	Tumour Necrosis Factor-Alpha
TSL	Thermal Sensory Limen
VAS	Visual Analog Scale
VDT	Vibration Detection Threshold
WAD2	Whiplash-Associated Disorders grade 2
WDT	Warm Detection Threshold

"The rapidity of the movement, the momentum of the person injured, the suddenness of its arrest, the helplessness of the sufferers, and the neural perturbation of the mind that must disturb the bravest, are all circumstances that of a necessity greatly increase the severity of the resulting injury to the nervous system, and that justly cause these ceases to be considered as somewhat exception from the ordinary accidents."

Professor John Erichsen, 1867,
'Railway and Other Injuries of the Nervous System'

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Background

1.1. Introduction of whiplash injuries

1.1.1. History

The evolution of modern transportation brought unparalleled opportunities and unforeseen difficulties. Pain and disability are major challenges resulting from such transportation-related injuries. The initially reported pain from transportation injuries was from locomotor accidents, which was aptly named 'Railway Spine'[1]. First described by physicians in the nineteenth century, Railway Spine brought a puzzling array of symptoms and a shroud of controversy in the medical community.

Professor John Erichsen first spoke of Railway Spine in his 1866 lecture entitled 'Railway and Other Injuries of the Nervous System'[1]. In this seminal series of lectures, Professor Erichsen described multiple case reports of people who experienced a collection of complex and disabling symptoms sustained from railway accidents. Such symptoms included widespread pain, paraesthesia, weakness, and psychological distress. Professor Erichsen initially hypothesised this array of symptoms to be originating from a 'concussion of the spine and of the spinal cord'.

Professor Erichsen's hypothesis was not however universally accepted. The gradual development of symptoms and the absence of apparent physical injury led other

physicians to describe Railway Spine as a type of psychosis rather than physical injury[2]. The latter theory considered pain from Railway Spine to be hysterical in nature and was conceptualised as a 'hallucination' resulting from the mind's reaction to abnormal excitable pain pathways.

The perplexity of Railway Spine continued with the advancement of transportation-related technology. An early case report of neck injury following a motor vehicle collision was described by Marshall in 1919. This reported symptoms of pain and distress in the presence of 'normal cervical spine x-rays in the injured patients'[3]. The advancement in both transport and medicine also brought a change in clinical nomenclature for these conditions. The sudden movement experienced from motor vehicle collisions was described by Crowe in 1928 'like cracking a whip' and was thus named 'whiplash'[4]. The term 'whiplash' became predominant and was solidified by its use in a key medical textbook in 1946[5].

1.1.2. Biomechanics of whiplash injury

The term whiplash, as defined by the rapid acceleration and deceleration of the cervical spine, has been consistently used since its inception. The foundational biomechanical understanding of whiplash injuries has been simulated from rear-end impact models[6,7]. Although there are biomechanical variations from different directions of impact, I will use the classical rear-end impact model for all following biomechanical review of whiplash injuries.

The initial impact phase after a motor vehicle collision includes the rapid forward acceleration of the occupants' thorax. This movement creates an initial sigmoid formation of the cervical spine[8,9]. The momentum from the thorax is then transferred to the cervical vertebrae creating relative cervical extension potentially impacting the zygapophyseal joints. The final phase of movement involves a rebound of forward momentum moving into cervical flexion before returning to a neutral posture[8]. The external forces acting on the cervical spine during the whiplash movement can place non-physiological strain on various structures of the spine, described in detail below.

1.1.3. Classification of whiplash injuries

An international task force was formed to tackle the growing clinical challenge of whiplash injuries. In 1995, the Quebec Task Force convened to improve the outcomes of whiplash injuries[10]. Several important implications came from this meeting. First, the task force adapted a new nomenclature for pain and symptoms arising from whiplash injuries entitled 'Whiplash Associated Disorders (WAD). WAD is defined as the signs and symptoms reported by people following an acceleration/deceleration injury to the neck[10,11]. The task force also introduced a new classification system to grade the severity of WAD using an ordinal scale from zero (no pain or physical injury) to four (fracture/dislocation), as shown in Table 1.1.

Table 1.1. Classification of WAD from the Quebec Task Force Grading System

QUEBEC TASK FORCE CLASSIFICATION[10]

GRADE	PRESENTATION
0	No complaints of neck pain or physical signs of injury
1	Complaints of neck stiffness or tenderness only No physical signs of injury
2	Complaints of neck pain Musculoskeletal signs: Decreased range of motion Point tenderness
3	Complaints of neck pain Neurological signs: Decreased or absent reflexes Muscle weakness Sensory deficits
4	Complaints of neck pain Signs of fracture or dislocation

The Quebec Task Force grading system is still commonly used around the world today, likely due to its simplicity. However, the validity of this classification system has long been questionned[12]. The most discussed challenge associated with the grading system is the heterogenous symptoms present in the most common type of injury (whiplash grade 2 [WAD2])[12-14]. WAD2 includes musculoskeletal findings in the absence of a frank nerve injury. Although the criteria to reach the higher classifications are rare (cervical radiculopathy or fracture)[15], it is suggested that people with WAD2 may have less apparent signs of nerve pathology[16,17].

Newer classifications have been created to grade the complexity of WAD more comprehensively. These updated classifications incorporate greater considerations for the severity of pain and disability, psychosocial contributions, and pre-existing health status[14,18]. Although these classifications incorporate the known clinical challenge

and complexity of whiplash injuries, they have not yet been as widely adopted as the original grading system.

1.2. Whiplash-Associated Disorders

1.2.1. Epidemiology

Sequalae following motor vehicle collisions have broad epidemiological impacts across the globe. Recent estimates by the World Health Organization reported the global burden of non-fatal road traffic collisions affects between 20-50 million people each year[19]. In the UK alone, 500,000 incidences of whiplash are reported annually[20]. Previous estimates in North America and Europe show similarly high rates of whiplash-related injuries, with more than 300 persons per 100,000 seen in the emergency department annually[21]. Sadly, up to half of all people who experience a whiplash injury report symptoms lasting more than 1 year and 20-30% report moderate levels of disability[21,22].

1.2.2. Socioeconomic burden

WAD is also associated with high socioeconomic costs that have continued to increase over the last 30 years. Socioeconomic costs of WAD exceed £5 billion annually in the UK, which includes healthcare costs, insurance claims, and days lost from work[20]. Similar estimates of exceedingly high costs have been estimated throughout the world, including the United States[23], Europe[24], Australia[25], and Japan[26].

1.2.3. Compensation culture

The litigious nature of whiplash injuries has long led to controversy regarding the actuality of patients' claims. The primary evidence supporting the malingering hypothesis comes from two highly impactful studies comparing systems with and without financial compensation[27,28]. Both studies unfortunately suffer from significant methodological flaws that limit the ability to conclude compensation significantly influenced people's recovery. Detailed commentary on the methodological shortcomings have previously been discussed[29-32]. For example, one landmark study did not include any measures of symptoms or disability and instead used a proxy for recovery based on time-loss payments. Additionally, people who reopened an injury claim were excluded from data analysis, which may account for up to 32% of their cohort. These confounding factors do not provide conclusive evidence for malingering or recovery. Overall, these studies suggest that changes to litigation policy create changes in the closure of insurance claims after motor vehicle collision, but they do not clearly show differences in recovery of the people involved.

More recent evidence from longitudinal cohort studies throughout the world lend support to the legitimacy of whiplash symptoms[33-35]. Although malingering is possible, mounting clinical evidence suggests similar symptomatology from WAD, regardless of language, country, or healthcare system. This evidence is recapitulated in a recently updated systematic review of WAD. This review suggests there is

limited evidence to support compensation and legal factors are prognostic for whiplash injuries and legitimises symptoms experienced following whiplash[36].

1.2.4. Symptomatology

The cardinal feature of WAD is neck pain[37]. However, reports of widespread pain and symptoms can be present. Pain reported in the thoracic and lumbar spine have been reported to be as high as 60% after whiplash injury[38,39]. It has also been reported that some people can experience radiating upper extremity pain, including paraesthesia[17,40-42]. Additional non-painful symptoms can include dizziness, difficulty balancing, and visual disturbance[43,44].

Psychological factors are commonly present after motor vehicles collisions and include feelings of distress, pain-related worrying, and posttraumatic stress. A recent systematic review of psychological sequelae identified a relative frequency of clinical depression occurring in approximately 33% of participants six months after the collision[45]. Diagnosis of posttraumatic stress disorder (PTSD) ranged from 9% - 23% at 3 months, and roughly 15% at 6 and 12 months after injury[45]. Though many studies do not reach the cut-off threshold for a clinical diagnosis of PTSD, a recent systematic review indicated posttraumatic stress symptoms are a clinical concern and may be related to participants' prognosis[46].

1.2.5. Prognosis

The high rate of persistent symptoms (~50%) create significant clinical challenges for patients with WAD. One key feature is identifying factors related to recovery. One of the most common tools for defining recovery in WAD is done using the Neck Disability Index (NDI). The NDI is a self-reported questionnaire that includes ten sections: pain intensity, personal care, lifting, reading, headaches, concentration, work, driving, sleeping, and recreation[47]. Previous longitudinal cohort studies following patients for one year suggest that those who recover typically do so in the first three months after injury[22,48]. Trajectory modelling suggests that acute NDI scores follow three primary trajectories of disability: mild (45% of participants), moderate (39% of participants), and severe (16% of participants)[49].

The primary prognostic factors assessed for WAD include clinical measures and questionnaire-based variables (summarised in Table 1.2). Factors consistent with poor recovery include high initial disability scores (NDI >15/50), negative expectations of recovery, high pain catastrophisation, cold hyperalgesia, and posttraumatic stress symptoms (Impact of Events scores >25)[21,36,46,50]. Factors that have consistently been shown not to be prognostic are accident-related parameters (speed, direction of impact, seatbelt use), imaging findings related to cervical joint alignment, disc protrusions, canal narrowing, alar and transverse ligament changes, motor dysfunction, education status, and older age. Lastly, prognostic factors that

included insufficient evidence include female sex, neck range of motion, and compensation factors.

There is limited evidence for biological or mechanistic prognostic factors for WAD. Preliminary evidence for biological factors related to poor prognosis from single studies in WAD include serum inflammatory markers (TNF-α and c-reactive protein)[51], lower levels of vitamin D in African Americans[52], genetic polymorphisms (FKBP5[53] and CRHBP[54]), elevated expression levels of MicroRNA-19b[55], and DNA methylation related to hypothalamic-pituitary-adrenal (HPA) axis genes[56]. **There is a current gap in the literature regarding direct biological measures of nerve pathology and recovery in WAD.**

Although numerous prognostic factors have been identified, there are few validated prognostic models for WAD. The inconsistencies of such models are summarised in a recent systematic review, which identified 58 whiplash-related prognostic models with only two reporting external validation[57]. The lack of clinical translation of known prognostic factors may be contributing to the difficulty to adequately assess and treat patients after whiplash injury. The most studied clinical prediction tool for WAD is the WhipPredict, which includes initial NDI scores, age, and posttraumatic stress symptoms, to stratify the probability of recovery. Internal and external validation indicate the WhipPredict to be an accurate and reliable measure to predict recovery after whiplash injury[58,59]. In contrast, physiotherapist-selected

clinical prognostic factors, such as range of motion and previous physiotherapy treatment, were shown to have low agreement with the validated prognostic factors from the WhipPredict[60].

Table 1.2. Summary of prognostic factors related to poor recovery in WAD.

Prognostic factors of recovery [21,36,46,50]		
Consistent prognostic factor	**Consistent to not be prognostic**	**Factors with inconsistent evidence**
High initial disability rating (>15/50 NDI)	Accident-related parameters (direction, speed, seat belt use)	Female sex
Negative expectations of recovery	Imaging findings (degenerative/minor pathological changes)	Neck range of motion
High pain related worrying	Motor dysfunction	Compensation factors
High frequency of acute healthcare utilisation	Education status	
Posttraumatic stress (IES >25)	Older age	
Cold hyperalgesia	Higher BMI	

Abbreviations: NDI: Neck Disability Index; IES: Impact of Events Scale; BMI: body mass index.

1.2.6. Management

The first line treatment for acute whiplash injuries without fracture includes conservative management (WAD grades 0-3). International treatment guidelines suggest acute management is comprised of patient education reassuring a favourable prognosis, pain-free exercise, and progressive return to activity[61-63]. Systematic reviews of the treatment effectiveness for whiplash injuries however show at best only small effects[64-66]. I will summarise the current state of the literature for conservative and pharmacological treatment for whiplash injuries below.

Conservative management

Exercise is a common treatment strategy for both acute and persistent WAD. The design of exercise interventions for WAD often incorporates exercises targeting cervico-thoracic mobility, strength, and sensorimotor dyfunction[64-66]. The pragmatic designs of most clinical trials make it difficult to analyse the effectiveness of individual aspects of a treatment programme (e.g., exercise, advice, reassurance). This is highlighted by the overall minimal effectiveness of exercise for WAD[64-66]. However, more specific, and stratified exercise trials may be beneficial in WAD. A recent study using neck-specific exercise for people with persistent whiplash-related arm pain and neurological deficits showed significant long-term improvements in pain and paraesthesia compared to generic physical activity prescription[67]. Taken together, exercise interventions may be beneficial for a subgroup of individuals with WAD, but additional research is needed.

Physiotherapy interventions for WAD are commonly used in both the acute and chronic stages and often incorporate a package of interventions (e.g., exercise, manual therapy, education). The effectiveness of physiotherapy interventions for WAD is similarly difficult to interpret due to the inclusion of numerous interventions which often show limited effectiveness[65]. The largest randomised controlled trial of acute WAD from the UK (N=3,851) demonstrated no difference in patient-reported disability comparing a package of six physiotherapy sessions to a single session of advice[35]. More recent physiotherapy interventions including a stress and disability-

stratified treatment approach also did not show significant differences in neck-related disability or recovery compared to usual care[68]. Although the overall quality of physiotherapy interventions for WAD is improving[69], there is minimal evidence for the effectiveness of contemporary physiotherapy for WAD[65,70].

The combination of physiotherapy combined with psychological-based stress reduction techniques may provide additional benefit. A randomised controlled trial of physiotherapist delivered stress reduction techniques paired with exercise showed significant reductions in pain-related disability for acute WAD2 compared to exercise alone[65,71]. However, another trial using more broadly defined cognitive behavioural therapy combined with exercise did not show benefit over supportive therapy and exercise for people with persistent WAD[72]. In summary, the use of physiotherapist delivered stress reduction techniques combined with exercise shows promise, but more evidence is needed to confirm these findings.

Pharmacological management

There is limited treatment guidelines or evidence for medication effectiveness for people with WAD. There are no randomised controlled trials for the use of medication for WAD[73-75]. The effectiveness of medication for WAD has primarily been examined in underpowered pilot and feasibility studies. Nevertheless, prescription of an analgesic medication is common following acute whiplash injury, especially at discharge from emergency departments[35,76,77].

The two most common medications prescribed for acute whiplash injury are non-steroidal anti-inflammatory drugs (NSAIDs) and opioids[78]. The effectiveness of opioids versus NSAIDs was assessed in a secondary analysis taken from a large prospective cohort of people after acute whiplash injury in the United States[79]. Overall, this study did not identify any significant difference in the risk for persistent pain between groups at 6-weeks. Not only was there no difference in effectiveness between medications, but there was also a 17% increased risk of continued opioid use at follow-up compared to those only receiving NSAIDs[79]. Similar results are corroborated in a more recent study of participants attending the emergency department after acute traumatic injury, which suggests that emergency department opioid exposure led to three times greater risk of later opioid use[80]. The increasing prevalence and limited efficacy for acute opioid use in WAD provides significant concerns for its use as an acute analgesic for WAD[76,79,81-83].

As WAD is perceived as primarily a musculoskeletal condition, the efficacy of neuropathic pain medications for acute WAD is less known. Current international guidelines for the treatment of WAD do not include neuropathic pain medications. First-line medications for the treatment of neuropathic pain include serotonin-noradrenaline reuptake inhibitors, tricyclic antidepressants, gabapentin, and pregabalin[84]. Increasing evidence for nerve involvement in WAD has led to recent developments assessing the efficacy of neuropathic pain medications after whiplash injury. This includes a feasibility trial for pregabalin which suggests reduced neck

pain compared to placebo[73]. Similarly, a recent pilot study using duloxetine showed tolerability and efficacy in acute WAD compared to placebo[74]. Despite these intriguing initial results, large scale randomised controlled trials are needed to further elucidate the benefits of neuropathic pain medications for WAD.

The overall current ineffective treatment strategies highlight the need to better understand the underlying causes and pathomechanisms of WAD.

1.3. Pathology

The primary pathological findings for WAD come from functional measures (e.g., clinical and quantitative sensory testing) with limited evidence for structural dysfunction. A summary of the currently identified pathological findings in WAD is summarised in Figure 1.1. I will highlight the predominant acute and persistent pathological findings related to the musculoskeletal and neurological systems below.

1.3.1. Musculoskeletal System

One of the most studied aspects of whiplash injuries is the musculoskeletal system. Structural musculoskeletal pathology in WAD can include the cervical muscles, zygapophyseal joints, cervical discs, and ligaments. Functional musculoskeletal dysfunction for WAD includes reduced movement in the cervical[85], thoracic[38], and lumbar spine[86].

Bone & ligament

The most studied structural musculoskeletal findings after whiplash injuries include the zygapophyseal joints. Strain to the facet joint and its capsule after whiplash injuries have been identified in humans through both cadaveric and PET imaging studies[87,88]. Injury to the cervical facet joints have been the predominant hypothesised mechanism for whiplash-related neck pain. This stems from numerous studies showing blinded diagnostic nerve blocks to the zygapophyseal joints, most commonly at C5-6, relieve chronic whiplash-related neck pain[89-91]. Numerous preclinical studies modelling zygapophyseal joint pain also highlight its role in contributing to persistent pain[92-95].

Injury to the cervical discs and ligaments have been suggested from post-mortem studies in participants with fatal motor vehicle collisions. This includes signs of tearing of the anterior longitudinal ligament and strains to the annulus fibrosis[96,97]. Such extreme injuries are suggested sources of whiplash-related neck pain, however, have not been studied outside of the fatal crash models. Pain emanating from musculotendinous structures is expected to be much more common.

Muscle

Whiplash-related muscle injury is postulated to occur from rapid lengthening of muscle fascicles. Modelling studies of whiplash injury suggest abnormal loading can occur, especially to the semispinalis capitis and sternocleidomastoid muscles[98,99].

Additionally, muscle tears, hematomas, strains, and peri-muscular fluid have been visualised in MRI in patients with WAD[100]. Though these studies highlight muscular injury may occur in WAD, a previous study using a marker of muscle injury (serum creatine kinase) did not show a relationship to persistent whiplash pain[101].

Functional changes to the muscular system after whiplash injury include reduced cervical muscle strength and endurance[102]. Studies using surface EMG have shown that individuals with chronic WAD exhibit altered muscle activation patterns compared to healthy controls. These changes included increased activity in superficial neck flexors and decreased activation of the upper trapezius muscles[103,104]. MRI has also demonstrated increased fatty muscle infiltration in the cervical spine in a subgroup with worse recovery[105,106]. However, measures of muscular dysfunction have limited association with recovery[107]. It remains unclear how this dysfunction relates to pain experienced by patients with WAD.

1.3.2. Nervous System

Central nervous system

The current evidence for pathophysiological changes to the central nervous system are incompletely understood. Although possible, there is limited evidence for acute central nervous system pathology in non-catastrophic WAD[108,109]. The most commonly identified CNS-related symptoms include reports of concussion or mild traumatic brain injury (e.g., headache, dizziness, fatigue)[110]. Dysfunction to the

vestibulo-ocular system after whiplash injury has also been shown to cause dizziness, unsteadiness, and disequilibrium following whiplash injuries[111,112].

Findings from chronic WAD share mixed results for CNS pathology predominantly from cross-sectional studies with small sample sizes. Pathological indications of CNS pathology in chronic WAD include altered cerebral blood flow[113] and changes in brain glucose metabolism[114]. Spinal cord MRI of persistent WAD also present mixed evidence from limited studies. One study using magnetization transfer imaging suggested white matter changes within the cervical spine one year after injury.[115] However, a separate magnetic resonance spectroscopy study did not show significant differences in metabolic changes in the spinal cord between participants with persistent WAD and controls[116].

Peripheral nervous system

The primary structures of the peripheral nervous system hypothesised to be affected by whiplash injuries include the dorsal root ganglia and spinal nerve roots. Akin to the central nervous system, evidence for acute peripheral nerve pathology in WAD is limited. Evidence suggestive of peripheral nervous system pathology has predominantly been done in small cross-sectional studies of chronic whiplash pain. One study using QST in chronic WAD showed signs of sensory hypoaesthesia, as evidenced by reduced cold, vibration, and electrical detection thresholds within C6-

8 dermatomes[16]. Importantly, such findings from QST suggest dysfunction to the nervous system but cannot localise whether the source of dysfunction.

Structural neurological changes have only been shown in one study in chronic WAD. This study identified reduced intraepidermal nerve fibre densities at the index finger in participants with persistent WAD2 compared to age-matched controls[40]. Another study assessing peripheral nerve roots in a small number of patients with persistent WAD (N = 10) demonstrated increased T2 MRI signal intensity compared to healthy controls[117]. Increased T2 signal intensity is thought to reflect signs of neural inflammation. Though intriguing, these studies highlight the need for further studies assessing potential structural nerve injury in WAD.

Peripheral nerve mechanosensitivity has been suggested through increased sensitivity to elongation and mechanical pressure in nerves of the upper extremity[104,118]. Participants with acute WAD have shown decreased movement to upper limb neural tension, which was more dysfunction in the group with more severe symptoms[104,118]. Acute WAD participants have also demonstrated increased sensitivity to pressure using an algometer over the median nerve at the cubital fossa. These studies suggest that participants with more severe WAD have higher levels of sensitivity to mechanical stimuli over the nerves[119,120]. Overall, there is emerging evidence for peripheral nervous system pathology in WAD, however, this has not

been carefully reviewed and summarised. As such, I will aggregate this data using a systematic review and meta-analysis of the literature in Chapter 2.

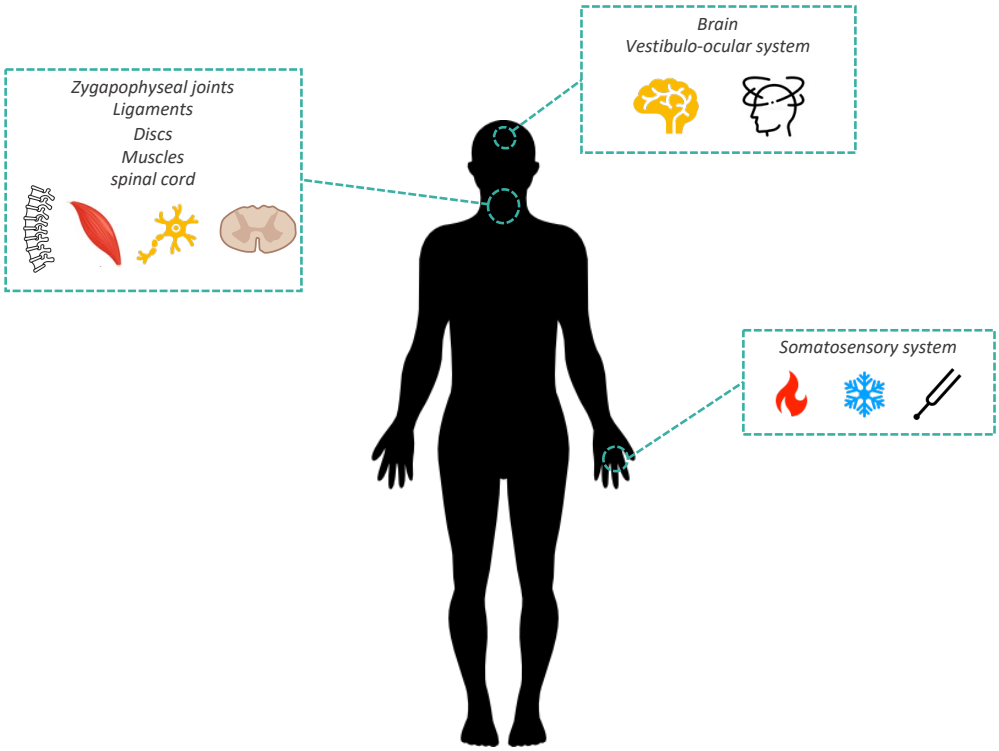


Figure 1.1. Overview of the pathological findings in WAD, encompassing primary areas and types of identified dysfunction. Post-WAD sequelae can manifest through various pathological mechanisms identified from a range clinical assessments. These findings can span abnormalities within the cervical spine, brain, and peripheral somatosensory system.

1.4. Aetiology of pain after whiplash injury

1.4.1. Definition of pain types

Pain is the most common symptom for people with WAD[37]. I will describe the current evidence and hypotheses related to the aetiological mechanisms of pain from whiplash injury.

To begin, it would be prudent to discuss the difference between nociception and pain. Nociception is the process of encoding noxious stimuli through the activation

of small diameter nerve fibres (A-delta and C-fibre types)[121]. The peripheral primary sensory neurons then transduce and transmit these signals from the periphery to the spinal cord, brainstem, and cortex[122]. The process of nociceptive information being perceived as a painful response requires interpretation at the cortical level. This is underscored by the current definition of pain, which is an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage[121].

The traumatic nature of whiplash-related pain brings with it a complex array of biopsychosocial considerations. As such, the aetiological mechanisms are difficult to individually separate. The rapidity of the head and neck movement can involve numerous anatomical structures. In the following sections, I will discuss the potential mechanisms contributing to both acute and persistent pain related to WAD (summarised in Figure 1.2).

1.4.2. Hypothesised mechanisms for acute whiplash pain

Acute nociceptive pain

The cervical spine is frequently reported as the primary site of pain in WAD and is commonly characterised as nociceptive in nature[37]. Potential sources of nociceptive pain after whiplash injuries can involve structures including the cervical vertebrae and zygapophyseal joints, spinal ligaments, cervical discs, and surrounding musculature[123]. Clinical indications linked to nociceptive pain may involve localised

and transient pain, decreased range of motion, and the absence of frank nerve damage.

Pain arising from the zygapophyseal joints and their capsules are arguably the most well-studied cause of acute nociceptive whiplash pain. Evidence for zygapophyseal joint pain comes from studies using diagnostic nerve blocks[89,124], positron emission tomography/computed tomography[125], and pain relief following radiofrequency neurotomy[126,127]. The hypothesis for zygapophyseal joint pain has been replicated using animal neck pain models[89,90,92,128,129]. Biomechanical models of simulated whiplash injuries have also highlighted the potential role of cervical spinal ligaments[9] and surrounding cervical musculature[98,130] to contribute to this nociceptive pain.

The traumatic nature of whiplash injuries may not only include actual but also threatened tissue damage. Posttraumatic stress symptoms are commonly present after acute whiplash injury, are associated with poor recovery, and may contribute to acute whiplash-related pain[46]. Animal models of stress-induced hyperalgesia suggests stress can modulate nociception in various pathways along the neuraxis[131]. Similarly, multiple stress-related biological pathways have been suggested in the modulation of pain in humans. These findings include, but are not limited to, changes to the hypothalamic-pituitary-adrenocortical axis (HPA), catecholaminergic and adrenergic systems, and serotonergic dysregulation[132]. Recent studies in patients with WAD have suggested HPA axis gene dysregulation, including FKBP5 and

CRHBP, are potential stress-related mechanisms that may increase pain sensitisation after whiplash injury[54,56,133].

Acute neuropathic pain

The rapid movement of the head and neck during a whiplash injury may not only affect musculoskeletal, but also neuronal structures. Pain caused from a lesion or disease of the somatosensory nervous system is known as neuropathic pain[121]. As highlighted above, the spectrum of whiplash injury could theoretically affect both the central and peripheral nervous system. Potential causes of whiplash-related lesions to the nervous system involve direct nerve compression or elongation, changes to spinal gradient pressures, or secondary mechanisms including inflammation[134-138].

Whiplash causing injury to the central nervous system is possible but shown to be biomechanically less likely than peripheral nervous system injuries[139]. A study using the classical rear-impact biofidelic model demonstrated that spinal canal diameter narrowing is not likely[139]. A similar model was then assessed for frontal impact showing a comparably low likelihood of central spinal canal narrowing[140]. However, an in vitro study using simulated impact models indicated an increased potential for spinal cord injury in the presence of cervical canal stenosis[141].

Potential injury to the peripheral nervous system is suggested to primarily affect the cervical dorsal root ganglia and nerve roots[123]. The primary preclinical evidence

supporting spinal ganglia injury was done using a porcine model. In this study, anesthetised pigs received a simulated whiplash injury which created blood flow resistance gradients applying direct pressure to the nerve roots and spinal ganglia[136,137]. This model was suggested to cause a breakdown of the spinal ganglia plasma membrane, which correlated with increased pressure amplitudes in the spinal canal and histopathologic findings of nerve injury[136,137]. A separate preclinical mouse model of non-traumatic cervical nerve injury suggested that loading the cervical dorsal nerve roots produced lasting nocifensive behaviours[142,143]. Murine induced cervical injury also showed signs of Wallerian degeneration and included disrupted axonal transport and mechanical hypersensitivity[142,143].

Evidence for human models of whiplash-related peripheral nerve injury have primarily been performed using theoretical modelling and cadaver studies. Nerve roots in the cervical spine lack an epineural sheath, which make them at increased risk of injury to external loading[138,144-148]. Simulated whiplash injury using cadavers and test mannequins demonstrated decreased neural foramina diameter during extreme neck motions[137,138]. This motion is thought to increase loading of the cervical ganglia and nerve roots[137,138], which increases in the presence of cervical stenosis[88,149,150]. These potential mechanisms may contribute to acute peripheral neuropathic pain after whiplash injuries.

1.4.3. Hypothesised mechanisms for persistent whiplash pain.

Theorised mechanisms for persistent pain in WAD include contributions, such as central and peripheral sensitization, systemic inflammation, and psychosocial factors. I will discuss the current evidence for the association of each category in the sections below. However, it is critical to emphasise that persistent pain in WAD likely stems from a complex array of numerous factors unique to each person.

Nociplastic pain

Nociplastic pain is defined as "pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing pain"[121]. A recent nociplastic pain grading system has been proposed to classify the certainty of nociplastic pain[151]. However, there is ongoing debate regarding the clinical classification of nociplastic pain. Such challenges include significant overlap with other pain classifications (nociceptive, neuropathic)[152-154], a limited understanding of underlying nocicplastic mechanisms[152,153], and difficulty in clinically assessing hypersensitivity and evoked pain related to nociplastic pain[152,155].

Indications of nociplastic pain in WAD include reports of diffuse pain, widespread hyperalgesia, signs of allodynia, decreased spinal reflex thresholds, and enhanced temporal summation[156]. While the mechanisms causing nociplastic pain remain

poorly understood, potential contributions in WAD may involve peripheral and central sensitisation, as well as reduced central inhibitory mechanisms[157].

Central sensitisation

A hallmark of central sensitisation is the increased responsiveness of nociceptive neurons in the CNS to normal or subthreshold input[121]. The specific mechanisms causing increased responsiveness after whiplash injury is currently unclear. However, the predominant evidence suggestive of central sensitisation in people with persistent WAD is from QST[156,158]. This includes reports of diffuse pain, widespread hyperalgesia, signs of allodynia, decreased spinal reflex thresholds, and enhanced temporal summation, and one study showing increased nociceptive flexion reflexes indicating spinal cord hyperexcitability in people who do not recover[156,158].

Peripheral sensitisation

Ongoing peripheral nerve irritation from mechanical or inflammatory mediators may also contribute to peripheral nervous system sensitisation[159,160]. Clinical signs of peripheral sensitisation in persistent WAD include peripheral nerve mechanosensitivity to pressure and elongation[16,118,161,162], upper extremity warm and cold hyperalgesia[118,119], reduced median nerve excursion using ultrasound[163], and increased brightness of T2-weighted MRI of the proximal brachial plexus suggestive of peripheral neuroinflammation[117]. These findings may indicate increased peripheral nerve excitability and activation contributing to persistent pain.

Systemic inflammation

Elevated systemic inflammation may contribute to persistent pain affecting both the central and peripheral nervous system[159,160]. A previous systematic review with meta-analysis of persistent WAD showed no significant difference for increased TNF-alpha and IL-1B, but low-level evidence for increased c-reactive protein in persistent WAD compared to controls[164]. Increased levels of systemic inflammation have been associated with other persistent pain conditions, including fibromyalgia[165], complex regional pain syndrome[166], and carpal tunnel syndrome[167]. Similarly, a recent study using transcriptional profiling of people transitioning to persistent low back pain suggested an immune-mediated response for recovery[168]. Thus, ongoing systemic inflammation could induce maladaptive physiological processing of the nervous system contributing to persistent pain after whiplash injury. Specifically, inflammation has been shown to contribute to neuropathic pain[160,169], sensory hypoaesthesia[170,171], and neural injury[172,173].

Psychosocial factors

As highlighted above, the experience of persistent pain after whiplash injury is a complex sensory and emotional experience. It is important to consider not only the biological but also psychological sequelae. Modelling from longitudinal cohorts of patients after whiplash injury have implicated posttraumatic stress, especially hyperarousal symptoms, to contribute to persistent whiplash pain[49,174,175]. Both preclinical and clinical models of persistent posttraumatic stress have demonstrated

increased levels stress-related hormones known to affect the nervous systems (e.g., cortisol, glucocorticoids, catecholamines)[132,175-178]. Persistent stress-related dysregulation may contribute to those experiencing persistent pain after whiplash injury.

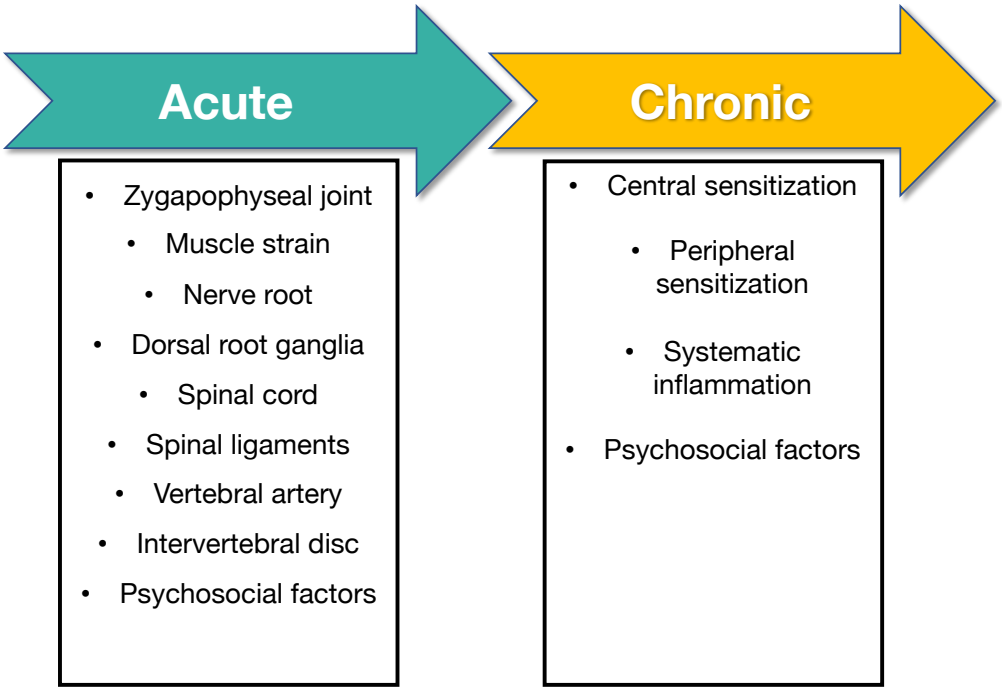


Figure 1.2. Proposed factors contributing to acute and chronic whiplash pain. Acute whiplash pain may arise from numerous anatomical structures, alongside psychosocial factors, including posttraumatic stress symptoms. Chronic whiplash pain is suggested to be influenced by a combination of physiological factors such as peripheral and central sensitization, indications of systemic inflammation, and ongoing psychosocial influences. Specific mechanisms underlying chronic whiplash pain likely varies among individuals, reflecting a complex interplay of factors.

1.5 THESIS OVERVIEW

The high rate of persistent pain and disability from whiplash injuries create significant challenges for individuals and society. Unfortunately, little is still known about the pathomechanisms or prognosis of persistent pain following whiplash injury. As highlighted above, there is increasing evidence that signs of nerve pathology may be present in the most common type of whiplash injury (WAD2). However, it is not

currently known if signs of nerve pathology are present acutely or if they contribute to the development of persistent pain.

1.5.1. Aims

Therefore, the overall aim of my thesis is to evaluate the temporal development of nerve pathology in a longitudinal cohort of participants with WAD2. I will then assess if biological or clinical measures of nerve pathology are prognostic for neck-related disability.

1.5.2. Objectives

I will achieve these aims using a deep phenotyping approach and robust assessment strategies, including:

1. Synthesising the current evidence for measures of nerve pathology and neuropathic pain in WAD (Chapter 2).
2. Identifying blood-based biomarkers of nerve pathology for selection and analysis in my WAD2 cohort (Chapter 3).
3. Developing a detailed clinical testing protocol to evaluate signs of nerve pathology and neuropathic pain in a longitudinal cohort of WAD2 (Chapter 4).
4. Evaluating signs of nerve pathology in the acute stage of WAD2 (Chapter 5).
5. Assessing the temporal patterns of nerve pathology and factors related to recovery in WAD2 (Chapter 6).

6. Summarising my chief results and discussing the current implications and future studies (Chapter 7).

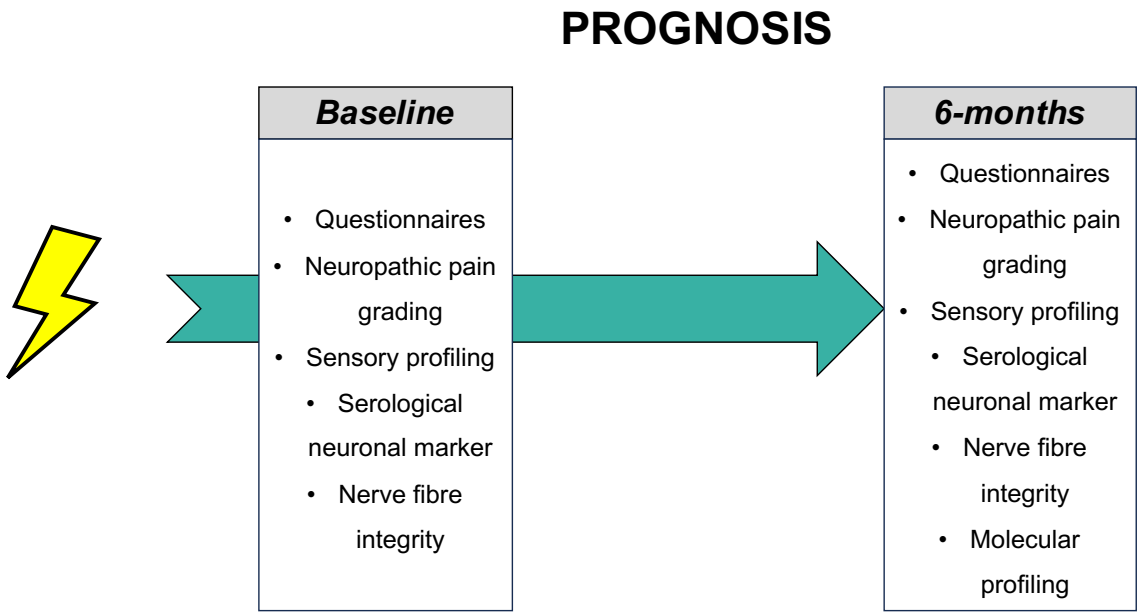


Figure 1.3. Summary of the deep phenotyping approach to assess nerve pathology in my cohort of WAD2.

1.5.3. Primary thesis methodologies

Meta-analysis to synthesise the current evidence.

I used systematic reviews and meta-analyses of the literature to amalgamate the current evidence to incorporate in my testing of participants with WAD2. First, I sought to synthesise the evidence for nerve pathology and neuropathic pain after whiplash injury (Chapter 2). Second, I evaluated blood-based biomarkers of nerve pathology in people with peripheral neuropathy (Chapter 3). These findings shaped my protocol through my selection of a nerve-related biomarker to measure in my cohort of people with WAD2. Additionally, I published a narrative summary of analytical approaches to using meta-analysis[179] and included this in Appendix A.

Deep phenotyping to assess nerve pathology in WAD2.

Deep phenotyping is a technique that includes comprehensive analysis of phenotypic characteristics. A detailed understanding of complex conditions is required to improve treatment and outcomes. The ultimate goal of deep phenotyping is to enable the stratification of patients to identify distinct subgroups. This approach has previously been used to improve our understanding and management in a number of painful conditions[180-182], including neuropathic pain[183-185].

I used a deep phenotyping approach to better understand the underlying pathological mechanisms in people with WAD2. Phenotypic information in my study includes participant reported outcome measures, a detailed clinical assessment, and sensory and molecular profiling (Figure 1.3).

Discovery of novel prognostic factors in WAD2

A prognostic factor is defined as a measure that is present at the start of health conditions and is associated with the clinical endpoint[186]. Prognostic factors provide critical information to better understand a diagnosis, improve an individual's risk prediction, and advance associated management strategies. Our understanding of clinical or biological prognostics factors for WAD2 is currently limited. To address this challenge, I assessed novel prognostic factors related to nerve pathology to better understand the trajectory of recovery after whiplash injury.

2

Systematic review and meta-analysis of nerve pathology and neuropathic pain in WAD

2.1. Introduction

Whiplash associated disorders (WAD) commonly occur after motor vehicle crashes and often include signs and symptoms of pain, psychological distress, and sensory/motor dysfunction[107]. Currently, there is not a clear understanding of the mechanisms of persistent pain in WAD. This is important as up to 50% of patients experience persistent symptoms after whiplash injuries. Additionally, routine clinical testing and imaging do not typically identify a specific structural lesion causing pain or symptoms[123]. These clinical challenges are reflected by the overall small effects of current treatment strategies for these patients[65].

WAD is commonly classified using the Quebec Task Force severity grading scale [10] that grades severity from O (no pain and physical signs of injury) to IV (neck fracture/dislocation). The most common type is WAD2[187,188], which includes neck symptoms and musculoskeletal signs (e.g., tenderness and impaired neck movement) in the absence of a frank nerve injury on routine diagnostic testing (electrodiagnostic tests, traditional neurological examination). However, individual WAD grades can include a diverse range of clinical signs and symptoms[187-189].

There is increasing evidence of nerve involvement and neuropathic pain in patients with chronic WAD. This includes sensory hypoaesthesia[17,120], signs of nerve inflammation on magnetic resonance imaging (MRI)[117], and structural degeneration of small nerve fibres in skin biopsies[40]. Additionally, clinical questionnaires have identified some patients reporting neuropathic pain characteristics after whiplash injury[190,191]. In line with these findings, a recent feasibility trial using a first-line neuropathic pain medication (pregabalin) for patients after acute whiplash injury showed short-term improvements in neck pain intensity when compared to placebo[192].

The presence of nerve pathology would have important implications for the management of patients with WAD. Compared to other chronic pain conditions, people with neuropathic pain experience greater impairments to quality of life and emotional wellbeing[193,194]. Neuropathic pain and nerve pathology would also require targeted treatment approaches (e.g., neuropathic pain medication, specific physiotherapy methods) compared to non-neuropathic pain conditions[195]. Although there is emerging evidence, the involvement of nerve injury and neuropathic pain in WAD is not well understood. Thus, this systematic review aimed to assess whether there are indications of nerve pathology and neuropathic pain in patients after a whiplash injury.

2.2. Methods

This review was preregistered on Prospero CRD42020211255; https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020211255) and was reported following the updated guidance for the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA 2020) statement[196].

2.2.1 Eligibility

This review included observational studies (cross-sectional, cohort, and case-control) including measures of neuropathic pain and/or peripheral nerve pathology following motor vehicle crashes resulting in whiplash injuries. Studies were included if they reported on both 1) participants with WAD of any severity grade or duration; and 2) participants in whom measures of peripheral nerve pathology or neuropathic pain were reported. These could include a. Electrodiagnostic testing (e.g., nerve conduction, electromyography studies); b. Clinical examination findings of nerve pathology (e.g., bedside neurological examination including muscle testing, sensory testing, reflexes); c. Quantitative sensory testing (specifically sensory measures of loss of function: mechanical, thermal, electrical detection); d. sympathetic reflexes (e.g., sympathetic skin responses); e. tests evaluating nerve mechanosensitivity (e.g., neurodynamic tests, pressure pain thresholds over peripheral nerves); f. imaging of neural structures (e.g., MRI, ultrasound); g. clinical questionnaires indicative of neuropathic pain (e.g., Self-complete Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS), Douleur Neuropathique 4 (DN4), Neuropathic Pain Symptom Inventory (NPSI)); h. grading systems or diagnostic codes suggesting the presence

of nerve injury or neuropathic pain (e.g., NeuPSIG grading system, International Classification of Diseases (ICD) codes). Measures of peripheral nerve pathology or neuropathic pain had to be reported such that they could be either interpreted as stand-alone measures (e.g, bedside neurological testing, diagnostic codes), compared to a control group (e.g., QST) or previously published normative values (e.g., electrodiagnostic testing).

Exclusion criteria comprised studies not published in English, case series, conference abstracts and randomised controlled trials. Additionally, articles reporting on any of the following participant characteristics were excluded: 1) participants diagnosed with a central nervous system disorder or pathology (e.g., spinal cord injury, traumatic brain injury); 2) participants less than 18 years old; 3) participants with a previous diagnosis of peripheral neuropathy.

2.2.2. Search Strategy

EMBASE, PubMed, CINAHL (EBSCO), and MEDLINE were searched from inception to 1st September 2020. A search strategy was developed by the study team in consultation with a medical librarian. The search strategies are provided in Supplemental Table 2.1.

2.2.3. Screening

Initial study eligibility was screened by one reviewer (Joel Fundaun) using titles/abstracts. Full texts were then reviewed by two independent reviewers (Joel Fundaun and Melissa Kolski). Disagreements in selection were resolved by consensus or consultation with a third reviewer (Annina Schmid). Grey literature was searched for any additional articles by screening reference lists, theses (EThOS database), and policy documents. All studies were downloaded into EndNote referencing software (Clarivate, US) and duplicates were removed.

2.2.4. Data extraction

Data were extracted into a standardised excel spreadsheet developed and piloted by the study team. Extracted data included study characteristics (author, year, study design), sample size (WAD and controls), type and chronicity of WAD, the instrument or tool used to identify neuropathic pain/nerve pathology, as well as the type of outcome measures of neuropathic pain/nerve pathology in patients and healthy controls.

When possible, mean values and standard deviations (SD) relating to measures of neuropathic pain and nerve pathology were extracted for patients and healthy controls. Extracted data lacking a control group was compared to published normative values (e.g, questionnaire cut-off scores, electrodiagnostic testing) or to referenced diagnostic criteria (e.g., ICD codes). Where included information was

unclear, we attempted to contact the authors to obtain the necessary information. If studies reported alternative summary statistics, means and SD were transformed using recommended calculations[197]. Graphically reported means and SD were estimated using Plot Digitizer software[198], as recommended by the Cochrane Handbook[199]. Data were extracted by one reviewer (Joel Fundaun) and independently checked by another reviewer (Melisa Kolski).

We further categorised studies (not individual patients) according to the Neuropathic Pain Grading System published by the Neuropathic Pain Special Interest Group of the International Association for the Study of Pain[194] to gather information about the certainty of neuropathic pain. Data extraction included details regarding the criteria for neuropathic pain. Possible neuropathic pain included a neuroanatomically plausible pain distribution and history suggesting relevant neurologic lesion. Probable neuropathic pain included negative sensory signs in the same neuroanatomically plausible distribution, such as quantitative sensory tests or bedside neurological examination. Definite neuropathic pain included a diagnostic test confirming a lesion or disease of the somatosensory nervous system explaining the pain, such as electrodiagnostic tests and imaging of neural structures. A grading of the next higher category could only be reached if the previous categories were met. If diagnostic tests confirmed a nerve lesion but sensory signs were not assessed, we classed these studies into a separate category of 'nerve pathology'.

2.2.5. Quality assessment

Study quality and risk of bias were assessed using the Newcastle-Ottawa Quality Assessment Scales for case-control and longitudinal cohort studies. These are scored from zero to nine for the categories of study selection, comparability, and exposure or outcome. For cross-sectional studies, an adapted Newcastle-Ottawa Quality Assessment Scales[200] was used, which is scored out of 10. The Newcastle-Ottawa Quality Assessment Scales classifies the risk of bias of observational studies on an increasing scale, with higher scores reflecting a lower risk of bias. Whereas no recommended cut-offs exist for case-control and cohort studies, Newcastle-Ottawa Quality Assessment Scales cross-sectional studies were interpreted using a previously described method[200] with scores from 0–3 indicating high risk, 4–7 as moderate risk, and 8–10 as low risk. Two independent reviewers assessed each study for risk of bias (Joel Fundaun and Melissa Kolski). Disagreements between reviewers were resolved through consensus or by mediation of a third reviewer (Annina Schmid).

2.2.6. Data synthesis and analysis

Results not included in the meta-analyses are described using narrative synthesis of nerve pathology or neuropathic pain measures. We used the Guidance on the Conduct of Narrative Synthesis in Systematic Reviews: A Product from the ESRC Methods Programme (2006) to report our findings[201].

If data were available for the same outcome measure from at least 2 studies using similar assessment methodology, meta-analysis was performed. Two meta-analyses were performed: 1) summarising overall data from all studies independent of WAD grade and 2) summarising studies only including patients with WAD 1-2 who per definition should not demonstrate nerve pathology[10]. If outcome measures from at least two studies examined more than one anatomical site (e.g., detection thresholds at finger and neck), each site was meta-analysed separately. If studies reported outcome measures for both right and left sides in the same participants, pooled means and SD were reported to avoid inflation during meta-analysis.

All statistical calculations were performed using the freely available software R[202] and RStudio[203] using the packages 'Meta' and 'Metafor'[204]. For estimated prevalence data, means and ranges were reported. For continuous data, group means, SD, and sample sizes were used to calculate standardised mean differences (SMD) and 95% confidence intervals (CI). P-values and I^2 heterogeneity were also reported.

Random effects models and inverse variance weighting methods were used to account for the variability of included studies. Statistical significance between patients and healthy control participants was determined using t-tests with a pre-registered significance cut-off of p-value < 0.05. The Hartung-Knapp adjustment for random effects model and Hedges' g bias correction for standardised mean difference were used. Sidik-Johnkman estimator for tau^2 adjusted for between study variance. As a

very small number of studies can make it impossible to estimate the between-studies variance with precision, a fixed effects model was used if only 2 studies were meta-analysed[205]. Heterogeneity was calculated using I^2 statistics and interpreted as 'might not be important' (0-40%), 'moderate' (30-60%), 'substantial '(50-90%), and 'considerable' (75-100%)[199].

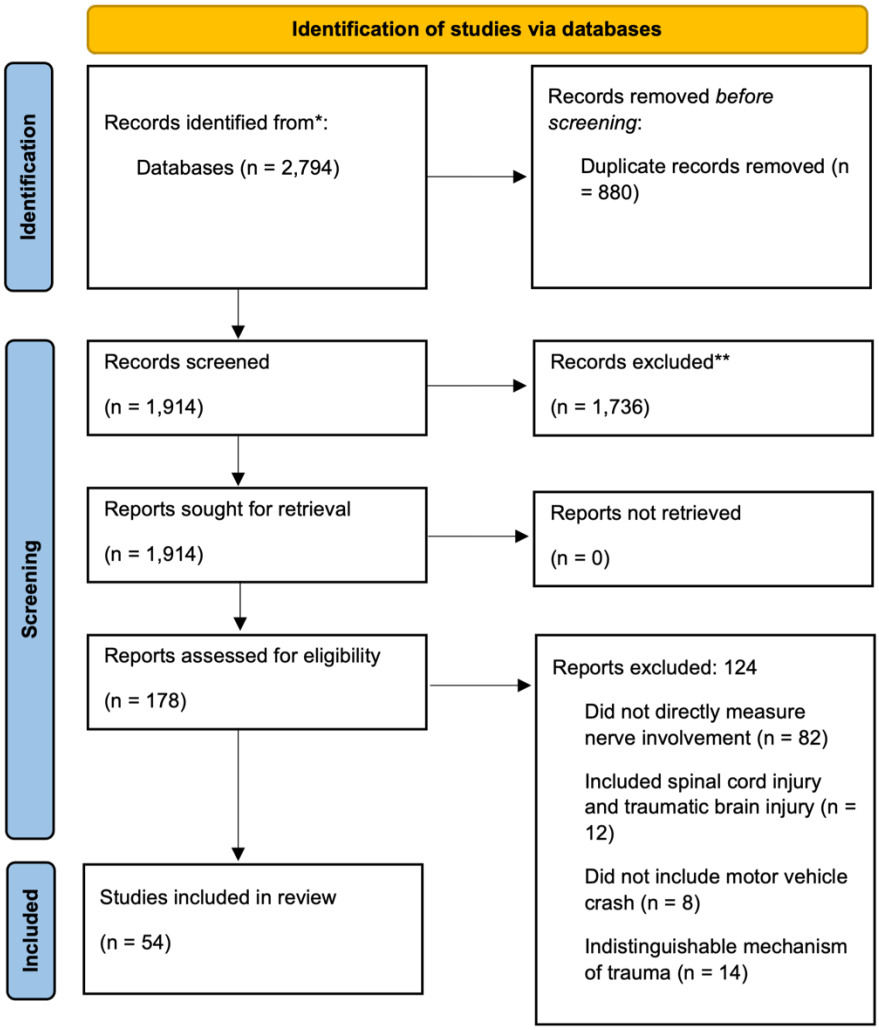


Figure 2.1. PRISMA Flow Diagram

2.3. Results

The search identified 1,914 non-duplicate citations for abstract/titles screening. A total of 178 articles were screened for full-text eligibility. A total of 54 studies reporting on n=390,644 patients and n=918 controls were included in this review (Figure 2.1). The main reason for study exclusion was the absence of a direct measure of nerve pathology or neuropathic pain (82 studies). We attempted to contact the authors of two studies for details regarding inclusion criteria and study methodology[206,207]. As we did not receive any responses, these studies were not included in this review.

Detailed study characteristics can be seen in Table 2.1 and Supplemental Table 2.2. The studies include a range of observational designs (22 cross-sectional, 28 cohort, four case-control), and report on sample sizes between n=9 and n=384,539 patients/controls. The average age of WAD participants was 37.67 (SD 2.25) years and 42.7% were female.

Thirty-two of the 54 included studies (59%) report the grade of WAD severity using the Quebec Task Force grading scale (0-4)[10]. The most commonly reported is WAD2 (7 studies, n=307 total patients) followed by the combination of grades 2-3 (6 studies, n=408 total patients) and grades 1-3 (5 studies, n=283 total patients).

Sensory detection measures are identified for six major body sites. We group outcomes recorded over the thenar emminence, phalange I and metacarpophalangeal joint I into a meta-analysis for 'thumb'; the phalanges II and metacarpophalangeal joint II into a meta-analysis for 'index finger'; and the phalanges V and hypothenar muscle into a meta-analysis for 'little finger'. Two studies[208,209] report outcome measures using separate values for right and left sides, which are pooled to avoid inflation during meta-analysis.

Table 2.1. Study characteristics and outcome measures

Author & Date	Study Participants	WAD Grade (QTF)	Study Measures	Outcomes: mean (SD)	
Sterling (2009)	WAD n=85	I-III	S-LANSS (0-24)	n=12 patients scoring ≥12	
Smith (2013)	WAD n=90, controls n=30	II	S-LANSS (0-24)	WAD (R; n=58): 11 (IQR 8-17), (NR; n=32): 13 (IQR 8-16)	
			ULNT (degrees of elbow flexion)*	WAD (R): 29.33 (16.30), NR: 34 (14.81)	Controls: 4 (6.67)
			PPT (kPa)*	WAD (R): 236 (78.15), WAD (NR): 229.34 (85.92)	Controls: 375.17 (134.38)
Karlsborg (1997)	WAD n=34	II-IV	Neurological Examination	n=5 patients (positive findings of nerve pathology)	
Henrikson (2013)	WAD n=20, controls n=10	II-III	Thermal detection (Thermotest)	No group mean values provided. n=6 with reduced temperature sensitivity; n=5 with increased temperature sensitivity (facial skin)	
Chuang (2002)	WAD n=85	NA	Electrodiagnostic testing	n=7 patients (positive findings of nerve pathology)	
Smith (2014)	WAD n=53, controls n=30	II	ULNT (degrees of elbow flexion)*	WAD: 28.67 (15.56)	Controls: 4 (6.67)
			PPT (kPa)*	WAD: 185.33 (63.70)	Controls: 365.33 (98.27)
Sterling (2004)	WAD n=80, controls n=20	II-III	ULNT (degrees of elbow flexion)	mild: 26.7 (17.7), moderate: 31.3 (14.9), severe: 36.5 (11.8)	Controls: 21.4 (10.8)
Serrano-Munoz (2019)	WAD n=20, control n=15	I-III	DN4 (0-10)	No Pain (n=5): median 3 (IQR 1.5), Pain (n=15): median 4 (IQR 3) out of 10.	
			NPSI (0-10)	No Pain (n=5): median 0 (IQR 2), Pain (n=15): median 3 (IQR 6) out of 10.	
Sterling (2010)	WAD n=62, controls n=22	II-III	PPT (kPa)	(R) 197.6 (71) Mild: 220 (77) Moderate/severe: 140.3 (77) (measures from 3 weeks)	Controls: 235 (70) (time 1)
Bowles (2004)	WAD n=25	NA	Electrodiagnostic testing	N=25 patients (positive findings of nerve pathology)	
Greening (2018)	WAD n=9, controls n=13	II	S-LANSS (0-24)	Mean: 12.7 (7.5) n=4 scores ≥12	
			ULNT	Symptomatic side: median n=9, ulnar n=6; Less symptomatic side: median n=4, ulnar n=1 (symptom reproduction)	

			Neurologic examination	n=4 (44.4%) reduced cutaneous sensation in the median nerve	
			Magnetic resonance imaging	Greater T2 signal intensity (brachial plexus, median nerve -wrist): WAD mean= 0.52 ± 0.13 and 2.09 ± 0.33, respectively) compared to the control group (mean= 0.45 ± 0.07 and 1.38 ± 0.31, respectively; p<.05)	
			Nerve palpation	symptomatic side: brachial plexus n=8, median nerve n=5, cubital tunnel n=7, Guyon's canal n=6. less symptomatic side: brachial plexus n=4, median nerve n=1, cubital tunnel n= 3, Guyon's canal n=2 (+ for local or referred pain &/or paresthesia)	
Hashish (2017)	WAD n=903	NA	Electrodiagnostic testing	cervical radiculitis: n= 315; lumbar radiculitis n= 216	
Chien (2009)	WAD n=31, controls n=31	II	ULNT (degrees of elbow flexion)	WAD: 22.3 (27.4)	Controls: 11.0 (5.9)
			PPT (kPa)	WAD: 212.67 (99.17)	Controls: 00.97 (61.26)
			Thermal detection thresholds (°C)	WAD heat index finger: 34.91 (2.29), little finger: 34.43 (2.2). Cold index finger: 28.99 (1.55), little finger: 28.62(2.05)	Controls heat index finger: 32.35 (1.43), little finger: 32.32 (1.12). Cold index: 29.58 (.85), little finger: 29.56 (.82)
			Vibration detection thresholds (μm)	WAD dorsal 5th: 0.48 (0.4), dorsal 2nd: 0.4 (0.27), palmar 2nd: 0.46 (0.31), palmar 1st: 0.79 (0.62)	Controls dorsal 5th: 0.29 (0.12), dorsal 2nd: 0.26 (0.09), palmar 2nd: 0.28 (0.16), palmar 1st: 0.41 (0.25)
			Current detection threshold 2,000 Hz (mA)	WAD elbow: 106.9 (26.64), index finger: 254.44 (55.84), little finger: 193.53 (40.96), tibialis anterior: 186.92 (78.15)	Controls elbow: 88.82 (22.33), index finger: 180 (45.08), little finger: 145.46 (31.88), tibialis anterior: 151.52 (56.24)
			Current detection threshold 250 Hz (mA)	WAD elbow: 41.84 (34.1), index finger: 84.79 (32.23), little finger: 83.65 (40.31), tibialis anterior: 37.26 (14.64)	Controls elbow: 32.61 (8.68), index finger: 62.16 (25.88), little finger: 60.5 (21.89), tibialis anterior: 41.94 (14.45)
			Current detection	WAD elbow: 22 (9.15), index finger:	Controls elbow: 22.16 (10.15), index

			threshold 5 Hz (mA)	46.35 (20.49), little finger: 42.53 (25.79), tibialis anterior: 27.89 (17.38)	finger: 35.23 (16.36), little finger: 34.84 (14.02), tibialis anterior: 23.11 (10.03)
Chien (2008b)	WAD n=52, controls n=31	NA	ULNT (degrees of elbow flexion)	WAD high risk: 51.65 (21.15), low risk: 29.72 (21.83)	Controls: 11.62 (5.96)
			PPT (kPa)	WAD high risk: 173.21 (68.45), low risk: 246.66 (91.57)	Controls: 300.97 (61.26)
			Thermal detection thresholds (°C)	WAD index finger heat: low risk 32.65 (1.42), high risk 32.78 (1.98) Little finger heat: low risk 33.20 (1.94), high risk 33.14 (2.10) Index finger cold: low risk 28.93 (0.75), high risk 28.73 (0.84), little finger cold: low risk 28.68 (0.90), high risk 28.63 (0.93)	Controls index finger heat: 32.35 (1.43), little finger heat: 32.32 (1.12) Index finger cold: 29.32 (0.52), little finger cold: 29.29 (0.50)
			Vibration detection thresholds (μm)	WAD dorsal 5th: low risk 0.41 (0.24), high risk 0.56 (0.57). Palmar 2nd: low risk 0.38 (0.23), high risk 0.56 (0.69). Palmar 1st: low risk 0.51 (0.33), high risk 0.64 (0.54)	Controls dorsal 5th: 0.29 (0.12), Palmar 2nd: 0.28 (0.16), Palmar 1st: 0.41 (0.25)
			Current detection thresholds 250 Hz (mA)*	WAD elbow: low risk 40.96 (10.19), high risk 50.44 (29.62). Index finger: low risk 101.16 (26.84), high risk 124.88 (50.59). Little finger: low risk 99.14 (22.59), high risk 125.09 (82.12).	Controls elbow: 32.61 (8.68), index finger: 62.16 (25.88), little finger: 58.82 (22.40), tibia: 41.94 (14.45)

				Tibia: low risk 80.23 (24.62), high risk 93.68 (60.96)	
Vaegter (2018)	WAD n=108	NA	Warm detection thresholds (°C)	WAD PTSD group = 34.0 (1.3) WAD Non-PTSD group = 34.7 (1.1)	
Greening (2005)	WAD n=9, controls n=8	NA	ULNT (degrees of elbow flexion)	WAD: n=9 (positive for symptom reproduction)	Controls: n=0 (positive for symptom reproduction)
			Nerve palpation	WAD carpal tunnel: n=6, proximal carpal tunnel n=5, brachial plexus n=7 (positive for symptom reproduction)	Controls: n=0 (positive for symptom reproduction)
			Ultrasound	WAD: significantly reduced longitudinal (mean=0.38 (0.08) mm, (95% CI=0.20–0.56 mm)) and transverse nerve movement (2.57 (0.80) mm, (95% CI=0.61–4.54 mm)) on the symptomatic side compared to the control group.	
Pedler (2013)	WAD n=64, controls n=24	I-II	PPT (kPa)	No group mean values provided. Reported significant difference between the left and right sides for median nerve PPT ($p<0.01$)	
Radanov (1995)	WAD n=117	I-III	Neurological examination	n=17 (tests positive findings of nerve pathology)	
Alpar (2002)	WAD n=38, controls n=30	NA	Neurological examination	n=38 with hypoaesthesia to light touch and pin prick (median nerve distribution)	
			Nerve palpation	n=36 (positive symptom reproduction)	
			Electrodiagnostic testing	n=11 patients had abnormal EMG and NCV results	
Pettersson (1994)	WAD n=39	NA	Neurological examination	Trigeminal nerve hypoaesthesia n=9 Reduced myotomal strength n=4 UE hypoaesthesia light touch n=15 UE hyporeflexia n=6 At least one abnormal finding n=19	
Midha (1997)	WAD n=16	NA	Electrodiagnostic testing	n=16 positive studies for nerve pathology	
Miranda (2016)	WAD n=20	NA	Electrodiagnostic testing	n=20 positive studies for nerve pathology	
Jonsson (1994)	WAD n=24	NA	Neurological examination	n=19 patients (positive neurologic findings)	
Braddom (2009)	WAD n=1,334	NA	Electrodiagnostic testing	n=1,248 positive for nerve pathology	
Kaiser (2014)	WAD n=12	NA	Electrodiagnostic testing	n=12 positive for nerve pathology	
Coert (1994)	WAD n=157	NA	Electrodiagnostic testing	n=157 total positive for nerve pathology:	

				carpal tunnel syndrome=68, cubital tunnel syndrome = 64, radial sensory nerve = 25	
Sterling (2006b)	WAD n=65	II-III	PPT (kPa)	Median nerve (recovered, mild, moderate/severe; >1 month): 197.6 (70.6), 231.8 (65.1), 210.5 (74.7) 6 months: 244 (64.6); 140.9 (50.5), 169.9 (54.7)	
			Sympathetic vasoconstrictor reflex	QI (recovered, mild, moderate/severe; >1 month): 58.4 (17.2), 55.7 (16.9); 52.19 (16.9) 6 months: 56.1 (15); 69.68 (18.2), 69.44 (17) SRF (recovered, mild, moderate/severe; >1 month): 0.75 (0.17), 0.75 (0.2); 0.76 (0.18) 6 months: 0.76 (0.17); 0.61 (0.15), 0.63 (0.14)	
Sterling (2005)	WAD n=76	II-III	PPT (kPa)	Recovered, mild, moderate/severe, (>1 month): 197.6 (70.6), 210.5 (74.7), 140.9 (50.5) Recovered, mild, mod/severe, (6 months): 231.8 (65.1); 244 (64.6); 169.9 (54.7)	
			Sympathetic vasoconstrictor reflex	QI (recovered, mild, moderate/severe; >1 month): 58.4 (17.2), 52.19 (16.9), 69.68 (18.2) 6 months: 55.7 (16.9); 56.1 (15); 69.44 (17) SFR (recovered, mild, moderate/severe; >1 month): 0.75 (0.17), 0.76 (0.18), 0.61 (0.15) 6 months: 0.75 (0.2); 0.76 (0.17); 0.63 (0.14)	
Sturzenegger (1994)	WAD n=137	I-III	Neurological examination	N=17 patients (positive findings of nerve pathology)	
Goudman (2020)	WAD n=21, controls n=18	I-III	Laser evoked potential	WAD hand (amplitudes,μV): n1: -4.67 (2.81), N2: -2.54 (1.70), P2: 4.27 (3.11), N2P2: 6.81 (4.32) Latency (msec): N1: 224 (53), N2 (225 (50), P2: 388 (70)	Controls hand (amplitudes,μV): N1 -4.47 (2.37), N2: -3.41 (3.25), P2: 5.56 (2.83), N2P2: 8.97 (5.29) Latency (msec): N1: 252 (56), N2: 229 (54), P2: 374 (59)
Sterner (2001)	WAD n=43	NA	Thermal detection threshold	n=14 patients with abnormal results (trigeminal nerve)	

			Vibration detection threshold	n=11 patients with abnormal results (trigeminal nerve)	
Radanov (1994)	WAD n=117	NA	Neurological examination	N=17 patients (positive findings of nerve pathology)	
Sterling (2002)	WAD n=156, controls n=95	II-III	ULNT (degrees of elbow flexion)*	WAD: 26.21 (11.73)	Controls: 12.92 (14.78)
			Neurological examination	n=23 patients (positive findings of nerve pathology)	
Bekelis (2014)	WAD n=384,539	NA	ICD-9 codes	n=3,086 patients (peripheral nerve injury)	
Lo (2007)	WAD n=20	I-III	Neurological examination	n=10 patients (positive findings of nerve pathology)	
			Electrodiagnostic testing	n=2 patients (positive findings of nerve pathology)	
			Cutaneous silent period	n=18 patients with abnormal findings of at least one recording (measured at hand and foot).	
Sterling (2003)	WAD n=76, controls n=20	II-III	ULNT (degrees of elbow flexion)*	WAD: 26.21 (11.73)	Controls: 12.92 (14.78)
			Sympathetic vasoconstrictor reflex*	WAD recovered QI: 54 (149.98), SRF: 0.79 (1.48) mild QI: 53.1 (147.37), SRF: 0.79 (1.57), mod/severe QI: 64.8 (158.70), SRF: 0.69 (1.31)	Controls QI: 52.3 (82.25) SRF: 0.71 (0.80)
Chien (2010)	WAD n=50, controls n=31	II	Thermal detection threshold (°C)*	WAD heat detection index finger: 34.93 (4.81), little finger: 34.70 (4.94) Cold detection index finger: 28.24 (1.20), little finger: 27.80 (1.98)	Controls heat detection index finger: 32.32 (3.29) little finger: 32.32 (3.73) Cold detection index finger: 29.30 (3.23), little finger: 29.28 (3.28)
			Vibration detection threshold (μm)*	WAD palmar 1st: 0.83 (0.92), palmar 2nd: 0.54 (0.57), dorsal 5th: 0.51 (0.57)	Controls palmar 1st: 0.41 (0.45), palmar 2nd: 0.28 (0.33), dorsal 5th: 0.42 (0.45)
			Current detection threshold 250 Hz (mA)*	WAD elbow: 46.93 (87.60), index finger: 94.27 (170.46), little finger: 87.06 (159.08), tibialis	Controls elbow: 32.48 (29.97), Index finger: 32.38 (34.53), little finger: 58.88 (59.26), tibialis

				anterior: 44.30 (79.04)	anterior: 41.84 (38.32)
			PPT (kPa)	WAD: 187.9 (87.9)	Controls: 301.0 (45.0)
Farrell (2020)	WAD n=24, controls n=24	II	S-LANSS (0-24)	7.5 (6.5)	
			NPSI (0-100)	26.1 (18.3)	
			Neurological examination	N=0 patients (positive for nerve pathology)	
			Thermal detection threshold (°C)	WAD cold index finger: 30.17 (1.16), warm detection index finger: 35.02 (1.55)	Controls cold index finger: 30.75 (0.36), warm index finger: 33.85 (0.47)
			Vibration detection threshold (disappearance)	WAD index: 7.88 (0.27)	Controls index: 7.96 (0.16)
			Mechanical pain threshold (mN)	WAD index: 205.42 (142.47)	Controls index: 161.68 (96.41)
			Mechanical detection threshold (mN)	WAD index: 1.06 (0.82)	Controls index: 0.48 (0.18)
			Intraepidermal nerve fibre density (fibres/mm)	WAD index finger (median (IQR)): 4.5 (4.9) WAD ankle: 7.3 (3.7)	Controls index (median (IQR)): 7.3 (3.9) Ankle: 9.3 (3.8)
			Dermal innervation	WAD index finger (median (IQR)): 3.7 (2.8) bundles/ mm^2 Meissner corpuscles density: (median (IQR)): 0.41 (0.51) corpuscles/mm	Controls index finger (median (IQR)): 4.9 (2.1) bundles/ mm^2 Meissner corpuscles density: (median (IQR)): 0.61 (0.52) corpuscles/mm
Squires (1996)	WAD n=37	NA	Neurological examination	n=4 patients (positive for nerve pathology)	
Chuang (1998)	WAD n=14	NA	Electrodiagnostic testing	n=14 patients (positive for nerve pathology)	
Sturzenegger (1995)	WAD n=117	NA	Neurological examination	n=17 patients (positive for nerve pathology)	
Saadat (2011)	WAD n=78	NA	ICD-9 codes	n=78 patients (positive peripheral nerve injury)	
Moog (2002)	WAD n=43, controls n=43	I-II	Sensation detection	n=0 patients with inability to detect light touch, punctate pressure, warm and cold detection	
			Vibration detection	No mean values provided. All participants reported detection within 10-15% of available frequency.	
Sterling (2006a)	WAD n=76	I-III	PPT (kPa)*	Median nerve (mean/SEM):	

				<1 month, PTSR: 155.12 (80.82), resPTSR: 187.55 (105.77), nonPTSR: 201.72 (76.25) 6 months, PTSR: 166.53 (82.12), resPTSR: 242.78 (76.25), nonPTSR: 230.56 (74.79)	
			Sympathetic vasoconstrictor reflex*	QI (<1 month), PTSR: 70.78 (19.62), resPTSR: 59.75 (20.1), nonPTSR: 55.42 (19.61) QI (6 months), PTSR: 70.66 (19.14), resPTSR: 57.48 (19.86), nonPTSR: 57.48 (19.86) SFR (<1 month), PTSR: 0.56 (.20), resPTSR: 0.70 (.20), nonPTSR: 0.75 (0.20) SFR (6 months), PTSR: 0.60 (0.20), resPTSR: 0.74 (0.20), nonPTSR: 0.74 (0.20)	
Wallin (2012)	WAD n=28,	II-III	Thermal detection threshold (°C)*	WAD cold: thenar 29.8 (1.3), trapezius=29.8 (2.3), tibialis anterior= 28.2 (3.6) Warm thenar: 34.1 (2.0), trapezius: 35.9 (2.5), tibialis anterior: 37.8 (4.7)	Controls cold: thenar: 30.7 (0.4), trapezius=30.9 (1.4), tibialis anterior=29.1 (1.4) Warm thenar: 33.5 (0.4), trapezius: 35.1 (1.4), tibialis anterior: 37.2 (2.7)
Raak (2006)	WAD n=17, controls n=18	NA	Thermal detection threshold (°C)	WAD thenar warm: 35.03 (2.67), cold 29.42 (2.12). trapezius warm 37.94 (4.39), cold 28.75 (4.94)	Controls thenar warm: 33.71 (0.57), cold: 29.88 (1.26). trapezius warm: 35.80 (3.13), cold: 30.28 (1.17)
Mailis (1995)	WAD n=32	NA	Nerve palpation	N=32 patients (positive for symptom reproduction upon pressure)	
Kaiser (2012)	WAD n=75	NA	Electrodiagnostic testing	n=75 studies (positive for nerve injury)	
Chien (2008a)	WAD n=50, controls n=31	II	ULNT (degrees of elbow flexion)	WAD: 21.3 (25.5)	Controls: 11.0 (5.21)
			Thermal detection threshold (°C)*	WAD (mean, 95% CI), heat, index finger: 34.91 (34.05, 35.63), little finger: 34.71 (33.78, 35.63) Cold index finger: 28.27 (27.32, 29.22), little finger: 27.82 (26.75, 28.90)	Controls (mean, 95% CI), heat, index finger: 32.35 (31.83, 32.88), Little finger: 32.32 (31.91, 32.73) Cold index finger: 29.32 (29.13, 29.51), little finger: 29.29 (29.10, 29.47)

			Current detection threshold 250 Hz (mA)*	WAD (mean, 95% CI) elbow: 47.13 (36.24, 58.02). index finger: 94.15 (80.78, 107.52) little finger: 86.81 (74.98, 98.64) tibialis anterior: 44.43 (37.03, 51.83)	Controls (mean, 95% CI) elbow: 32.61 (29.43, 35.80), index finger: 62.16 (52.67, 71.65), little finger: 58.82 (50.61, 67.04), tibialis anterior: 41.94 (36.64, 47.24)
			Vibration detection threshold (μm)*	WAD (mean, 95% CI): palmar 1st: 0.83 (0.64, 1.02), palmar 2nd: 0.54 (0.38, 0.65), dorsal 5th: 0.51 (0.36, 0.65)	Controls (mean, 95% CI): palmar 1st: 0.41 (0.32, 0.50),palmar 2nd: 0.28 (.022, 0.34), dorsal 5th: 0.29 (0.43, 0.71)
			PPT (kPa)*	WAD (mean, 95% CI): 196.00 (171.35, 220.66)	Controls (mean, 95% CI): 300.97 (278.5, 323.44)
Maimaris (1988)	WAD n=102	NA	Neurological examination	n=18 patients (positive for nerve pathology)	
Ovadia (2002)	WAD n=866	NA	Neurological examination	n=20 patients (positive for nerve pathology)	
			Electrodiagnostic testing	n=127 studies with abnormal findings (EMG)	
Steinberg (2005)	WAD n=330	I-II	Electrodiagnostic testing	n=104 studies with abnormal findings (EMG)	
Terzis (2009)	WAD n=25	NA	Electrodiagnostic testing	n=25 studies positive test for nerve pathology	
Scott (2005)	WAD n=29	II	PPT (kPa)*	WAD median: 162.68 (243.90), ulnar: 281.55 (263.94), radial: 191.04 (243.90)	Controls median: 274.55 (255.47), ulnar: 373.22 (285.19), radial: 296.80 (232.17)

*mean/sd estimated from graph or transformed from alternatively reported summary statistic.
Abbreviations: (NR): non-recovered; (R): recovered; DN4: Douleur Neuropathique 4**;** EMG: electromyography; ICD: International Classification of Diseases; IQR: interquartile range; NCV: nerve conduction velocity; nonPTSR: non-posttraumatic stress reaction; NPSI: Neuropathic Pain Symptom Inventory; PPT: pressure pain threshold; PTSD: Posttraumatic Stress Disorder**;** PTSR: Posttraumatic stress reaction; QI: quotient interval; QTF: Quebec Task Force; NA: not available; resPTSR: resolved posttraumatic stress reaction; S-LANSS: Self-complete Leeds Assessment of Neuropathic Symptoms and Signs; SEM: standard error of the mean; SRF: sympathetic reflex; UE: upper extremity**;** ULNT: upper limb neurodynamic test: WAD: whiplash associated disorders.

2.3.1. Quality assessment

Newcastle-Ottawa Quality Assessment Scales is summarised in Supplemental Table 2.3. The median score is 7 (range 3 - 10) for cross-sectional studies, 5 (range 3 - 8) for cohort studies, and 5.5 (range 5 - 6) for case-control studies indicating a moderate risk of bias on average, with studies ranging from low to high risk of bias. The comparability of subjects and controls based on study design is the most common limitation. The total score agreement between raters was 87.7%.

2.3.2. Evidence of nerve pathology and neuropathic pain in WAD 1 - 4.

In total, 19 assessments are utilised to assess neuropathic pain or peripheral nerve pathology. The use of normative values is not required as all meta-analysed studies included their own control groups.

The findings of studies including all WAD severity grades (1 -4) are categorised by type of outcome measure (Figure 2.2 and Supplemental Table 2.4). Mechanical, current, and thermal detection thresholds are measured at multiple sites including the thumb, index finger, little finger, upper trapezius muscle, and anterior tibialis muscle and were meta-analysed separately. Neural mechanosensitivity of the median nerve include data on upper limb neurodynamic testing (measured as degrees of elbow flexion) and pressure pain thresholds measured over peripheral nerves (PPT; using an algometer). Individual studies that report participant subcategories (e.g.,

mild pain vs moderate/severe pain, recovered vs non-recovered, etc) are indicated in the analyses.

The most commonly used assessments for nerve pathology after whiplash injury are PPT over peripheral nerves and nerve palpation (17 studies,[13,16,17,104,117-120,126,163,189,209-214]), electrodiagnostic testing (16 studies,[211,215-229]), and clinical neurological examination (16 studies[40-42,117,162,211,223,227,230-237]). Four studies[13,118,212,213] assess sympathetic vasoconstrictor responses. Two studies use diagnostic ICD-9 coding for nerve injury and involvement[225,238]. Additional assessments of nerve pathology from single studies include cutaneous silent periods[223], laser evoked potentials[239], intraepidermal nerve fibre density[40], MRI[117], and ultrasound[163] (Table 2.1 and Supplemental Table 2.4).

2.3.3. Prevalence of neuropathic pain

The prevalence of neuropathic pain signs and symptoms is determined in five studies by two questionnaires (S-LANSS and DN4). The prevalence scores indicating the presence of neuropathic pain characteristics have a mean of 34% (range 25-75%, n=208 in all grades of WAD severity[40,117,190,191,209]. Two studies use the NPSI to evaluate the severity of neuropathic pain symptoms with a median score of 3 out of 10 (interquartile range: 6, n=20)[190] and mean score of 26.1 out of 100 (SD 18.3, n = 24)[40]. See Table 2.1 and Supplemental Table 2.4 for a summary of study assessments and outcomes.

Table 2.2 includes a summary of the certainly of neuropathic pain for each study according to the neuropathic pain grading system. Seven studies (13%) include sufficient tests so that a grading of definite neuropathic pain can be reached in a subgroup of patients. Nineteen studies (35%) reach a grading of probable and 18 of possible neuropathic pain (33%). Ten studies (19%) are classed as 'nerve pathology' as the absence of sensory testing prevent a firm conclusion of definite neuropathic pain.

2.3.4. Prevalence of nerve pathology

The mean prevalence of nerve pathology identified by clinical examination varies according to the assessment used: neurological examination is 13% (range 0 - 100%, n = 1,885)[40-42,117,162,211,223,227,230-237] and electrodiagnostic testing is 32% (range 10 - 100%, n = 3,921)[211,215-229]. ICD-9 codes related to nerve pathology and nerve injury include n = 384,617 patients from two studies with a nerve injury mean prevalence of 1% (range 1 - 100%)[225,238].

Mechanical Detection

All three locations where vibration detection thresholds are reported demonstrated significantly impaired vibration thresholds in patients compared to controls (Figure 2.2a). This difference is significant at all locations measured in the hand, including the thumb (SMD 0.51 [0.29; 0.74] p=0.0032, $I^2 = 0\%$), index finger (SMD 0.65 [0.30;

1.00] p<0.005, $I^2 = 25\%$), and little finger (SMD 0.45 [0.13; 0.78] p=0.0183, $I^2 = 7\%$) compared to controls with heterogeneity that may not be considered important. One study shows a statistically significant decrease in mechanical detection thresholds using von Frey hairs but not mechanical pain threshold at the index finger compared to healthy controls (Table 2.1)[40].

Current Detection

Studies measuring current detection thresholds find significant differences at the index finger (SMD 0.82 [0.25; 1.39] p=0.0165, I^2 =67%), little finger (SMD 0.84 [0.05; 1.64] p=0.0425, I^2 =82%), and elbow (SMD 0.49 [0.06; 0.92] p=0.0337, $I^2 = 43\%$). However, the current detection threshold over the tibialis anterior muscle is not statistically significant between patients and controls (SMD 0.58 [-0.60; 1.75] p=0.2435, $I^2 = 91\%$). All current detection measures have moderate to considerable between study heterogeneity (Figure 2.2b).

Thermal Detection

In total, six studies measure thermal detection in multiple upper extremity locations (Figure 2.2c). Cold detection thresholds are significantly impaired at the thumb (SMD -0.66 [-1.08; -0.24] p=0.0023, I^2=57%), index finger (SMD -0.43 [-0.73; -0.13] p=0.0204, I^2 =0%), and trapezius muscle (SMD -0.51 [-0.93; -0.10] p= 0.0154, I^2=0%), but not at the little finger (SMD -0.46 [-0.96; 0.04] p=0.0574, $I^2 = 0\%$) in patients compared to controls.

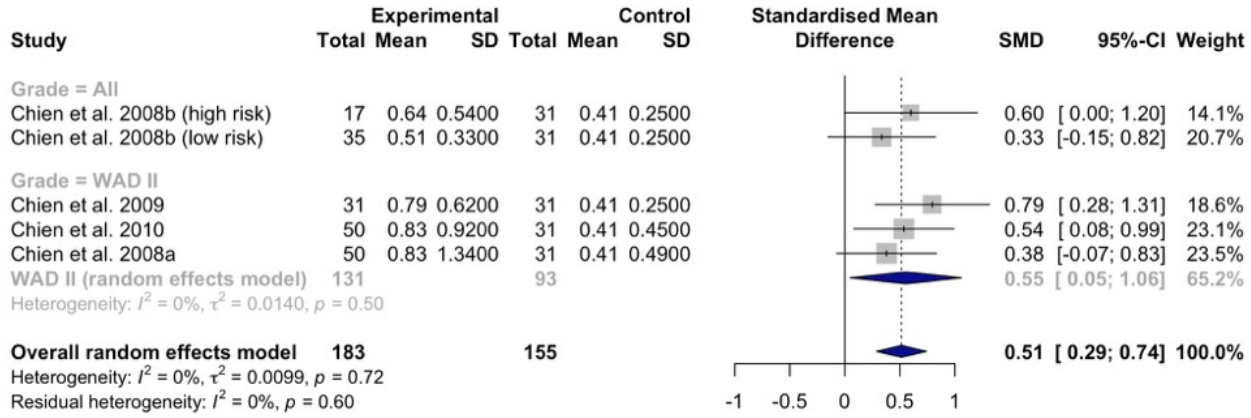
Warm detection thresholds show significant impairments at the thumb (0.51 [0.10; 0.93] p=0.0161, I^2=0%), index finger (SMD 0.84 [0.25; 1.42] p=0.0200, I^2 = 49%), and trapezius muscle (SMD 0.45 [0.04; 0.87] p=0.0329, I^2=0%), but not at the little finger (SMD 0.68 [-0.24; 1.61] p=0.0866, I^2 = 53%). Between-study heterogeneity ranges from not considered important to moderate. Thermal detection thresholds at the tibialis anterior muscle are measured in one study[208], which find a significant impairment in left-sided but not right-sided warm detection compared to controls.

Neural Mechanosensitivity

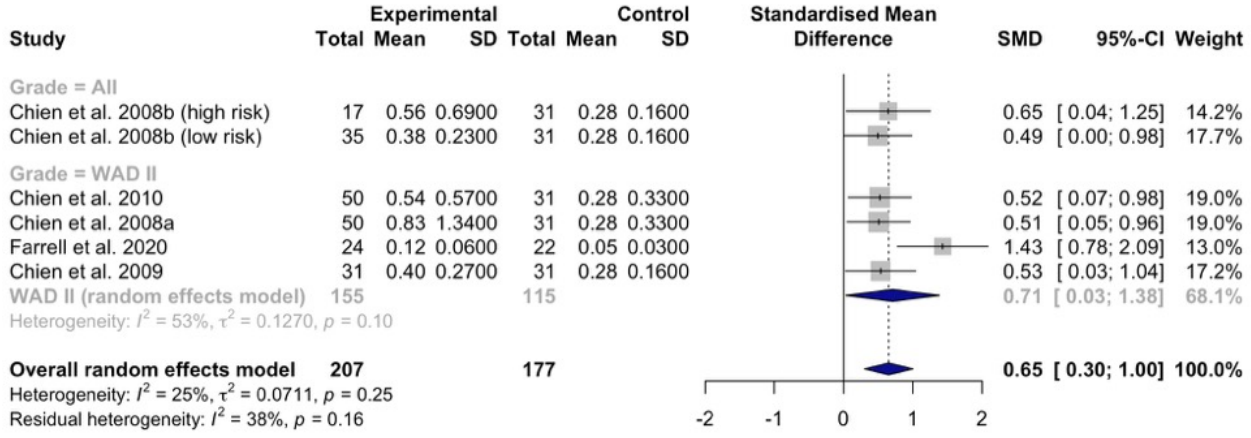
Eight studies and a total of n = 527 patients and n = 389 healthy controls are included in the neural mechanosensitivity meta-analysis. There is a significant difference in both elbow range of motion during median nerve neurodynamic testing (SMD 1.68 [0.92; 2.44], p=0.0004, I^2 = 91%) and PPT over the median nerve at the elbow (SMD -1.10 [-1.50; -0.70], p<0.0001, I^2 =78%) compared to controls (Figure 2.2d); both with considerable between-study heterogeneity. The average proportion of patients who report symptom reproduction upon median nerve palpation was 91% (range 67% -100%, n = 56 total patients)[117,163,211] and 94% (range 78 - 100%, n = 50 total patients) upon brachial plexus palpation[117,163,240].

a. Mechanical Detection

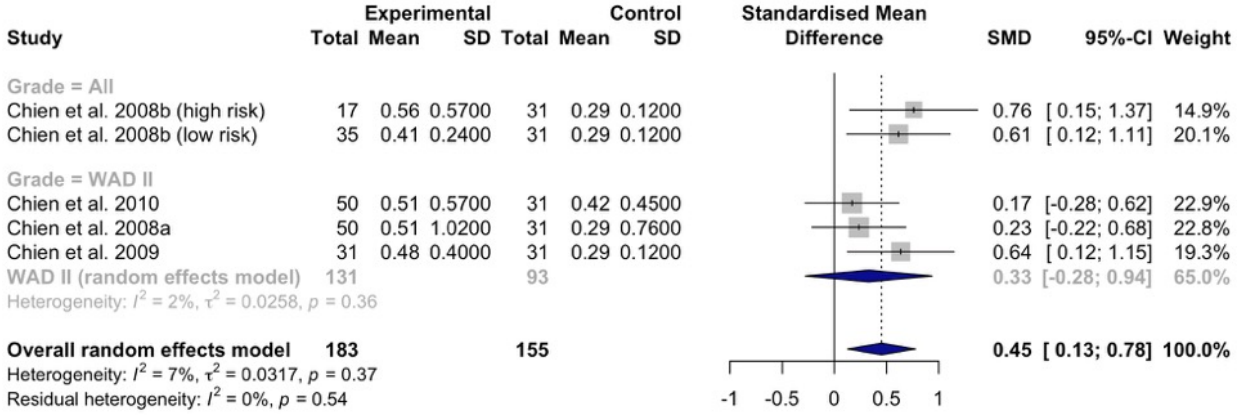
Vibration detection threshold – thumb



Vibration detection threshold – index finger

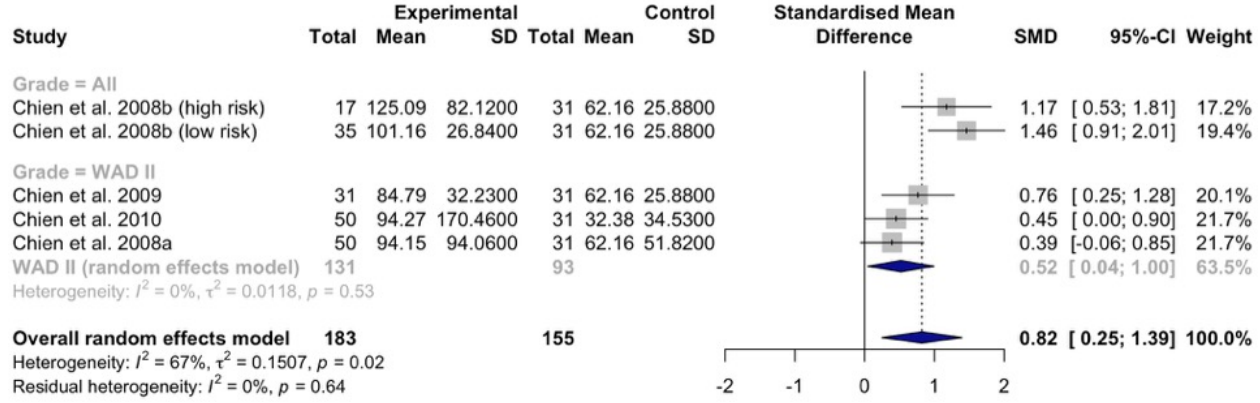


Vibration detection threshold – little finger

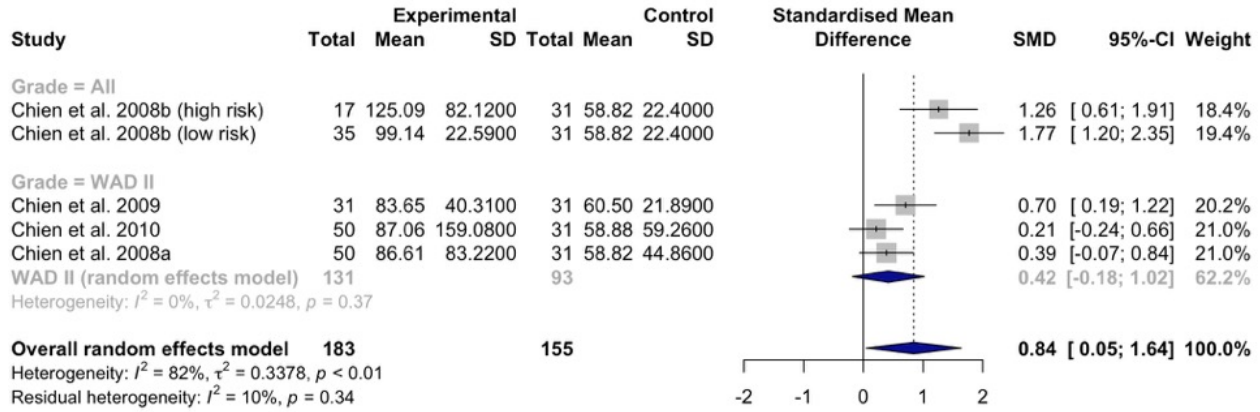


b. Current Detection

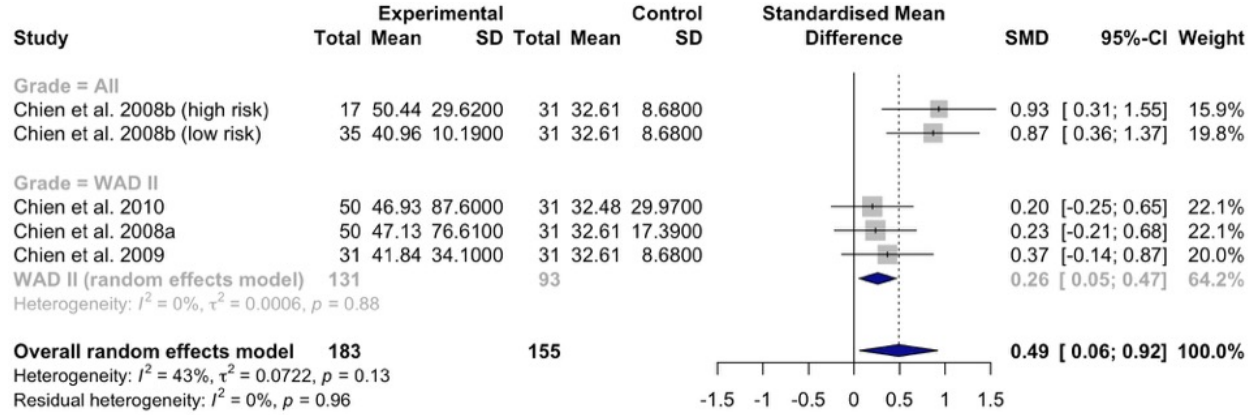
Current detection threshold – index finger



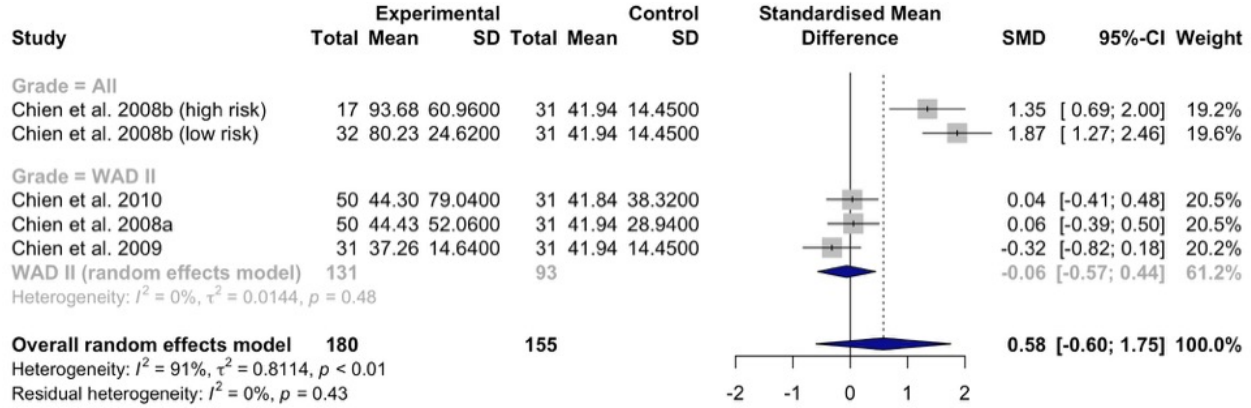
Current detection threshold – little finger



Current detection threshold - elbow

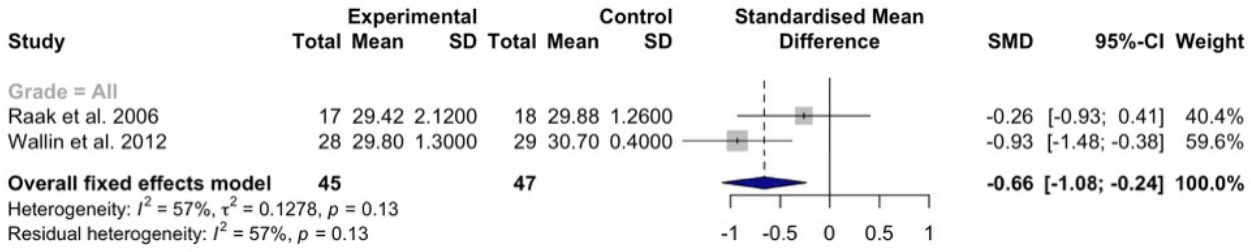


Current detection threshold – tibialis anterior

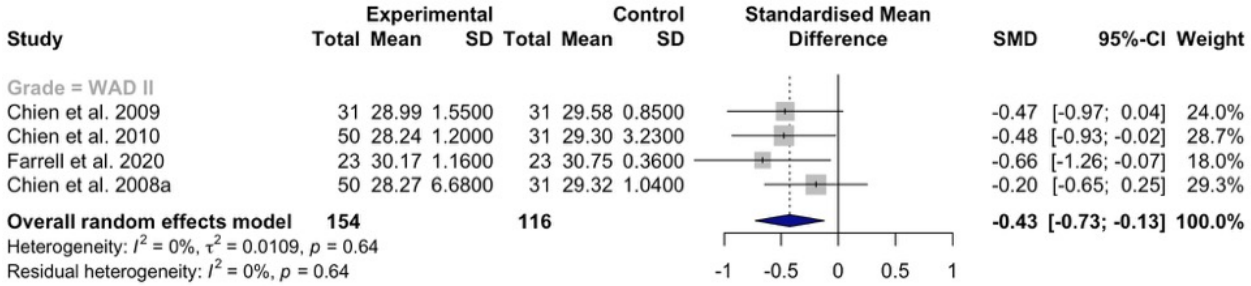


c. Thermal Detection

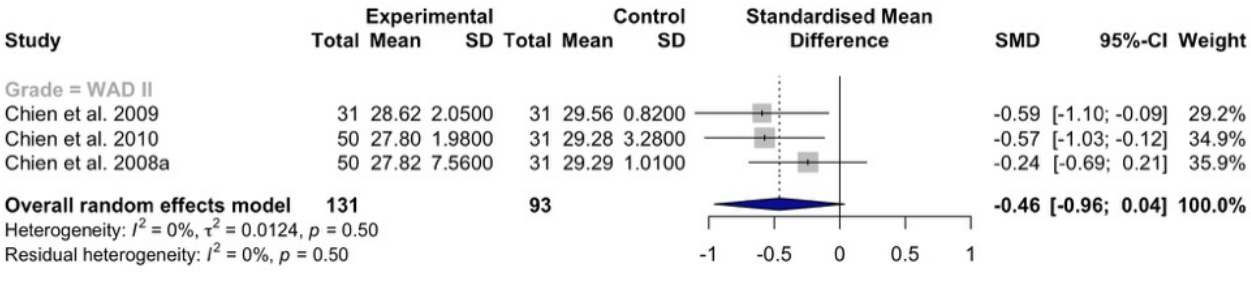
Cold detection threshold – thumb



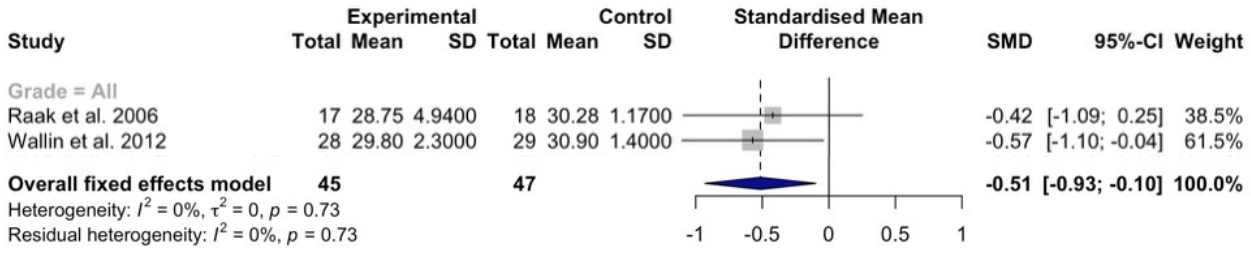
Cold detection threshold – index finger



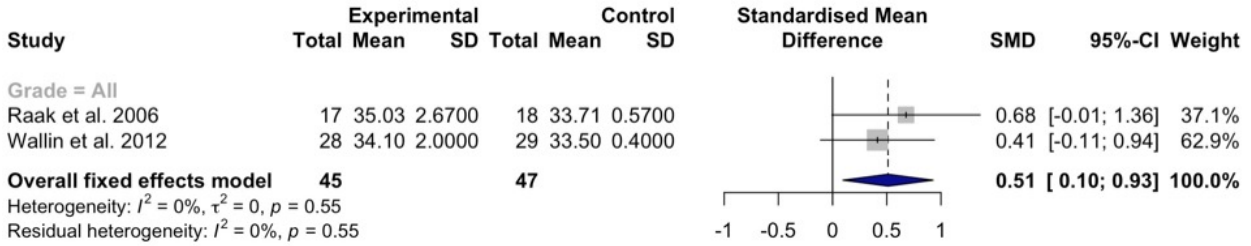
Cold detection threshold – little finger



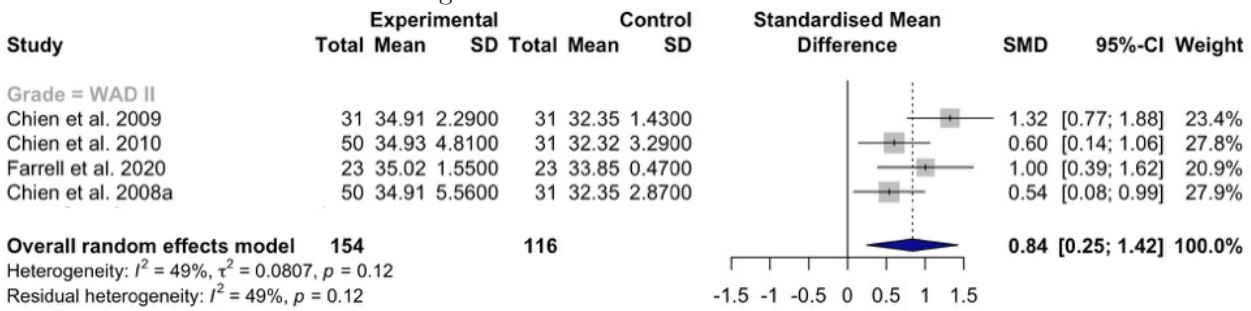
Cold detection threshold – trapezius muscle



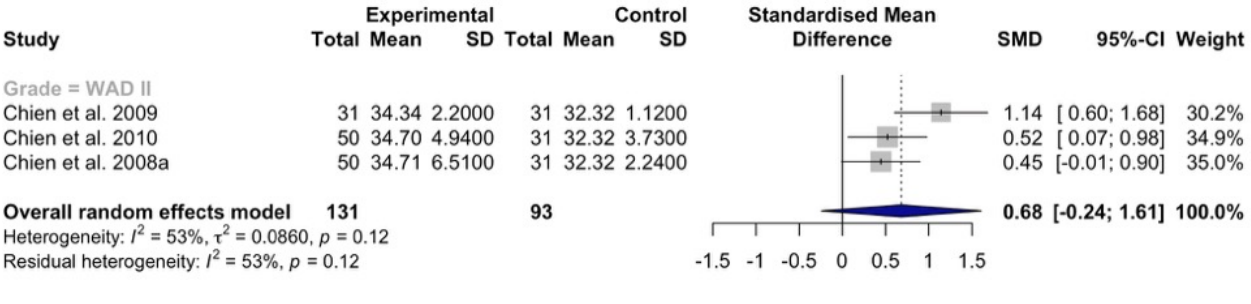
Warm detection threshold – thumb



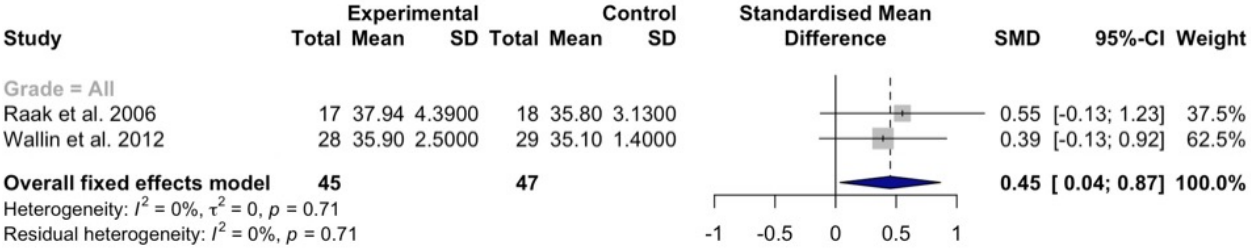
Warm detection threshold – index finger



Warm detection threshold – little finger



Warm detection threshold – trapezius muscle



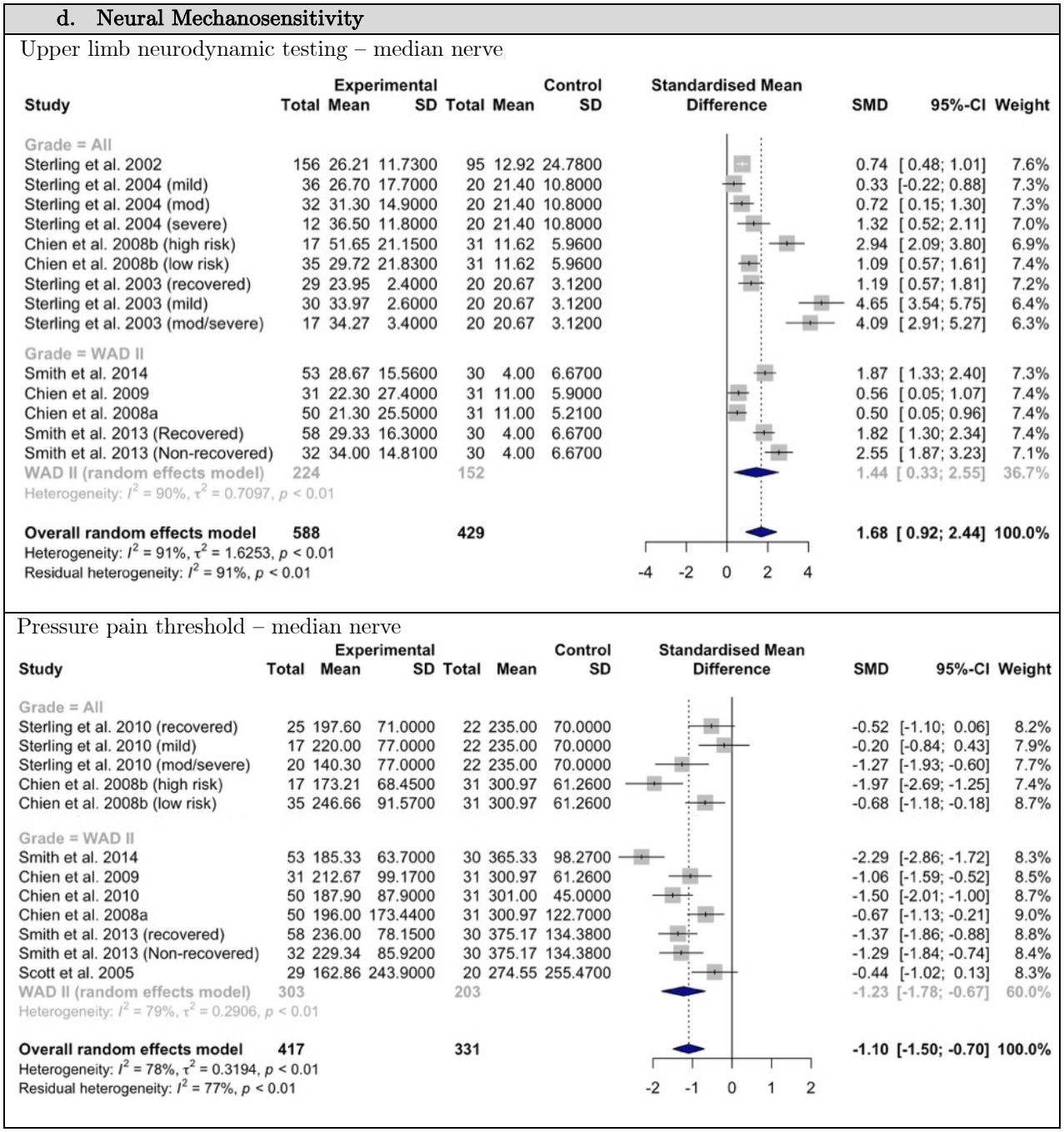


Figure 2.2. Meta-analysis of detection threshold measures and neural mechanosensitivity.
Studies are subgrouped based on the Quebec Task Force grading scale. Overall effects, standardised mean differences (SMD), 95% confidence intervals (CI), and I^2 heterogeneity are summarised for two meta-analyses: 1) including the overall data from all studies independent of WAD grades ("All") and 2) for studies only including patients with grade 2 ("WAD2").

2.3.5. Other assessments

Four studies (n = 293)[13,118,212,213] assess sympathetic vasoconstrictor response with a mean quotient of integral of 59.42 (SD 7.13) and sympathetic reflex quotient of 0.72 (SD 0.70) listed in Supplemental Table 2.4. One study (n = 20) assessing cutaneous silent periods find abnormalities suggestive of peripheral nerve involvement[223]. In contrast, another study (n = 21) measuring laser evoked potentials does not find a difference between patients with WAD 1-3 and healthy controls[239]. Five additional studies use sensory testing parameters that are not comparable for meta-analysis[210,234,241-243] but most findings are consistent with the presence of a sensory deficit; complete outcome details provided in Table 2.1.

Two imaging studies both report signs of nerve involvement. Using MRI, one study finds greater T2-weighted signal intensity of the brachial plexus and median nerve at the wrist compared to controls[117]. Another imaging study using high frequency ultrasound identifies biomechanical changes to median nerve excursion at the forearm and wrist[163]. Lastly, a significant decrease in intraepidermal nerve fibre and dermal nerve bundle densities are apparent in skin biopsies of the index finger compared to controls[40].

2.3.6. Evidence of nerve pathology and neuropathic pain in WAD2.

Eight studies report separate data for patients classified as only WAD2 and are sub-grouped for meta-analysis (Figure 2.2 and Supplemental Table 2.4). Additional

assessments of peripheral nerve pathology in WAD2 include mechanical detection using von Frey hairs[40]; T2-weighted signal intensity of the peripheral nerves using MRI[117]; biomechanical changes to nerve excursion using high frequency ultrasound[163]; and structural intraepidermal nerve fibre and dermal nerve bundle density using skin biopsies [40].

Prevalence of Neuropathic Pain

Using the S-LANSS, mean prevalence scores indicating the presence of neuropathic pain characteristics are 34% (range 25% - 36%, n = 123) in WAD2[40,117,209]. One study used the NPSI and reports a mean (SD) of 26.1 (18.3) out of 100 (n = 24)[40]. Using the IASP neuropathic pain grading system, two studies (25%) have sufficient tests to reach the grade of definite neuropathic pain in at least a subgroup of patients. Three studies (38%) reach a grade of probable neuropathic pain. Three studies (38%) are classified as possible neuropathic pain. As all studies include reports of pain and sensory testing results, no studies are classed as 'nerve pathology' (Table 2.2).

Table 2. Certainty of neuropathic pain at study level according to the IASP Neuropathic Pain Grading System.

	Possible	**Probable**	**Definite**	
Article	History neurologic lesion & neuroanatomically plausible	Sensory signs	Diagnostic tests	**Outcome**
Sterling 2009	Patients after whiplash injury reporting neck pain, S-LANSS (34% positive)	NA	NA	Possible
Smith 2013	Patients after whiplash injury reporting neck pain, S-LANSS (36% positive)	NA	NA	Possible
Karlsborg 1997	Patients after whiplash injury reporting neck pain	n=5/34 patients with upper extremity sensory loss (light touch)	NA	Probable
Henrikson 2013	Patients after whiplash injury reporting neck pain	n=5/20 reduced temperature sensitivity	NA	Probable
Chuang 2002	Included patients after whiplash injury measuring the brachial plexus	NA	n=7/85 positive NCV and EMG findings of nerve pathology	Nerve pathology
Smith 2014	Patients after whiplash injury reporting neck pain, 21% reporting upper extremity symptoms	NA	NA	Possible
Sterling 2004	Patients after whiplash injuring reporting neck pain	NA	NA	Possible
Serrano-Munoz 2019	Pain after whiplash injury, DN4 (n=15/20 indicating neuropathic pain), and NPSI questionnaires (median score pain group: 3/10)	NA	NA	Possible
Sterling 2010	Patients after whiplash injuring reporting neck pain, 48% reporting upper limb symptoms	NA	NA	Possible
Bowles 2004	Included patients after whiplash injury measuring the brachial plexus	NA	n=25/25 patients with positive EMG findings of nerve pathology	Nerve pathology

Greening 2018	Patients after whiplash injury reporting painful symptoms in upper limb, S-LANSS questionnaire (n=4/9 patients indicating neuropathic pain)	n=4/9 (44.4%) reduced cutaneous sensation in median nerve	MRI: increased T2 signal intensity brachial plexus and median nerve at wrist	Definite
Hashish 2017	Patient after whiplash injury referred to pain clinic for cervical and lumbar nerve assessment	NA	Positive EMG testing: cervical radiculitis: n= 315/903; lumbar radiculitis n= 216/903	Nerve pathology
Chien 2009	Patients after whiplash injury reporting neck pain, 45% reporting arm pain	Abnormal upper extremity thermal, vibration, and current detection thresholds	NA	Probable
Chien 2008b	Patients after whiplash injury reporting neck pain	Abnormal upper extremity thermal, vibration, and current detection thresholds	NA	Probable
Vaegter 2018	Patients after whiplash injury reporting spinal pain	Abnormal upper extremity warm detection thresholds	NA	Probable
Greening 2005	Patients after whiplash injury reporting neck and arm pain	NA	NA	Possible
Pedler 2013	Patients after whiplash injury reporting pain	NA	NA	Possible
Radanov 1995	Patients with pain after whiplash injury, 49% reporting shoulder pain, 92% reporting neck pain, 15% reporting dermatomal paraesthesia, tingling	n=17/117 patients neurologic deficit (sensory loss, reflex loss, paresis)	NA	Probable
Alpar 2002	Patients after whiplash injury reporting neck and shoulder pain	n=38/38 with hypoaesthesia to light touch and pin prick (median nerve distribution)	n=11/38 patients had abnormal EMG and NCV results	Definite

Pettersson 1994	Patients after whiplash injury reporting neck pain, 69% shoulder pain	Trigeminal nerve hypoaesthesia n=9/39 Reduced myotomal strength n=4/39 UE hypoaesthesia light touch n=15/39 UE hyporeflexia n=6/39	NA	Probable
Midha 1997	Included patients after whiplash injury measuring the brachial plexus	NA	n= 16/16 abnormal EMG or NCV results	Nerve pathology
Miranda 2016	Included patients after whiplash injury	NA	n=20/20 abnormal EMG and NCV studies for nerve pathology	Nerve pathology
Jonsson 1994	Patients after whiplash injury reporting neck pain, 79% radiating arm pain	n=19/24 patients with decreased strength, sensation, or reflexes	NA	Probable
Braddom 2009	Patients after whiplash injury referred to pain clinic for cervical and lumbar nerve assessment	NA	n=1,248/1,334 abnormal EMG studies for nerve pathology	Nerve pathology
Kaiser 2014	Included patients after whiplash injury measuring the brachial plexus	NA	n=12/12 abnormal EMG and NCV studies for nerve pathology	Nerve pathology
Coert 1994	Included patients after whiplash injury measuring the median, radial, or ulnar nerves	NA	n=157/157 abnormal EMG or NCV for nerve pathology	Nerve pathology
Sterling 2006b	Patients after whiplash injury reporting neck pain, 20% reported shoulder pain	NA	NA	Possible
Sterling 2005	Patients after whiplash injury reporting neck pain, 30% reporting shoulder pain	NA	NA	Possible
Sturzenegger 1994	Patients after whiplash injury reporting pain, 35% reported neurologic symptoms, 49% reported shoulder pain	N=17/137 patients had neurologic deficit (sensory loss, reflex loss, or paresis with radicular distribution)	NA	Probable

Goudman 2020	Patients after whiplash injury reporting pain	NA	No significant differences in laser evoked potentials	Possible
Sterner 2001	Patients after whiplash injury reporting pain, 47% report radiating pain in arms and hands, 59% report paraesthesia in arms and hands	n=14/43 patients abnormal thermal detection and n=11/43 patients abnormal vibration detection (trigeminal nerve)	NA	Probable
Radanov 1994	Patients after whiplash injury reporting neck pain, 49% reported shoulder pain	N=17/117 patients neurologic deficit in radicular pattern (weakness, hyporeflexia, or hypoaesthesia)	NA	Probable
Sterling 2002	Patients after whiplash injury reporting pain	n=23/156 patients (weakness, hyporeflexia, or hypoaesthesia)	NA	Probable
Bekelis 2014	Patients after whiplash injury, n=3,086 patients ICD-9 codes for peripheral nerve injury	NA	NA	Possible
Lo 2007	Patients after whiplash injury reporting neck pain	n=10/20 patients (weakness, hyporeflexia, or hypoaesthesia)	n=2/20 with abnormal EMG testing, n=18 with at least one abnormal recording of cutaneous silent periods	Definite
Sterling 2003	Patients after whiplash injury reporting neck pain	NA	NA	Possible
Chien 2010	Patients after whiplash injury reporting neck pain, 45% reported radiating arm pain	Reduced thermal, vibration, and current detection thresholds	NA	Probable

Farrell 2020	Patients after whiplash injury reporting neck pain, 46% shoulder or arm pain, 17% forearm or hand pain, S-LANSS and NPSI questionnaires	N=0/24 patients with abnormal strength, reflexes, and light touch sensation. Findings of reduced thermal and mechanical pain thresholds	Reduced dermal and intraepidermal nerve fibre density (skin biopsy)	Definite
Squires 1996	Patients after whiplash injury reporting pain, 45% report paraesthesia	n=4/37 patients (weakness, hyporeflexia, or hypoaesthesia)	NA	Probable
Chuang 1998	Included patients after whiplash injury measuring the brachial plexus	NA	n=14/14 abnormal EMG and NCV studies for nerve pathology	Nerve pathology
Sturzenegger 1995	Patients after whiplash injury reporting neck pain	n=17/117 patients (weakness, hyporeflexia, or hypoaesthesia)	NA	Probable
Saadat 2011	Included patients after whiplash injury, n=78 patients positive for peripheral nerve injury using ICD-9 codes	NA	NA	Possible
Moog 2002	Patients after whiplash injury reporting pain	n=0/43 patients with inability to detect light touch, punctate pressure, warm and cold detection	NA	Possible
Sterling 2006a	Patients after whiplash injury reporting neck pain	NA	NA	Possible
Wallin 2012	Patients after whiplash injury reporting neck and shoulder pain	Reduced thermal detection thresholds	NA	Probable
Raak 2006	Patients after whiplash injury reporting pain	Reduced thermal detection thresholds	NA	Probable
Mailis 1995	Patients after whiplash injury reporting pain, 84% reported paraesthesia	NA	NA	Possible

Kaiser 2012	Included patients after whiplash injury measuring the brachial plexus	NA	n=75/75 abnormal EMG or NCV studies for nerve injury)	Nerve pathology
Chien 2008a	Patients after whiplash injury reporting neck pain, 45% reported radiating arm pain	Reduced thermal, vibration, and current detection thresholds	NA	Probable
Maimaris 1988	Patients after whiplash injury reporting pain, 46% reported shoulder pain	n=18/102 patients (weakness, hyporeflexia, or hypoaesthesia)	NA	Probable
Ovadia 2002	Patients after whiplash injury reporting pain, 25% reporting shoulder pain, 36% reporting upper limb pain	n=20/866 patients (weakness, hyporeflexia, or hypoaesthesia)	n=127/866 with abnormal EMG findings	Definite
Steinberg 2005	Patients after whiplash injury reporting pain, 7% reported radiating shoulder pain	NA	n=104/330 with abnormal EMG findings	Nerve pathology
Terzis 2009	Included patients with pain after whiplash injury measuring the brachial plexus	NA	n=25/25 abnormal NCV and EMG findings for nerve pathology	Nerve pathology
Scott 2005	Patients after whiplash injury reporting pain	NA	NA	Possible

Abbreviations: Douleur Neuropathique 4 (DN4), Electromyography (EMG), Not available (NA), Nerve conduction velocity (NCV), Neuropathic Pain Symptom Inventory (NPSI), Self-complete Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS). *Nerve pathology indicates studies that reported outcomes of diagnostic tests confirming a lesion of the somatosensory nervous system (definite neuropathic pain) but did not report sensory signs (probable neuropathic pain).*

Mechanical Detection

Vibration detection thresholds are measured at the thumb, index and little fingers (Figure 2.2a). Overall, there are significantly impaired vibration detection thresholds at the thumb (SMD 0.55 [0.05; 1.06] p=0.0422, $I^2 = 0\%$) and index finger (SMD 0.71 [0.03; 1.38] p= 0.0446, $I^2 = 53\%$), but no difference at the little finger (0.33 [-0.28; 0.94] p=0.1448, $I^2 = 2\%$) compared to controls. Heterogeneity ranges from might not be important to moderate. As previously reported, one study including only WAD2

finds a significant reduction in mechanical detection using von Frey hairs but preserved mechanical pain at the index finger compared to controls[40].

Current Detection

Current detection thresholds of WAD2 are significantly higher at the index finger (SMD 0.52 [0.04; 1.00] p=0.0427, I^2 = 0%) and elbow (SMD 0.26 [0.05; 0.47] p=0.0332; I^2 = 0%), but not at the little finger (SMD 0.42 [-0.18; 1.02] p=0.0961, I^2 = 0%) or tibialis anterior muscle (SMD -0.06 [-0.57; 0.44] p=0.6537, I^2 = 0%) compared to healthy controls (Figure 2.2b). Overall heterogeneity was very low.

Thermal Detection

The previously described thermal detection thresholds for the index and little fingers include only WAD2 and can be seen in Figure 2.2c.

Neural Mechanosensitivity

Six studies report PPT of the median nerve at the elbow and four studies report median nerve neurodynamic testing (Figure 2.2d). Compared to controls, there are significantly restricted elbow range of motion during median nerve neurodynamic testing (SMD 1.44 [0.33; 2.55] p=0.0225, I^2 = 90%) and lower median nerve PPT (SMD -1.23 [-1.78; -0.67] p=0.0016, I^2 =79%) in patients with WAD2. Both analyses demonstrate substantial heterogeneity. The proportion of patients who report symptom reproduction upon nerve palpation of the brachial plexus and median nerve

range from 78% - 88.9% and 55.6% - 66.7%, respectively in two studies (n = 18)[117,163].

Other assessments

Single studies using MRI, high frequency ultrasound and skin biopsies all find indications of nerve involvement (Table 2.1 and Supplemental Table 2.4).

2.4. Discussion

My systematic review including 54 studies in 390,644 patients suggests that after whiplash injury, a subset of people demonstrate signs of peripheral nerve injury and neuropathic pain. These findings were seen irrespective of whiplash severity grading, and importantly, were also present in WAD2. These data contradict the traditional definition of WAD2, which is defined by an absence of nerve involvement. The included studies utilised a varied set of clinical measures and questionnaires to identify signs of nerve pathology and neuropathic pain. The mean prevalence estimates of nerve pathology in WAD ranged from 1% (ICD-9 codes) to 32% (electrodiagnostic testing). The prevalence of neuropathic pain measured with questionnaires ranged from 34% to 75%. Measures of nerve function revealed abnormalities in large nerve fibre apparent by the presence of muscle weakness, hyporeflexia, hypoaesthesia to light touch and vibration, and abnormal electrodiagnostic testing. Small nerve fibre pathology was recognised via reduced temperature, pin prick, current detection thresholds, and decreased intraepidermal

nerve fibre density. Several studies demonstrated heightened nerve mechanosensitivity, and imaging studies suggested altered nerve movement and structural abnormalities using high frequency ultrasound and MRI, respectively.

Neuropathic pain is reported by a significant group of patients with WAD.

Pooled from four studies and 208 patients, the S-LANSS identified 34% of patients with predominant neuropathic pain characteristics. When using the DN4 questionnaire, one study found estimates of neuropathic pain as high as 75% in a smaller sample size (n = 20)[190]. The prevalence of neuropathic pain appears in contrast to the low prevalence of nerve pathology from ICD-9 codes (1%). This disparity, though, is primarily based on one large retrospective study (n = 384,539) using ICD-9 codes which only included peripheral nerve injuries in WAD that were present with an accompanying upper or lower extremity fracture[238]. Conversely, estimates of neuropathic pain from questionnaires closely align with nerve pathology using electrodiagnostic testing (32%).

The neuropathic pain grading system[194] helps to determine the certainty of neuropathic pain. Unfortunately, no study used the grading system at patient level. We therefore performed retrospective grading at study level, thus providing information about at least a subset of patients. Most studies met the possible neuropathic pain category, assuming the history of a whiplash injury itself has the potential to include nerve involvement for a subset of patients[10] and that pain

referral to the neck or upper limbs is neuroanatomically plausible as it is unlikely that single root levels are involved[16,123,136]. Fourteen studies provided evidence of sensory signs in the upper extremity or neck predominantly though quantitative sensory testing. Although quantitative sensory testing cannot conclusively demonstrate neuropathic pain, they are considered a confirmatory diagnostic test in the grading system to reach the category of 'probable' neuropathic pain[194,244]. Lastly, 18 studies confirmed a lesion of the somatosensory nervous system through diagnostic tests (e.g., electrodiagnostic tests, MRI). As many of these studies (10) did not include sensory testing, we took a conservative approach and only classified eight as 'definite' neuropathic pain.

Taken together, the data from questionnaires and retrospective neuropathic pain grading suggest that a significant portion of patients with WAD experience at least possible neuropathic pain. This illustrates the importance of clinical screening for neuropathic pain symptoms.

Sensory loss of function is apparent across a range of modalities.

A hallmark of nerve pathology and peripheral neuropathic pain is the presence of sensory loss of function in the anatomical territory of the suspected lesion of the peripheral nervous system[194]. We did not include gain of function measures (thermal and mechanical pain thresholds, wind-up ratios, etc) as hyperalgesia is not only a feature of neuropathic but also nociceptive[245,246] or nociplastic pain[247,248]. Overall,

the sensory testing results show a loss of function affecting both large (vibration, light touch) and small nerve fibres (temperature) in patients with WAD compared to healthy controls. Sensory dysfunction was present throughout the entire upper extremity, but most consistently seen in thumb and index finger. Lower extremity sensory assessment included current and thermal detection thresholds at the tibialis anterior, which was not significantly different from controls. This suggests there is altered sensory processing in at least a subset of patients after whiplash injury. One explanation for these functional sensory deficits may be structural nerve fibre loss, which was identified in one study taking skin biopsies[40].

Similar findings of loss of function dominate a range of focal nerve injuries, including lumbar radiculopathy[249], carpal tunnel syndrome[250], and various traumatic peripheral nerve lesions[251]. As such, a direct nerve injury resulting from the collision may explain the identified loss of function. The theory that whiplash injury causes peripheral nerve injury is supported by sensory testing, neurological examination, and electrodiagnostic testing[16,217,231]. Both preclinical and clinical data suggest sensory hypoaesthesia[252] can occur as early as one week after peripheral nerve injury. These sensory abnormalities may indicate functional or structural nerve pathology, such as ischaemia[253,254], demyelination or axon degeneration[255,256].

Alternatively, upper extremity sensory loss of function may be a downstream effect that develops from secondary mechanisms rather than from a direct nerve injury.

Indeed, subtle sensory hypoaesthesia has been identified in non-neuropathic conditions [257,258] and in non-painful limbs of unilateral peripheral neuropathies[259]. It has been speculated that such hypoaesthesia in the absence of an apparent nerve lesion could be attributed to central mechanisms[259], which are known to not only modulate painful but also non-painful sensory input[258,260].

Another potential secondary mechanism explaining sensory loss of function is inflammatory processes triggered after a motor vehicle crash[51,261,262]. Elevated systemic inflammation has previously been linked with widespread sensory hypoaesthesia in other painful conditions such as fibromyalgia[165] and complex regional pain syndrome[166]. Indeed, preclinical models of traumatic nerve injury suggest that pathological neuroinflammation has a role in inducing axonal degeneration[263,264]. This hypothesis is supported by radiological findings of increased T2 signal intensity of the brachial plexus and median nerve in patients with chronic WAD[117], which has been interpreted as a clinical correlate of neuroinflammation[265]. Additionally, increased levels of serum inflammatory markers have been identified from patients with chronic WAD[51,262].

As such, systemic or central mechanisms, in addition to direct traumatic nerve injury, may explain the reported sensory abnormalities. Further studies evaluating the temporal development and spatial distribution of neural loss of function could shed light on the nature of mechanisms driving the sensory hypoaesthesia in WAD. In individual patients, the findings of sensory hypoaesthesia need to be considered

in the context of the patient's history, clinical presentation, and additional assessments.

Clinical findings of nerve mechanosensitivity are present in some patients after whiplash injury.

This review identified the presence of heightened median nerve mechanosensitivity to nerve elongation or pressure. Such nerve mechanosensitivity in patients is consistent with findings of nociceptive axonal mechanical sensitivity reported in animal models of localised peripheral neuroinflammation[266-268]. Although these findings may demonstrate nerve involvement, they do not necessarily confirm direct nerve pathology or neuropathic pain as nerve mechanosensitivity can also be present in patients without apparent nerve injury. Consistent with this, PPT over peripheral nerves has shown heightened sensitivity in both neuropathic[189,269,270] and traditionally non-neuropathic pain conditions, such as tension-type headache[271] and epicondylalgia[269]. Furthermore, upper limb neurodynamic tests do not demonstrate diagnostic accuracy in detecting peripheral neuropathic pain[272] as they can be negative in patients with clear nerve involvement[273] or positive in patients with traditionally non-neuropathic conditions such as non-specific neck and arm pain[274] and fibromyalgia[275]. Therefore, although the findings of heightened nerve mechanosensitivity in WAD are intriguing and warrant further exploration, care must be taken in their interpretation regarding neuropathic pain or structural nerve pathology.

Neuropathic pain and nerve dysfunction is present irrespective of WAD severity grading.

Whereas nerve pathology and neuropathic pain may not be surprising in patients with WAD3 (defined by the presence of neurological signs), our findings strongly suggest there is nerve involvement even in some patients with WAD2. This was apparent by the self-reports of neuropathic pain in 34% of WAD2 patients (LANSS)[40,117,209]. In addition, multiple measures showed abnormal findings, including reduced neural excursion on ultrasound[163] and increased T2 weighted signal intensity on MRI[117], reduced nerve fibre density from skin biopsy [163], and measures of sensory hypoaesthesia[17,40,120,189]. Of note, the findings in the WAD2 cohort were comparable to the analysis including all WAD grades, suggesting that the findings are not purely driven by more severe WAD grades.

Our findings directly challenge the widely used Quebec Task Force definition, in which patients with WAD2 are characterised by musculoskeletal signs including decreased range of motion and point tenderness in the absence of neurological deficits[10]. Although still utilised, the Quebec Task Force classification system has long received criticism regarding its over-simplified classifications[276,277] with suggestions to modify grade 2 for over twenty years[187]. More modern classifications have been proposed incorporating recent advances in psychological and physiological variables related to recovery[14,188]. Nevertheless, the original Quebec Task Force grading system remains popular because of its simplicity[11]. This may be contributing

to the diagnostic difficulties and poor clinical outcomes of WAD and importantly WAD2, which is the most prevalent group of WAD severity[188]. Taking our findings into account, the current grading system likely oversimplifies a heterogenous group of patients which may require distinct treatment approaches.

Clinical implications

This review suggests that not all patients may fit the traditionally defined categories of WAD 1 – 4[10]. As we identified dysfunction in both the large and small nerve fibres, a comprehensive clinical neurological examination extending beyond the traditional light touch, muscle strength and reflex testing and including small fibre tests (e.g., thermal thresholds) is critical for these patients. Small fibre pathology has been shown to precede findings of inherent large fibre pathology in patients with focal nerve injury[278,279], but this remains to be shown for patients with WAD. Furthermore, we may have to consider the sensitivity of the traditional neurological examination in detecting sensory loss. Our findings suggest that quantitative sensory testing methods demonstrate dysfunctions in patients who are classified as having no neurological deficit upon routine clinical neurological examination. It remains to be explored whether more sensitive detection of sensory changes impacts the prediction of patient outcomes or choice of intervention.

An incomplete clinical assessment may also create dissonance between subjective reports of neuropathic symptoms that lack corresponding objective findings.

Qualitative reports of patient challenges highlight difficulties with feeling understood or properly treated, which contribute to prolonged distress and trauma[280]. Similarly, some patients reported their WAD symptoms did not match the management strategies suggested by their healthcare provider[281]. Including a detailed evaluation may improve personal patient challenges and may also help direct more targeted management strategies.

Importantly, the management of neuropathic pain differs from nociceptive pain[282]. Current treatment guidelines for WAD2 do not include management strategies for nerve-related pathology or neuropathic pain[61,283]. Our findings suggest that this may need to be considered for a subset of patients. There are currently several efforts underway to examine the benefit of targeted neuropathic treatments for patients with WAD[192,284]. Such studies are required to determine whether interventions targeting neuropathic pain and nerve pathology may be beneficial in a subset of patients.

Limitations

The primary limitations of this study are the overall risk of bias and some data heterogeneity. Many studies had a risk of bias, which was often due to small sample sizes and comparability of selected outcome groups. High data heterogeneity was seen in some meta-analyses, particularly regarding nerve mechanosensitivity. It is also important to consider potential publication bias. Negative findings for nerve

pathology and neuropathic pain might be less likely to be reported. Lastly, limitations in generalisability involve the inclusion of only English language articles, single author screening for initial abstract eligibility, and that some meta-analyses included studies from only one research group.

Conclusions

Our data suggest that nerve pathology and signs of neuropathic pain are present in a subset of patients after whiplash injury. Importantly, this included patients categorised as WAD2, who are traditionally classified by the lack of neurological signs. Therefore, including detailed clinical assessments and clinically screening for neuropathic pain is recommended for patients with WAD. Future research including large prospective cohorts is needed to identify underlying mechanisms of nerve pathology and neuropathic pain and to evaluate whether targeting treatments at neuropathic pain and nerve pathology improves clinical outcomes of this specific subgroup of patients with whiplash injuries.

Supplemental Table 2.1. Example search strategies for all included search engines.

PubMed

S1	(MH "Whiplash") OR (MH "neck injuries)
S2	(MH Cohort studies) OR (MH Case-control studies) OR (MH observational study) OR (MH Cross-sectional studies)
S3	S1 AND S2
S4	(MH peripheral nervous system disease) OR (MH somatosensory disorder) OR (MH neuropathic pain) OR (MH nerve disorder)
S5	(MH magnetic resonance imaging) OR (MH diffusion tensor imaging) OR (MH ultrasonography)
S6	('painDETECT' OR 'Douleur Neuropathique en 4 question' OR 'DN4' OR 'S-LANSS' OR 'Neuropathic Pain Symptom Inventory' OR 'Neuropathic Pain Questionnaire' OR 'ID Pain' OR 'Neuropathic Pain Scale')
S7	(MH quantitative sensory testing) OR (neurological examination) OR (MH muscle strength) OR (MH electrodiagnosis) OR (MH neural conduction) OR ('dermatome OR 'myotome') OR (sympathetic reflexes) OR (small fibre neuropathy OR small fiber neuropathy OR small fibre sensory neuropathy OR small fiber sensory neuropathy OR small fibre pathology OR small fiber pathology) OR (intraneural change)
S8	(MH prevalence) OR (MH incidence) OR (MH epidemiology)
S9	S3 AND S4
S10	S3 and S5
S11	S3 AND S6
S12	S3 AND S7
S13	S3 AND S4 AND S8

Embase and MEDLINE

1	'whiplash'/exp OR 'traffic accident'/exp OR 'whiplash associated disorder'/exp
2	'cohort study'/exp OR 'case control study'/exp OR 'observational study'/exp OR 'cross-sectional study'/exp
3	1 and 2
4	'peripheral nervous system disease' OR 'somatosensory disorder'/exp OR 'neuropathic pain'/exp OR 'nerve disorder' OR 'neuralgia'/exp OR 'peripheral nerve injury'/exp OR 'cervical plexus'/exp OR 'spinal nerve roots'/exp
5	'neurological exam'/exp OR 'muscle strength'/exp OR 'electrodiagnosis'/exp OR 'neural conduction' OR 'dermatome' OR 'myotome' OR 'sympathetic reflex' OR 'small fibre neuropathy' OR 'small fiber neuropathy' OR 'small fibre sensory neuropathy' OR 'small fiber sensory neuropathy' OR 'small fibre pathology' OR 'small fiber pathology' OR 'intra neural change' OR 'quantitative sensory testing' OR 'muscle weakness' OR 'sensory testing'
6	'douleur neuropathique 4 questions' OR 'leeds assessment of neuropathic symptoms and signs' OR 'paindetect' OR 'dn4' OR 'lanss' OR 's-lanss' OR 'neuropathic pain symptom inventory' OR 'neuropathic pain questionnaire' OR 'id pain' OR 'neuropathic pain scale'
7	'magnetic resonance imaging' /exp OR 'diffusion tensor imaging'/exp OR 'ultrasonography'/exp
8	'prevalence'/exp OR 'incidence'/exp OR 'epidemiology'/exp
9	3 & 4
10	3 & 5
11	3 & 6
12	3 & 7

13	3, 4, & 8

Cinahl (EBSCO)

S1	Whiplash OR Whiplash injuries OR whiplash disorders OR motor vehicle collision
S2	MW cohort study OR case-control study OR observational study OR cross-sectional study
S3	(MW cohort study OR case-control study OR observational study OR cross-sectional study) AND (S1 AND S2)
S4	Peripheral nervous system disease OR somatosensory disorder OR neuropathic pain OR nerve disorder OR neuralgia OR peripheral nerve injury OR cervical plexus OR spinal nerve roots
S5	Neurological examination OR muscle strength OR electrodiagnosis OR neural conduction OR "dermatome" OR "myotome" OR "sympathetic reflexes" OR "small fibre neuropathy" OR "small fiber neuropathy" OR small fibre sensory neuropathy" OR "small fiber sensory neuropathy" OR "intraneural change" OR "quantitative sensory testing" OR muscle weakness OR sensory testing
S6	"Douleur Neuropathique 4 Questionnaire" OR "Leeds Assessment of Neuropathic Symptoms and Signs" OR "painDETECT" OR "DN4" OR "LANSS" OR S-LANSS" OR "Neuropathic Pain Symptom Inventory" OR "Neuropathic Pain Questionnaire" OR "ID Pain" OR "Neuropathic Pain Scale"
S7	Magnetic resonance imaging OR diffusion tensor imaging R ultrasonography
S8	Prevalence OR incidence OR epidemiology
S9	S3 AND S4
S10	S3 AND S5
S11	S3 AND S6
S12	S3 AND S7
S13	S3 AND S4 AND S8

Supplemental Table 2.2. Characteristics of included studies: design and population characteristics

Author & Date	Study Design	Study Participants	Female participants	Age of participants (years): mean (SD)
Sterling (2009)	Cross-sectional	WAD n=85	WAD n = 54	WAD=36.27 (12.69)
Smith (2013)	Cross-sectional	WAD n=90, controls n=30	WAD n=58, controls n=21	WAD recovered= 44.3 (10.4), Non-recovered=45.4 (9.7), controls=44.2 (9.7)
Karlsborg (1997)	Cohort	WAD n=34	WAD n=20	WAD=35.5
Henrikson (2013)	Cohort	WAD n=20, controls n=10	WAD n= 10	NA
Chuang (2002)	Cross-sectional	WAD n=85	NA	NA
Smith (2014)	Cohort	WAD n=53, controls n=30	WAD n=36, controls n=21	WAD=44.7 (10.9), controls=44.2 (9.7)
Sterling (2004)	Cohort	WAD n=80, controls n=20	WAD n=56, controls n=11	WAD=33.5 (14.7), controls= 39.5 (14.6)
Serrano-Munoz (2019)	Cohort	WAD n=20, controls n=15	WAD n=16, controls n=9	WAD No Pain (n=5)=40.8 (4.6), Pain (n=15)=39.7 (3.1), controls=40.5 (3.4)
Sterling (2010)	Cohort	WAD n=62, controls n=22	WAD n= 36, controls n= 14	WAD=36.1 (13.13), controls=40.1 (13.6)
Bowles (2004)	Cross-sectional	WAD n=25	WAD = 5	WAD = 37.5 (15.5)
Greening (2018)	Cross-sectional	WAD n= 9, controls n = 13	WAD n=6, controls n= 6	WAD =46.8 (2.9), controls=38.9 (10.8)
Hashish (2017)	Cross-sectional	WAD n=903	WAD n=488	WAD=34.3 (18.04)
Chien (2009)	Cross-sectional	WAD n=31, controls n=31	WAD n=25, controls n=25	WAD=35.3 (10.7), controls=31.4 (8.9)
Chien (2008b)	Cross-sectional	WAD n=52, controls n=31	WAD n=32, controls n=25	WAD=36.3 (13.1), controls=31.4 (8.9)
Vaegter (2018)	Cross-sectional	WAD n=108	WAD n=58	WAD=45.7 (11.6)
Greening (2005)	Cross-sectional	WAD n=9, controls n=8	WAD n=5, controls n=4	WAD=37.2 (9.87), controls=40.8 (8.2)
Pedler (2013)	Cross-sectional	WAD n=64, controls n=24	WAD n=35, controls n=14	WAD=44.7 (12.6), controls=40.3 (13.4)
Radanovf (1995)	Cohort	WAD n=117	WAD n=68	WAD =30.7 (9.6)

Alpar (2002)	Cohort	WAD n=38, controls n=30	WAD n=28, controls n=18	WAD=37.5, controls=34.2
Pettersson (1994)	Cohort	WAD n=39	WAD n=20	WAD=32 (range: 18-52)
Midha (1997)	Cross-sectional	WAD n=16	NA	NA
Miranda (2016)	Cross-sectional	WAD n=20	NA	NA
Jonsson (1994)	Cohort	WAD n=24	WAD n=13	WAD=36.5 (9.6)
Braddom (2009)	Case-control	WAD n=1,334	NA	WAD= 40
Kaiser (2014)	Cross-sectional	WAD n=12	NA	NA
Coert (1994)	Cross-sectional	WAD n=157	NA	NA
Sterling (2006)	Cohort	WAD n=65	WAD n=56	WAD=36.27 (12.69)
Sterling (2005)	Cohort	WAD n=76	WAD n=53	WAD=36.27 (12.69)
Sturzenegger (1994)	Cohort	WAD n=137	WAD n=81	WAD=30.8 (9.6)
Goudman (2020)	Case-control	WAD n=21, controls n=18	WAD n=10, controls n=11	WAD=45.8 (40.41–51.08), controls=46.8 (27.73 - 51.21)
Sterner (2001)	Cohort	WAD n=43	WAD n=24	WAD=36.2 (23–62)
Radanov (1994)	Cohort	WAD n=117	WAD n=68	WAD=30.8 (9.6)
Sterling (2002)	Cohort	WAD n=156, controls n=95	WAD n=127, controls n=50	WAD=37.43 (9.3), controls=38.95 (14.47)
Bekelis (2014)	Cohort	WAD n=384,539	WAD n=164,292	WAD=40.45 (19.33)
Lo (2007)	Cohort	WAD n=20	WAD n=10	WAD=42 (23–60)
Sterling (2003)	Cohort	WAD n=76, controls n=20	WAD n=54, controls n=12	WAD=36.27 (12.69), controls=40.1 (13.6)
Chien (2010)	Cross-sectional	WAD n=50, controls n=31	WAD n=39, controls n=25	WAD=37.2 (10.4), controls=31.4 (8.9)
Farrell (2020)	Cross-sectional	WAD n=24, controls n=24	WAD n=16	WAD=49 (15 IQR), controls=50 (17 IQR)
Squires (1996)	Cohort	WAD n=37	NA	WAD symptomatic=40.7, WAD asymptomatic=30.7
Chuang (1998)	Cross-sectional	WAD n=14	WAD n=6	WAD=38.26 (14.85)
Sturzenegger (1995)	Cohort	WAD n=117	WAD n=73	WAD=30.8 (9.5)
Saadat (2011)	Cross-sectional	WAD n=78	NA	NA

Moog (2002)	Case-control	WAD n=43, controls n=43	WAD n=28, controls n=28	WAD=37 (12-66), controls=NA
Sterling (2006)	Cohort	WAD n=76	WAD n=53	WAD=33.5 (14.7)
Wallin (2012)	Cohort	WAD n=28,	WAD n=28	WAD=40.1 (7.1)
Raak (2006)	Cohort	WAD n=17, controls n=18	WAD n=16, controls n=17	WAD=50.8 (11.3), controls=44.8 (10.2)
Mailis (1995)	Cohort	WAD n=32	WAD n=23	WAD=27.5 (23-55)
Kaiser (2012)	Cross-sectional	WAD n=75	WAD n=10	NA
Chien (2008a)	Cross-sectional	WAD n=50, controls n=31	WAD n=39, controls n=25	WAD=37.2 (10.4), controls=31.4 (8.9)
Maimaris (1988)	Cohort	WAD n=102	WAD n=58	WAD=37 (17-72)
Ovadia (2002)	Cohort	WAD n=866	WAD n=439	WAD=38 (26-50)
Steinberg (2005)	Cohort	WAD n=330	WAD n=193	WAD=19 (18)
Terzis (2009)	Cross-sectional	WAD n=25	NA	NA
Scott (2005)	Case-control	WAD n=29 controls n=20	WAD n=17 controls n=12	WAD=41.6 (10), controls=31.25 (10)

Supplemental Table 2.3. Quality Assessment using the Newcastle-Ottawa Scales

Author	Year	Selection	Comparability	Outcome	Total score
Cross-sectional		(/5)	(/2)	(/3)	(/10)
Sterling et al.	2009	4	0	3	7
Smith et al.	2013	4	2	2	8
Chuang et al.	2002	3	1	3	7
Bowles et al.	2004	2	0	3	5
Greening et al.	2018	4	2	3	9
Hashish et al.	2017	2	0	2	4
Chien A et al.	2009	4	1	2	7
Chien et al.	2008	4	1	2	7
Vaegter et al.	2018	5	0	2	7
Greening et al.	2005	4	1	3	8
Pedler et al.	2013	5	1	2	8
Midha	1997	3	0	2	5
Miranda et al.	2016	3	0	0	3
Kaiser et al.	2014	3	0	3	6
Coert et al.	1994	3	0	2	5
Chien et al.	2010	4	1	2	7
Farrell et al.	2020	5	2	3	10
Chuang et al.	1998	2	0	2	4
Saadat et al.	2011	2	1	2	5

Kaiser et al.	2012	3	0	3	6
Chien et al.	2008	4	1	3	8
Terziset al.	2009	3	0	0	3
Cohort		(/4)	(/2)	(/3)	(/9)
Karlsborg et al.	1997	2	0	3	5
Henrikson et al.	2013	0	1	3	4
Jull et al.	2014	3	2	3	8
Sterling et al.	2004	4	1	3	8
Serrano-Munoz	2019	3	1	2	6
Sterling	2010	3	1	3	7
Radanov et al.	1995	2	1	2	5
Alpar et al.	2002	2	0	3	5
Pettersson et al.	1994	2	0	3	5
Jonsson et al.	1994	2	0	3	5
Sterling et al.	2006	2	1	3	6
Sterling et al.	2005	2	1	3	6
Sturzenegger et al.	1994	3	1	1	5
Sterner et al.	2001	2	0	1	3
Radanov et al.	1994	3	1	2	6
Sterling et al.	2002	4	0	2	6
Bekelis et al.	2014	3	0	1	4
Lo et al.	2007	3	1	3	7
Sterling et al.	2003	2	1	3	6
Squires et al.	1996	1	0	2	3
Sturzenegger et al.	1995	3	1	2	6
Sterling et al.	2006	2	1	2	5
Wallin et al.	2012	1	1	2	4
Raak et al.	2006	1	0	2	3
Mailis et al.	1995	2	1	2	5
Maimaris et al.	1988	2	0	2	4
Ovadia et al.	2002	2	0	3	5
Steinberg et al.	2005	2	0	3	5
Case-control		(/4)	(/2)	(/3)	(/9)
Braddom et al.	2009	3	1	2	6
Goudman et al.	2020	3	1	1	5
Moog et al.	2002	4	1	1	6
Scott et al.	2005	3	1	1	5

Supplemental Table 2.4. Synthesis of results by type of outcome measure.
Please note this does not include the sensory testing studies which are reported in Figure 2.2.

Measure	Number of studies	Number of participants	WAD grade	Outcome	Interpretation
S-LANSS	4	208	I-III	A combined total n=71 WAD participants had positive scores ≥12. When subdivided by WAD2, 42 of 123 participants had scores ≥12.	34% of both the total WAD population as well as patients with WAD2 had scores suggestive of neuropathic pain (≥12)
DN4	1	20	I-III	No Pain (n=5): median 3 (IQR 1.5), Pain (n=15): median 4 (IQR 3) out of 10.	75% (of 20 participants) had scores suggestive of neuropathic pain (≥4).
NPSI	2	44	I-III	WAD I-III: No Pain (n=5): median 0 (IQR 2), Pain (n=15): median 3 (IQR 6) out of 10. WAD2: Mean (SD) score: 26.1 (18.3) out of 100.	Overall, participants had low severity (3/10 and 26.1/100) of neuropathic pain symptoms.
Neurological examination	16	1,885	I-IV	n=241 pooled patients had positive findings	13% of pooled WAD patients had positive findings
Electrodiagnostic testing	16	3,921	I-III	n=1,249 pooled participants had positive findings of peripheral nerve pathology	32% of pooled WAD participants had positive findings
Magnetic resonance imaging	1	9	II	Greater T2 signal intensity (brachial plexus, median nerve - wrist): WAD mean= 0.52 ± 0.13 and 2.09 ± 0.33, respectively) compared to the control group (mean= 0.45 ± 0.07 and 1.38 ± 0.31, respectively; P<.05).	These findings suggest that patients with chronic whiplash may have peripheral nerve pathology measured at the wrist and brachial plexus.
Ultrasound	1	9	II	WAD participants had significantly reduced longitudinal [mean=0.38 (0.08) mm, (95% CI=0.20–0.56 mm)] and transverse nerve movement [2.57 (0.80) mm, (95% CI=0.61–4.54 mm)] on the symptomatic side	The results suggest nerve sliding impairments at both the forearm and the wrist compared to healthy controls.

				compared to the control group.	
Sympathetic vasoconstrictor reflex	4	293	II-III	SVR results: Quotient of Integrals (QI) mean 59.42 (7.13). Sympathetic Reflex (SFR)= 0.72 (.70)	These findings may suggest sympathetic nerve dysfunction.
Laser evoked potentials	1	21	I-III	No between-group differences (WAD and controls) for stimulus intensity (A-delta fibers), amplitudes and latencies of laser evoked potential wave components.	This study did not find a functional deficit in sensory afferent a-delta nerve fibers.
ICD-9 codes (nerve injury)	2	384,617	NA	n=3,164 participants with ICD-9 codes for peripheral nerve injury	1% of pooled WAD data showed signs of nerve injury or neuropathic pain identified through ICD-9 codes.
Cutaneous silent periods	1	20	I-III	18 of 20 participants demonstrated abnormal findings of at least one CSP recording (measured at hand and foot).	These findings may indicate peripheral nerve dysfunction, but larger studies are needed.
Intraepidermal nerve fiber density	1	24	II	Index finger: WAD median (IQR) IENFD 4.5 (4.9) fibres/mm compared with 7.3 (3.9) fibres/mm for the control group (p = .010, r = .37.) Ankle: WAD mean [SD]: IENFD 7.3 [3.7] fibres/mm; control group: 9.3 [3.8] fibres/ mm (p = .09).	IENFD demonstrated structural peripheral nerve pathology at the index finger of participants with WAD2.
Nerve palpation	4	88	I-III	An average of 91% (range 67-100%, n=56 total patients) and 94% (range 78-100%, n=50 total patients) had symptom reproduction with palpation of the median nerve and brachial plexus respectively.	Median nerve and brachial plexus palpation reproduced pain and symptoms suggestive of neural mechanosensitivity in a majority of patients in the

					included four studies.

Abbreviations: CSP: Cutaneous silent period; DN4: Douleur Neuropathique 4**;** ICD: International Classification of Diseases; IENFD: intraepidermal nerve fibre density; IQR: interquartile range; NPSI: Neuropathic Pain Symptom Inventory; NA: not available; SD: standard deviation; S-LANSS: Self-complete Leeds Assessment of Neuropathic Symptoms and Signs; WAD: whiplash associated disorders.

3

Blood-based biomarkers of nerve pathology in peripheral neuropathy.

3.1. Introduction

Peripheral neuropathies are common conditions with an estimated prevalence up to 7% in the general population[285] and an increasing global burden with older age.[286] Patients with peripheral neuropathy often experience significant pain, weakness, and paraesthesia that can result in poor balance, limited mobility, increased risk of lower-limb amputation, and shorter mean survival compared to age- and sex-matched controls.[287] The complex clinical presentations of peripheral neuropathies can lead to delayed or missed diagnosis[288] and often have limited treatment strategies.[289]

For central neurological conditions, biomarkers have led to significant advances in the diagnosis, prognosis, and management of numerous conditions including Alzheimer's Disease,[290] traumatic brain injury,[291] Multiple Sclerosis,[292] and Amyotrophic Lateral Sclerosis (ALS).[293] Although biomarker research was initially limited to cerebrospinal fluid (CSF), advanced immuno-assays have enabled the identification of blood-based biomarkers.[294] These blood-based assays enable the detection of biomarkers related to the peripheral nervous system. Contrasting the expanding evidence for their efficacy in the central nervous system, the role of

biomarkers in peripheral nerve disorders is currently less well understood. Specifically, the identification and role of peripheral nerve biomarkers in peripheral neuropathies remains unclear.

The aim of this review was therefore to assess differences in the concentrations of blood-based biomarkers associated with nerve involvement in patients with peripheral neuropathy compared to control participants.

3.2. Methods

The reporting of this review followed the updated PRISMA 2020 guidelines[196] and was preregistered on PROSPERO (CRD42021288101).

3.2.1. Data sources

Ovid, MEDLINE, Embase, and CINAHL were searched from inception to September 23, 2021, for studies published in English. Search strategies were developed with a medical librarian and are provided in Supplemental Table 3.1.

3.2.2. Study selection

Observational studies reporting on a quantitative blood-based measure of a biomarker associated with nerve involvement in patients with peripheral neuropathy versus a comparative control group (healthy or comorbid control group without peripheral neuropathy or central nervous system diagnosis) were included. Patients

were considered to have peripheral neuropathy if the study reported the use of established diagnostic criteria or confirmed neurological contributions through a clinical neurological examination, electrodiagnostic, or quantitative sensory testing.

Exclusion criteria included: 1) participants less than 18 years old; 2) participants with a concomitant central nervous system disorder; 3) studies without a control group or only including a comorbid control group with neuropathy; 4) studies that did not include biomarkers associated with nerve involvement; 5) data from non-blood bio-samples (e.g., urine, CSF, saliva); 6) non-quantitative methods to assess biomarker concentrations (e.g., explorative proteomics or western blot); and 7) case series, conference abstracts, and randomised controlled trials. When studies met all inclusion criteria but included a mixture of paediatric and adult patients, we contacted the study authors to obtain separate biomarker data for the adult population.

Two reviewers (Joel Fundaun and Miguel Molina Álvarez) initially screened study eligibility using titles/abstracts, followed by full texts. Disagreements in selection were resolved by discussion or by mediation of a third reviewer (Annina Schmid).

3.2.3. Quality assessment

Study quality and risk of bias was assessed using the Newcastle-Ottawa Scale for observational studies including cohort, case-control, and cross-sectional study

designs. These scales assess selection, comparability, and outcome. Case-control and cohort studies are scored from 0 to 9 with higher scores suggesting lower risk of bias. This score does not include established cut-offs. An adapted Newcastle-Ottawa Quality Assessment Scales for cross-sectional studies[295] was used, which is scored out of 10 and has recommended cut-off scores from 0-3 indicating high risk, 4-7 as moderate risk, and 8-10 as low risk. Two independent reviewers assessed each study for risk of bias (Joel Fundaun and Melissa Kolski). Disagreements between reviewers were resolved through consensus or by mediation of a third reviewer (Annina Schmid).

3.2.4. Biomarker selection

Only biomarkers associated with nerve involvement were selected for inclusion and analysis. Biomarkers associated with neuropathy but not directly indicating nerve involvement were excluded (e.g., cytokines, chemokines). When the neural relationship of a protein biomarker was unclear, its physiological function and tissue expression were cross-referenced using the Human Protein Atlas[296] (https://www.proteinatlas.org/). Studies reporting the use of microRNA were screened for neural cell expression using the freely available database reporting expression of animal central nervous system tissue (https://mirna.wustl.edu/search/?name=mir-1825). Neural cell specificity was then cross-referenced with another available RNA database (rnacentral.org). Biomarkers

whose predominant role and function are outside of the nervous system were excluded.

3.2.5. Data extraction

Data were extracted into a standardised spreadsheet. Extracted data included study and participant characteristics (study design, participant age and sex, timing of sample collection); criteria and duration of peripheral neuropathy diagnosis; analytical platform for biomarker detection (e.g., Simoa, ELISA); biomarker concentration for patients and controls; and biomarker diagnostic accuracy (e.g., sensitivity, specificity, positive and negative predictive values). When patient populations were compared to multiple control groups, the healthy control group (without disease) was selected as the comparator.

Biomarker concentrations reported as means and standard deviations (SD) were extracted when possible. As per Cochrane Handbook,[199] alternative summary statistics were transformed to means and SD using recommended calculations[197] or estimated using Plot Digitizer Software[198] when only reported graphically. Data were extracted by one reviewer (Joel Fundaun) and independently checked by another reviewer (Melissa Kolski). Attempt to contact study authors was done to obtain any missing or unclear data.

3.2.6. Statistical analysis

All statistical calculations were performed in R[202] using the packages "Meta" and "Metafor".[204] Data were meta-analysed when at least two studies reported on the same biomarker, even if different assays were used. A random-effects model with restricted maximum likelihood and inverse variance weighting methods was used to analyse biomarker data included from more than two studies. The Knapp-Hartung adjustment was used to control for the standard error of the pooled effect. A fixed-effect model was used to meta-analyse biomarker data when only two studies were included to properly account for between-study variance.[205] Effect estimates using standardised mean differences (SMDs) and 95% confidence intervals [CIs] were calculated for biomarker concentrations using Hedge's g to correct for bias from small sample sizes. Statistical significance was set at $P < 0.05$. Heterogeneity was calculated using I^2 statistics and interpreted as "might not be important" (0% - 40%), "moderate" (30% - 60%), "substantial" (50% - 90%), and "considerable" (75% - 100%).[199]

Pre-planned subgroup meta-analysis according to type of peripheral neuropathy was performed when two or more studies analysed the same biomarker in a similar diagnosis of peripheral neuropathy. Like the primary analysis, overall effect estimates of the concentration of each biomarker were calculated within each sub-group of peripheral neuropathies compared to controls (SMD and 95% CI). In individual studies that compared two or more subgroups of patients with peripheral neuropathy

to one control group, the number of control participants were divided by the number of patient subgroups.[199]

A post-hoc analysis of primarily axonal and demyelinating peripheral neuropathy subtypes was performed. All included articles were assessed for distinct diagnostic classification of primarily axonal or demyelinating peripheral neuropathy subtypes. Subtype analysis was not performed if an article included peripheral neuropathy subtypes that were considered to be mixed (without separate group data provided), uncertain, or equivocal diagnostic classifications. As reported in the main-text methods, data extraction (section 2.5) and meta-analysis (section 2.6) were performed if two or more studies reported data of the same primary subtype (axonal or demyelinating) and the same biomarker.

Based on these criteria, the only biomarker that could be meta-analysed was neurofilament light chain. Supplemental Table 3.5 includes the concentrations and varying diagnostic criteria used to distinguish primarily axonal and demyelinating subtypes for each study. Results are presented in Supplemental Figure 3.1.

I also pre-planned to meta-analyse diagnostic accuracy data from at least two studies using the same biomarker. However, these data were not meta-analysed, as all included studies reporting diagnostic accuracy data used different diagnostic cut-off thresholds and had high between-study heterogeneity.

Results from single studies unable to be meta-analysed were narratively synthesised using the principles of the "Guidance on the Conduct of Narrative Synthesis in Systematic Reviews: A Product from the ESRC Methods Programme (2006).[201]

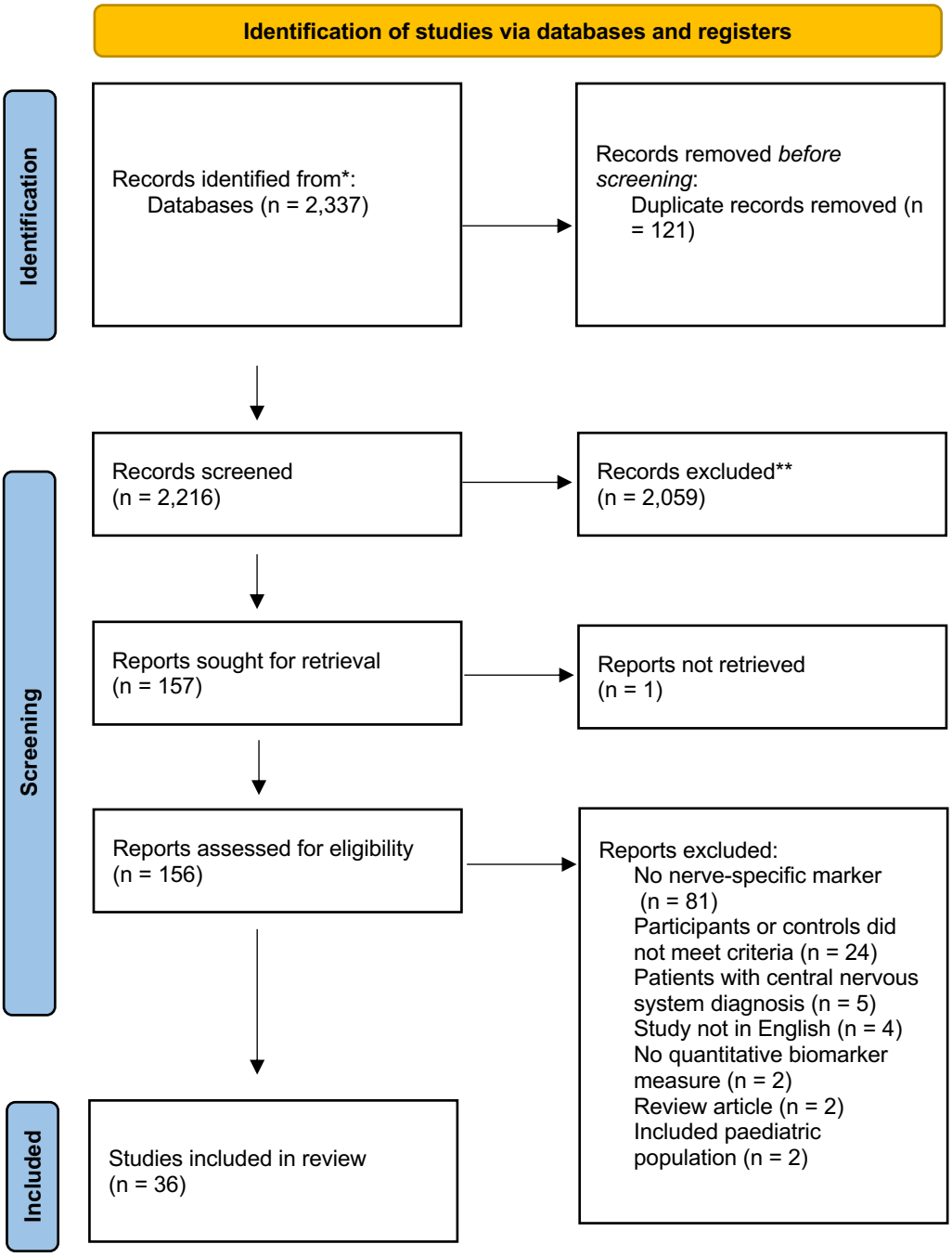


Figure 3.1. PRISMA flow diagram.

3.3. Results

2,216 non-duplicated records were screened resulting in 36 studies being included after eligibility assessment, including 2,301 patients with peripheral neuropathy and 2,113 control participants (Figure 3.1). Participant characteristics are detailed in Supplemental Table 3.2. The most commonly reported type of peripheral neuropathy is diabetic neuropathy (13 studies[297-309]), followed by Charcot-Marie-Tooth Disease (6 studies[310-315]), Guillain-Barre Syndrome (6 studies[302,316-320]), chronic inflammatory demyelinating polyneuropathy (5 studies[302,310,321-323]), acute inflammatory demyelinating polyneuropathy (3 studies[302,310,321]), and hereditary transthyretin-mediated amyloidosis with polyneuropathy (3 studies[324-326]). Single studies include hexane-induced neuropathy,[327] critical illness polyneuropathy,[328] acute motor axonal neuropathy,[310] axonal sensori-motor neuropathy,[302] vasculitic neuropathy,[329] rheumatoid arthritis with neuropathy,[330] leprosy with neuropathy,[331] and a cohort including various neuropathies[332] (Table 3.1). Data from three microRNA biomarker studies are excluded, as the examined markers are not exclusive to the nervous system and involve a diverse range of physiological functions in numerous disease states (e.g., cancer, cardiac, immune system disorders).

From all studies, 16 different blood-based biomarkers associated with nerve involvement measured in either serum or plasma are identified. Neurofilament light chain (NfL) is the most studied biomarker (17 studies), followed by brain derived neurotrophic factor, S100B, and neuron-specific enolase (3 studies each). Biomarkers

from 2 studies include nerve growth factor, neural cellular adhesion molecule, glial fibrillary acidic protein, transmembrane protease serine 5, and neurofilament heavy chain (Table 3.1). Biomarkers that are identified from single studies are listed and summarised in Supplemental Table 3.3.

Study risk of bias from median Newcastle-Ottawa Quality Assessment Scales scores is 6 (range 2-8) for case-control studies, 8 (range 5-9) for cross-sectional studies, and 7 (range 4-9) for cohort studies (Supplemental Table 3.4).

Table 3.1. Study characteristics including type of neuropathy and concentrations of blood biomarkers.

Source (author, year)	Diagnosis (number)	Control type (number)	Biomarker (blood measure, assay)	Patient concentration: mean (SD)	Control concentration: mean (SD)
Afarideh et al, 2019	Diabetic neuropathy, n=44	Healthy, n=45	S100B (serum, ELISA)	S100B: 212.42 (76.79) pg/mL*	S100B: 65.5 (30.59) pg/mL*
Altmann et al, 2020	GBS, n=27	Patients without neuropathy, n=22	Neurofilament light chain (serum, Simoa)	118.9 (141.29) pg/mL*	9.07 (3.24) pg/mL*
Azoulay et al, 2020	Diabetic neuropathy, n=23	Diabetic without neuropathy, n=67	BDNF (serum, ELISA)	14.34 (4.54) ng/mL	18.92 (5.47) ng/mL
Bischof et al, 2018	Vasculitic neuropathy, n=11	Healthy, n=30	Neurofilament light chain (serum, Simoa)	586.91 (784.51) pg/mL	25.62 (11.43) pg/mL*
Celikbilek et al, 2014	Diabetic neuropathy, n=37	Healthy, n=50	S100B, GFAP (serum, ELISA)	S100B: 9.79 (1.75) pg/mL*	S100B: 14.88 (9.16) pg/mL*
				GFAP: 0 (608.28) ng/mL*	GFAP: 0 (707.12) ng/mL*
Ghafouri-Fard et al, 2021	AIDP and , n=22 CIDP, n=31	Healthy, n=49	BDNF (serum, real-time PCR)	AIDP: -9.77 (1.61)* expression levels	-10.18 (6.35) expression levels
				CIDP: -7.51 (2.09) expression levels	
Frithiof et al, 2021	Critical illness polyneuropathy, n=11	Critical illness without neuropathy, n=7	Neurofilament light chain, tau, GFAP (plasma, Simoa)	NfL: 631.43 (545.88) pg/mL*	NfL: 156.83 (135.49) pg/mL*
				GFAP: 181.93 (121.65) pg/mL*	GFAP: 90.26 (45.36) pg/mL*
				Tau: 3.67 (4.16) pg/mL*	Tau: 1.27 (0.79) pg/mL*
Hayashi et al, 2021	CIDP, n=11	Healthy, n=7	Neurofilament light chain (serum, Simoa)	166.6 (337.8) pg/mL*	12.2 (6.3) pg/mL*
Jadhav et al, 2011	Leprosy with neuropathy, n=48	Healthy, n=160	S100 (serum, ELISA)	56.86 (39.6) au	28.7 (28.5) au
Kapoor et al, 2019	Hereditary transthyretin amyloidosis neuropathy, n=20	Healthy, n=16	Neurofilament light chain (plasma, Simoa)	68.4 (71.29) pg/mL	15.45 (7.262) pg/mL
Kim et al, 2019	AIDP, n=14 CIDP, n=36	Healthy, n=20	p75 neurotrophin receptor, NCAM (serum, ELISA)	p75 CIDP: 256 (31.26) pg/mL	p75: 73.69 (40.58) pg/mL
				AIDP: 207.3 (37.85) pg/mL	

	AMAN, n=20 CMT, n=39			CMT1a: 12.74 (5.315) pg/mL AMAN: 128.83 (88.76) pg/mL*	
				NCAM: CIDP: 4960 (476) pg/mL AIDP: 4729 (661) pg/mL CMT1a: 6663 (277) pg/mL AMAN: 3095 (2242.89) pg/mL*	NCAM: 2298 (303) pg/mL
Kortvelyessy et al, 2020	GBS-high, n=3 GBS-low, n=18	Non-neuropathy, n=19	Neurofilament light chain (serum, Simoa)	GBS-albumin low: 2871.6 (3,946.0) pg/mL GBS-albumin high: 397.4 (537.9) pg/mL	50.7 (32.75) pg/mL
Li et al, 2021	Diabetic neuropathy, n=56	Diabetic without neuropathy, n=24	GAP-43 (serum, real-time PCR)	0.821 (0.561) - expression levels	1.064 (0.367) - expression levels
Li et al,25 2013	Diabetic neuropathy, n=214	Healthy, n=136	NSE (serum, electrochemiluminescence immunoassay automatic analyser)	10.8 (2.8) ug/L	8.7 (1.7) ug/L
Lieverloo et al, 2019	CIDP – Induction, n=29 Maintenance, n=24 Remission, n=27	Healthy, n=30	Neurofilament light chain (serum, Simoa)	Induction: 47.33 (38.18) pg/mL* Maintenance: 29.7 (14.06) pg/mL* Remission: 30.67 (18.02) pg/mL*	24.03 (10.80) pg/mL*
Mariotto et al, 2020	Various peripheral neuropathies, n=37	Healthy, n=37	Neurofilament light chain (serum, Simoa)	22.93 (21.11) pg/mL*	6.63 (3.29) pg/mL*
Martin-Aguilar et al, 2021	GBS, n=98	Healthy, n=53	Neurofilament light chain (serum, Simoa)	93.86 (156.28) pg/mL*	9.67 (6.42) pg/mL*
Mateos-Hernande	GBS, n=8	Healthy, n=4	Piccolo (serum, ELISA)	4.0517 (1.2155) ug/mg	0.0005 (0.0001) ug/mg

z et al, 2016					
Millere et al, 2021	CMT, n=83	Healthy, n=56	Neurofilament light chain (plasma, Simoa)	13.86 (9.81) pg/ml	5.62 (1.97) pg/ml
Xiaowei et al, 2014	Hexane-induced peripheral neuropathy, n=18	Healthy, n=106	human myelin protein P0 (serum, ELISA)	407.21 (93.60) pg/mL*	203.04 (86.49) pg/mL*
Niezgoda et al, 2017	Demyelinating polyneuropathy, n=80 Axonal polyneuropathy, n=40 Diabetic polyneuropathy, n=20	Healthy, n=20	Neural cellular adhesion molecule (serum, ELISA)	Demyelinating: 4,588.7 (1,763.6) ng/mL Axonal: 3,138.4 (1,221.3) ng/mL Diabetic: 2,869.7 (923.5) ng/mL	1,549.1 (295.4) ng/mL
Ozuguz et al, 2016	Diabetic neuropathy, n=26	Non-neurological disease, n=70	Nerve growth factor (serum, ELISA)	13.1 (2.6) pg/mL	14.2 (4.7) pg/mL
Rossor et al, 2016	CMT, n=90	Healthy, n=79	Neurofilament heavy chain (plasma, ELISA)	27.4 (22.04) ng/mL*	Mean: 21.5 (18.64) ng/mL*
Maia et al, 2020	Hereditary transthyretin-mediated amyloidosis with polyneuropathy, group 1 n=18 Group 2 n=26	Healthy, n=16	Neurofilament light chain (plasma, Simoa)	1: 29.34 (47.37) pg/mL 2: 117.06 (98.18) pg/mL	5.57 (4.44) pg/mL
Salih et al, 2000	Rheumatoid arthritis with peripheral neuropathy, n=28	Healthy, n=28	Anti-neuroblastoma cell antibodies (serum, ELISA)	IgG: 4.86 (2.76) a.u.* IgM: 1.85 (2.49) a.u.*	IgG: 2.29 (1.58) a.u.* IgM: 1.06 (1.15) a.u.*
Sandelius et al, 2018	CMT, n=75	Healthy, n=67	Neurofilament light chain (plasma, Simoa)	25.63 (12.07) pg/mL*	15.57 (7.48) ng/mL*
Sandhu et al, 2008	Diabetic neuropathy, n=24	Healthy, n=26	NSE (whole blood, PCR)	0.0067 (0.0038) expression levels*	0.0094 (0.0048) expression levels*

Qiao et al, 2015	Diabetic neuropathy, n=23	Non-neurological disease, n=62	Neurofilament heavy chain (serum, ELISA)	739.98 (791.28) pg/mL*	378.56 (329.28) pg/mL*
Sessa et al, 1997	GBS, n=61	Healthy, n=40	Myelin-associated β4 integrin (serum, ELISA)	0.208 (0.606)	0.003 (0.020)
Sun et al,30 2018	Diabetic neuropathy, n=65	Healthy, n=110	Nerve growth factor, BDNF (serum, ELISA)	NGF: 42.7(4.9) pg/mL	NGF: 64.3 (11.7) pg/mL
				BDNF: 1,739.8 (132.9) pg/mL	BDNF: 2,246.7 (331.5) pg/mL
Ticau et al, 2021	Hereditary transthyretin-mediated amyloidosis with polyneuropathy, n=159	Healthy, n=57	Neurofilament light chain (plasma, Simoa)	69.43 (60.50) pg/mL	16.25 (45.87) pg/mL
Wang et al, 2020	CMT, Group 1 n=20 Group 2 n=31	Healthy, Group 1 n=20 Group 2 n=24	Transmembrane protease serine 5, Neurofilament light chain (plasma, Olink immuno PCR, Simoa)	TMPRSS5 (NPX): Group 1: 4.37 (0.62)*	TMPRSS5 (NPX) Control 1: 3.32 (0.50)*
				Group 2: 4.42 (0.49)*	Control 2: 3.43 (0.648)*
				NfL (NPX): Group 1: 3.60 (0.38)*	NfL (NPX) Control 1: 2.91 (0.43)*
				Group 2: 3.52 (0.76)*	Control 2: 2.84 (0.44)*
Wang et al, 2021	CMT, TMRSS5 n= 65 NfL n=41	Healthy, TMRSS5 n=52 NfL n=40	Transmembrane protease serine 5, Neurofilament light chain (plasma, Olink immuno PCR, Simoa)	TMPRSS5 (NPX): 4.63 (0.74)*	TMPRSS5 (NPX): 3.57 (0.52)*
				NfL (NPX): 3.5 (0.60)*	NfL (NPX): 2.83 (0.45)*
Ziegler et al, 2019	Diabetic neuropathy, n=304	Healthy, n=354	Neurotrophin-3 (serum, OLINK Inflammation multiplex immunoassay)	0.87 (0.40) NPX	1.03 (0.34) NPX
Morgenstern et al, 2021	Diabetic neuropathy, n=63	Healthy, n=30	Neurofilament light chain, Myelin protein zero (serum, Simoa)	NfL: 14.69 (8.5) pg/ml	NfL: 9.88 (3.72) pg/ml
				Myelin protein zero: 4.189 (2.528) expression levels mRNA	Myelin protein zero: 7.720 (2.609) expression levels mRNA
Celikbilek et al, 2014	Pre-diabetic neuropathy, n=22	Healthy, n=30	Neurofilament light chain, Neuron-specific enolase	NfL: 0.0163 (0.0158)* mRNA expression	NfL: 0.004 (0.009)* mRNA expression

			(serum, real-time PCR)	NSE: 0.209 (0.078)* mRNA expression	NSE: 0.145 (0.204)* mRNA expression

Legend: Acute inflammatory demyelinating polyneuropathy (AIDP); Acute motor axonal neuropathy (AMAN); Arbitrary units (AU); Brain derived neurotrophic factors (BDNF); Charcot-Marie-Tooth Disease (CMT); Chronic inflammatory demyelinating polyneuropathy (CIPD); Enzyme-linked immunosorbent assay (ELISA); Growth association protein 43 (GAP-43); Glial fibrillary acidic protein (GFAP); Guillain-Barre Syndrome (GBS); Neural cell adhesion molecule (NCAM); Neurofilament light chain (NfL); Neuron-specific enolase (NSE); normalised protein expression (NPX); Polymerase chain reaction (PCR); Single Molecule array (Simoa); Transmembrane protease serine 5 (TMPRSS5). *indicates means estimated from graphs/figures.

3.3.1. Biomarker meta-analyses

Blood-based biomarkers that are significantly increased in patients with peripheral neuropathy compared to control participants include NfL (SMD 0.93 [0.82; 1.05] P< 0.001, I^2 = 0%, Figure 3.2), neurofilament heavy chain (SMD 0.41 [0.15; 0.67] P < 0.01, I^2 = 54%), and transmembrane protease serine 5 (SMD 1.68 [1.43; 1.93] P < 0.01, I^2 = 0%) with between-study heterogeneity ranging from not important to moderate (Figure 3.3). In contrast, nerve growth factor is the only biomarker that shows a significant decrease in patients with peripheral neuropathy compared to controls (SMD -1.38 [-1.68; -1.09] P < 0.001, I^2 = 98%) with considerable heterogeneity (Figure 3.3).

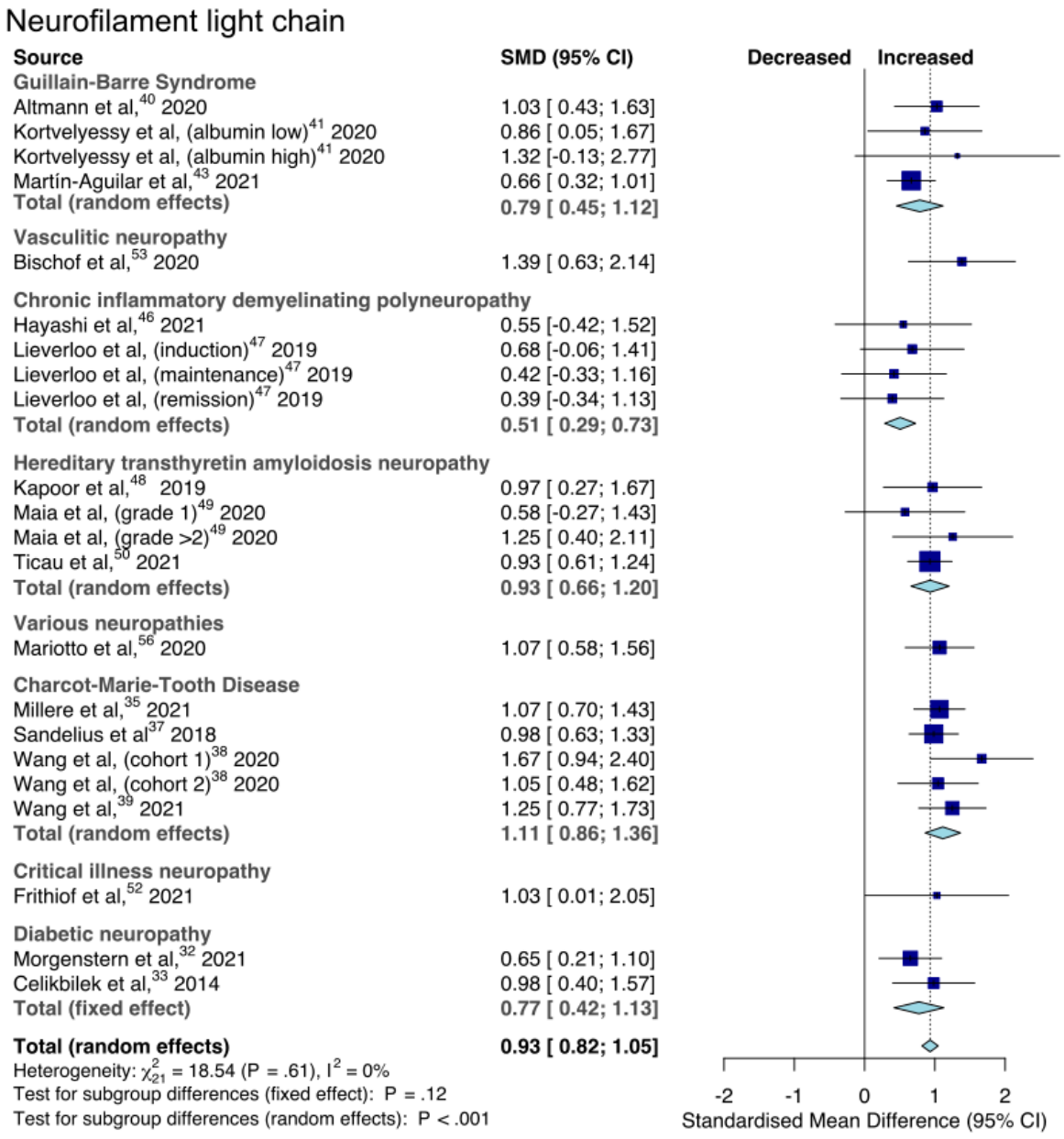


Figure 3.2. Meta-analysis of neurofilament light chain in patients with peripheral neuropathy compared to controls. Overall effects, standardised mean differences (SMDs), 95% confidence intervals (CIs), and heterogeneity (I^2) are summarised and further sub-grouped based on the type of peripheral neuropathy.

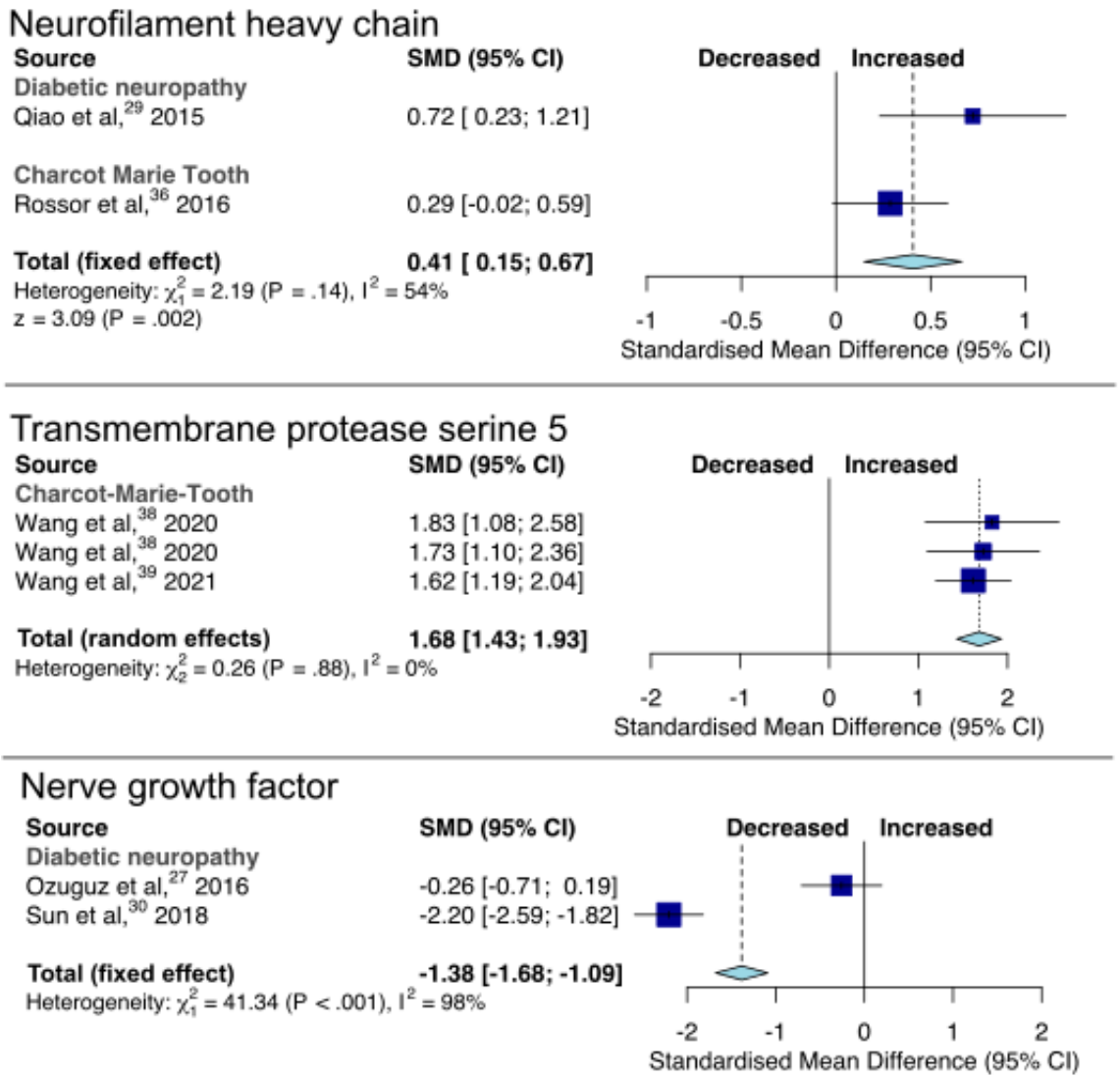


Figure 3.3. Meta-analyses of blood-based biomarkers that demonstrated a statistically significant difference of concentrations in patients with peripheral neuropathy compared to controls. Overall effects, standardised mean differences (SMDs), 95% confidence intervals (CIs), and heterogeneity (I^2) are summarised for each biomarker and further sub-grouped based on the type of peripheral neuropathy. The scales for standardised mean differences vary by biomarker.

Several biomarkers are not significantly different in patients with peripheral neuropathy compared to controls (Figure 3.4). This includes myelin protein zero (SMD 0.13 [-0.24; 0.50] P = 0.50, I^2 = 99%), S100B (SMD 1.10 [-3.08; 5.28] P = 0.38, I^2 = 98%), brain derived neurotrophic factor (SMD -0.52 [-2.23; 1.19] P = 0.40, I^2 = 95%), glial fibrillary acidic protein (SMD 0.13 [-0.26; 0.52] P = 0.50, I^2 = 60%), neural cellular adhesion molecule (SMD 4.09 [-0.54; 8.73] P = 0.07, I^2 = 94%), and neuron-specific enolase (SMD -0.00 [-1.99; 1.98] P = 0.10, I^2 = 94%). All studies have substantial to considerable heterogeneity.

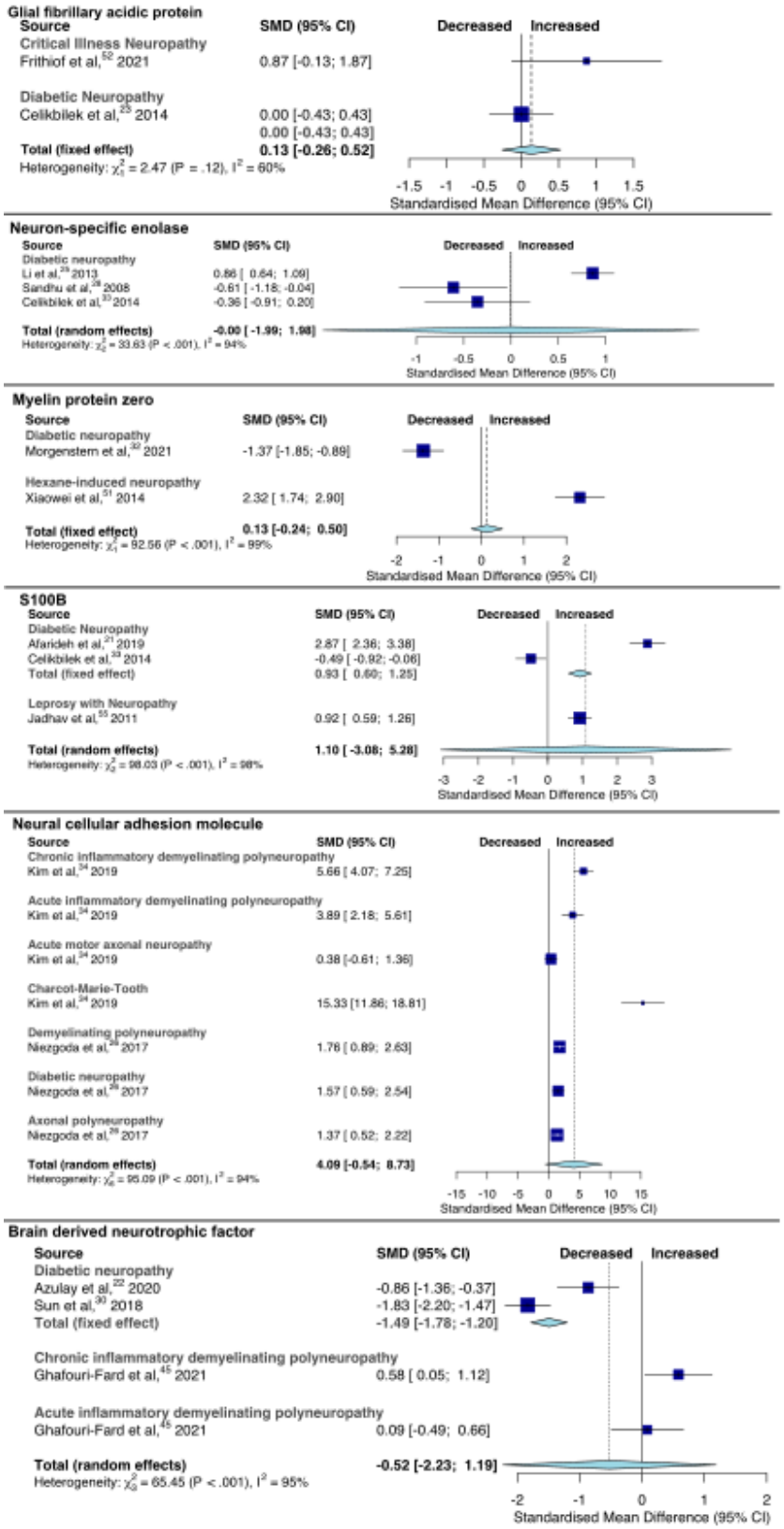


Figure 3.4. Meta-analyses of blood-based biomarkers that did not demonstrate a statistically significant difference of concentrations in patients with peripheral neuropathy compared to controls. Overall effects, standardised mean differences (SMDs), 95% confidence intervals (CIs), and heterogeneity (I^2) are summarised for each biomarker and further sub-grouped based on the type of peripheral neuropathy. The scales for standardised mean differences vary by biomarker.

3.3.2. Diagnostic subgroup meta-analyses

When separately analysed to detect subgroup differences based on type of peripheral neuropathy (Figures 2.2 -2. 4), diabetic neuropathy has increased NfL (SMD 0.77 [0.42; 1.13] $P < 0.001$, $I^2 = 0\%$) and S100B (SMD 0.93 [0.60; 1.25] $P < 0.001$, $I^2 = 99\%$). Nerve growth factor (SMD -1.38 [-1.68; -1.09] $P < 0.001$, $I^2 = 98\%$) and brain derived neurotrophic factor (-1.49 [-1.78; -1.20] $P < 0.001$, $I^2 = 90\%$) are decreased. No significant difference is identified in neuron-specific enolase compared to controls (SMD -0.0025 [-1.99; 1.98] $P = 0.10$, $I^2 = 94\%$, Table 3.1).

In patients with Guillain-Barre Syndrome, there is a significant increase in NfL (SMD 0.79 [0.45; 1.12] $P < 0.01$, $I^2 = 0\%$). Similarly, patients with chronic inflammatory demyelinating polyneuropathy have significantly increased NfL (SMD 0.51 [0.29; 0.73] $P < 0.01$, $I^2 = 0\%$). NfL concentrations of predominantly axonal and demyelinating peripheral neuropathy subtypes do not differ (Supplemental Table 3.5 and Supplemental Figure 3.1).

Patients with Charcot-Marie-Tooth Disease have significantly increased NfL (SMD 1.11 [0.86; 1.36] $P < 0.001$, $I^2 = 0\%$) and transmembrane protease serine 5 (SMD 1.68 [1.43; 1.93] $P < 0.01$, $I^2 = 0\%$) compared to controls.

Lastly, patients with hereditary transthyretin-mediated amyloidosis with polyneuropathy have significantly increased levels of NfL (SMD 0.93 [0.66; 1.20] $P < 0.01$, $I^2 = 0\%$) compared to controls.

3.3.3. Diagnostic accuracy

Meta-analyses of biomarker diagnostic accuracy cannot be performed due to significant differences in concentration cut-off values between studies (e.g., NfL range: 8.9 – 155 pg/mL) and varying peripheral neuropathy diagnoses. Biomarker concentration cut-off values and corresponding diagnostic accuracy data are listed in Supplemental Table 3.6.

3.4. Discussion

My systematic review with meta-analysis identified 16 blood-based biomarkers from 36 studies including 2,301 patients with peripheral neuropathy and 2,113 control participants. My meta-analyses identified four biomarkers that were significantly altered in patients with peripheral neuropathy compared to controls. Among those, NfL was consistently upregulated in peripheral neuropathy with a large effect size (based on 17 studies and 8 types of neuropathies). Neurofilament heavy chain, transmembrane protease serine 5, and nerve growth factor were also significantly dysregulated with magnitudes of effect ranging from moderate to large. However, these results were derived from only two studies with higher heterogeneity.

Neurofilament light chain seems a promising biomarker for peripheral neuropathies.

My findings, taken from 854 patients with peripheral neuropathy and 561 controls, strongly suggest that a blood-based measure of NfL is a useful biomarker in patients with peripheral neuropathy. Increased concentrations of NfL were consistently identified in each type of peripheral neuropathy using varying methods of blood collection (e.g., plasma, serum) and immuno-assays (e.g., ELISA, Simoa). These findings in peripheral neuropathies are aligned with strong evidence demonstrating increased concentrations of NfL in several central nervous system conditions, including ALS,[290,333] Alzheimer's Disease,[290,334] Multiple Sclerosis,[290,292] and traumatic brain injury.[291,335] NfL's functional role in axonal growth and stability and its high expression in neuronal tissue[336] (Supplemental Figure 3.2) make it a robust neuronal biomarker.[337] My data corroborate using NfL as a blood-based biomarker not only for central but also for peripheral neuropathies.

Additional biomarkers associated with peripheral neuropathies.

My meta-analyses highlighted additional biomarkers that may be useful in peripheral neuropathies. Neurofilament heavy chain, transmembrane protein serine 5, and nerve growth factor were significantly altered in patients with peripheral neuropathy compared to controls. Biomarkers that were not significantly altered (e.g., brain derived neurotrophic factor, glial fibrillary acidic protein, neural cellular adhesion molecule) may be more limited as a blood-based measure of nerve involvement due to their diverse neurological functions.[338-340] However, firm conclusions regarding the use of these

biomarkers cannot be drawn as all meta-analyses besides NfL were taken from a small number of studies with significant between-study heterogeneity. Further research is needed to understand the role of these biomarkers in the context of different types of peripheral neuropathies.

Potential clinical implications and considerations for future research.

Early detection

There is growing evidence for the use of biomarkers in the early detection of nerve involvement. Numerous studies have shown that NfL is a reliable measure of nerve involvement in the pre-symptomatic stage of central nervous system diseases, including frontotemporal dementia,[341] Alzheimer's disease,[342] Multiple Sclerosis,[343] and ALS.[333] To date, the role of blood biomarkers for the early or pre-symptomatic detection of peripheral neuropathy remains understudied. We only identified one such study assessing potential early signs of neuropathy in prediabetic patients.[309] This study identified increased expression of NfL in prediabetic patients compared to healthy controls. Therefore, more studies are needed to understand biomarkers' ability to detect pre-symptomatic pathology.

Diagnosis

Patients with peripheral neuropathy often have comorbid conditions (e.g., cardiovascular, immune, metabolic dysfunction) making it difficult to detect and diagnose neuropathy.[344] For example, it is currently estimated that physicians only

recognise neuropathy symptoms in less than one-third of patients presenting with symptomatic diabetic neuropathy;[345] supporting the need for improved diagnostic tools. Initial CSF-based diagnostic studies using NfL highlighted its discriminatory ability in multiple neurological conditions.[290] Advanced immuno-assays can now reliably detect lower concentrations of NfL in the blood and strongly correlate with CSF concentrations.[292,346] Similarly, a recent study using plasma NfL confirmed significant diagnostic implications in multiple neurodegenerative conditions.[334] Although study heterogeneity limited my ability to meta-analyse diagnostic cut-off thresholds, numerous studies in my systematic review demonstrated strong diagnostic accuracy. This included using NfL to discriminate between patients with and without vasculitic neuropathy,[329] symptomatic versus asymptomatic hereditary transthyretin-mediated amyloidosis with polyneuropathy,[325] and between Charcot-Marie-Tooth Disease and healthy controls.[311,314,315] Future research considering the optimal type of blood analysis (e.g., serum, plasma) and immunological assay (e.g., ELISA, Simoa) is needed to develop generally accepted clinical diagnostic cut-off scores for patients with different types of peripheral neuropathy. Of note, NfL did not seem to be a good marker to differentiate primarily axonal from demyelinating types of neuropathies with elevated levels in both types.

Prognosis

Biomarkers could serve important roles in evaluating the severity and prognosis of peripheral neuropathies. Although meta-analysing this was out of scope for this review,

several included studies showed a significant correlation between NfL and disease severity (Charcot-Marie-Tooth Disease,[313,314] Guillain-Barre Syndrome[316,319]). Additionally, two studies in patients with diabetic neuropathy identified significant correlations between increased NfL concentrations and neuropathic pain,[309] as well as a hyperalgesic pain phenotype.[308] Although few studies included long-term follow-up data, one study in Guillain-Barre syndrome highlighted NfL's ability to predict patients' ability to walk or run independently one year after disease onset.[319]

Treatment stratification

Biomarkers may also be used to improve treatment through patient stratification. The pathophysiology of neuropathies is diverse and requires unique, individualised treatments. Identifying robust clinical biomarkers could lead to improved quality and cost-effectiveness of care.[347] Further development and validation is currently required before biomarkers can be used for patient stratification in clinical neurology.[348] The identification of promising biomarker candidates in this review provides an important initial step to progress towards personalised management for people with peripheral neuropathies.

Limitations

There are limitations to consider when interpreting my results. First, publication bias may prevent the reporting of negative results of biomarker data. Additionally, only English language publications were included and between-study heterogeneity in

smaller studies (e.g., methodological heterogeneity) may limit the generalisability of my findings. The overall quality of included studies was high. Limited reporting or adjustment of confounding variables between participants and controls was the primary limitation, which was only identified in five of 36 studies. Lastly, it is important to consider the pathophysiological differences in peripheral neuropathies. This needs to be remembered when interpreting the overall meta-analyses of certain biomarkers, as some markers may be more useful in certain neuropathies than others.

3.5. Conclusions

In this systematic review and meta-analysis, my findings supported the use of NfL as a blood-based biomarker of nerve involvement in patients with peripheral neuropathy. When compared to other nerve-related biomarkers, NfL was consistently increased in patients with varying types of peripheral neuropathies compared to control participants. Neurofilament heavy chain, transmembrane protease serine 5, and nerve growth factor were also significantly altered in peripheral neuropathy, although these results are based on few studies. Future research is required to assess the temporal patterns, diagnostic accuracy, and prognostic ability of these biomarkers in patients with peripheral neuropathy and peripheral nerve injuries.

Supplemental Table 3.1. Search strategies for all included search engines

Medline

1.	“Peripheral Nervous System Diseases"[Mesh] OR "Autoimmune Diseases of the Nervous System"[Mesh] OR "peripheral neuropathy" OR "peripheral nerve disorder" OR "peripheral nervous system" OR "peripheral nerve"
2.	(("blood"[Subheading] OR "blood"[All Fields] OR "blood"[MeSH Terms]) AND ("biomarkers"[MeSH Terms] OR "biomarkers"[All Fields] OR "biomarker"[All Fields])) OR "Biomarkers"[Mesh] OR "biomarker" OR "biologic marker" OR “biological marker” OR "blood biomarker" OR "serum biomarker"
3.	Cohort studies OR Case-control studies OR observational study OR Cross-sectional studies
4.	"Central Nervous System Diseases"[Mesh] OR "Cranial Nerve Diseases"[Mesh] OR "Multiple Sclerosis"[Mesh]
5.	1 AND 2 AND 3 NOT 4

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1.	Exp Peripheral Nervous System Diseases/ OR peripheral nerve disorder* OR peripheral nerve disease* OR peripheral neuropath* OR poly neuropath* OR neuralgia OR peripheral nerve injur*
2.	Exp Biomarkers/bl OR (Blood/ and Biomarker/) or biomark* or biologic* marker* or blood biomarker* or serum biomarker*
3.	Exp Central Nervous System Diseases/ OR exp Cranial Nerve Diseases/ OR exp Multiple Sclerosis/
4.	1 AND 2 NOT 3

Cinahl

1.	Exp Peripheral Nervous System Diseases/ OR peripheral nerve disorder* OR peripheral nerve disease* OR peripheral neuropath* OR poly neuropath* OR neuralgia OR peripheral nerve injur*
2.	Exp Biomarkers/bl OR (Blood/ and Biomarker/) or biomark* or biologic* marker* or blood biomarker* or serum biomarker*
3.	*Exp Central Nervous System Diseases/ OR exp Cranial Nerve Diseases/ OR exp Multiple Sclerosis/*
4.	1 AND 2 NOT 3

Supplemental Table 3.2. Summary of diagnostic criteria and cohort characteristics at study-level

Source	Diagnosis	Diagnostic criteria (neuropathy group)	Mean duration of diagnosis at sample collection (neuropathy group)	Participant characteristics (mean age, number female)	Control characteristics (mean age, number female)
Afarideh et al, 2019	Diabetic neuropathy	NCV, EMG, h-reflex, strength, sensation (Mythili exam score)	10 years	56.5 years, n=17	38.1 years, n=45
Altmann et al, 2020	GBS	Neurologic examination, Brighton criteria (levels 1 or 2), NCV	Within 5 days of hospitalisation	55 years, NA	36 years, NA
Azoulay et al, 2020	Diabetic neuropathy	Fasting glucose levels, NCV, reduced neuropathy score, small nerve fibre testing	10.4 years	60.5 years, n=34 (total cohort)	60.5 years, n=34 (total cohort)
Bischof et al, 2018	Vasculitic neuropathy	Neurologic examination and symptom score, peripheral nerve biopsy, clinical presentation	At time of diagnosis	57.3 years, n=7	NA (age matched controls)
Celikbilek et al, 2014	Diabetic neuropathy	Fasting glucose, NCV, EMG	NA	56.2 years, n=50 (full diabetic cohort)	58.9, n=34
Ghafouri-Fard et al, 2021	AIDP and CIDP	Clinical examination, electrophysiology, CSF, NINDS classification	NA	36.2 years, n=6 AIDP, n=11 CIDP	35.3 years, n=11
Frithiof et al, 2021	Critical illness polyneuropathy	Critical Illness Neuropathy guidelines (Bolton 2005, Lacomis 2000), EMG, NCV	>11 days from admission	64 years (median), n=0	51 years (median), n=1
Hayashi et al, 2021	CIDP	EFNS/PNS 2010 criteria	16.0 weeks (median)	59.6 years (median), n=6	58.0 years (median), n=3
Jadhav et al, 2011	Leprosy with neuropathy	Ridley-Jopling classification, strength testing, monofilament testing	NA	NA	NA

Kapoor et al, 2019	Hereditary transthyretin amyloidosis neuropathy	Genetic testing, Rasch modified CMT examination, neuropathy impairment score	NA	65.8 years (median), n=8	67 years (median), n=1
Kim et al, 2019	AIDP, CIDP, AMAN, CMT	EFNS/PNS 2010 criteria; GBS classification criteria (Wakerley 2014; Ho 1995).	NA	NA, AIDP n=3, CIDP n=10, AMAN n=7, CMT, n=17	NA, n=14
Kortvelyessy et al, 2020	GBS-high GBS-low	Presenting with at least bilateral or flaccid weakness of limbs accompanied by decreased or absent tendon reflexes, CSF cell count under 50 cells/μl, monophasic course and time between onset-nadir of 12 h to 28 days, Brighton collaboration criteria 1 and 2	NA	GBS-high 58.4 years, n=0; GBS-low 60.7 years, n=6	64.7 years, n=9
Li et al, 2021	Diabetic neuropathy	ADA diagnosis criteria for diabetes, neurologic exam, NCV	9.9 years	61.6 years, n=25	50.1 years, n=13
Li et al, 2013	Diabetic neuropathy	WHO classification of diabetes, ADA diabetic neuropathy, neurologic examination, QST, NCV	7.3 years	52.3 years (median), n=70	52.5 (median), n=47
Lieverloo et al, 2019	CIDP – Induction Maintenance Remission	EFNS/PNS 2010 criteria, neurologic exam, NCV	Induction: 22 months (median) Maintenance: 71 months (median) Remission: 39 months in remission (median)	Induction 61 years, n=8 Maintenance 59 years, n=7 Remission 63 years, n=4	62 years, n=11
Mariotto et al, 2020	Various peripheral neuropathies	Neurologic examination, sural nerve biopsy,	NA	65.1 years, n=12	NA

		neurophysiological evaluation			
Martin-Aguilar et al, 2021	GBS	NINDS classification, Brighton diagnostic criteria, NCV	4 days (median time from symptom onset)	57.4 years, n=42	53.1 years, n=30
Mateos-Hernandez et al, 2016	GBS	Clinical examination	NA	57 years, n=4	37.3 years, n=3
Millere et al, 2021	CMT	Clinical/neurophysiological examination or positive genetic testing for a known pathogenic variant in the family, neurography, CMT Neuropathy Score	NA	38.6 years, n=51	35.7 years, n=41
Xiaowei et al, 2014	Hexane-induced peripheral neuropathy	Diagnostic Criteria of Occupational Chronic n-hexane Poisoning (GBZ84-2002)	NA	28.6 years, n=18	26.6 years, n=106 (total cohort)
Niezgoda et al, 2017	Demyelinating, axonal, and diabetic polyneuropathy	GBS from lab and electrophysiological testing, clinical assessment (overall neuropathy limitations scale), criteria type by EFNS/PNS 2010	NA	Demyelinating 39.3 years, n=30 Axonal polyneuropathy 49.5 years, n=19 Diabetic polyneuropathy 46.8 years, n=10	40.3 years, n=10
Ozuguz et al, 2016	Diabetic neuropathy	MNSI, vibration sensation, ankle reflexes, monofilament testing (10g)	18.1 years	38 years, n=14	28 years, n=39
Rossor et al, 2016	CMT	Genetically confirmed diagnosis of CMT, CMT Examination Score (CMTES, 2nd version)	NA	44 years, n=44	NA, n=23

Maia et al, 2020	Hereditary transthyretin -mediated amyloidosis with polyneuropathy	Genetic testing, neurologic examination, polyneuropathy disability score	Group 1 - PND1: 1.4 years, PND 2 and 3: 4.1 years Group 2 - PND 1: 2.0 years, PND 2 and 3: 3.9 years, PND 4: 5.4 years	Group 1 – PND 1: 41.0 years, n=7, PND: 2 and 3: 53.5 years,n=8 Group 2 -PND 1: 57.4, n=1, PND 2 and 3: 62.0 years, n=4, PND 4: 63.8 years , n=1	33.5 years, n=7
Salih et al, 2000	Rheumatoid arthritis with peripheral neuropathy	American College of Rheumatology revised criteria (1987), NCV, EMG, neurologic symptom and disability scores	11.4 years	64 years, n=18	61 years, n=22
Sandelius et al, 2018	CMT	Genetically confirmed CMT, NCV	NA	46.2 years, n=39	47.0 years, n=46
Sandhu et al, 2008	Diabetic neuropathy	Clinical examination	18.1 years	59.6 years, n=6	44.2 years, n=8
Qiao et al, 2015	Diabetic neuropathy	WHO diabetes diagnosis criteria, neuropathy symptom and disability scores, clinical examination, QST, NCV, EMG	11.0 years (median)	61.6 years, n=15	64.5 years, n=33
Sessa et al, 1997	GBS	GBS diagnostic criteria (Asbury and Cornblath, 1990)	2–7 days: n=36 patients, 8–14 days: n=19 patients, 15–21 days: n=6 patients	NA	NA
Sun et al, 2018	Diabetic neuropathy	WHO and ADA diabetes classification, clinical examination, NCV, EMG	12.7 years	58.2 years, n=35	57.3 years, n=63

Ticau et al, 2021	Hereditary transthyretin-mediated amyloidosis with polyneuropathy	Neuropathy Impairment Score 5-130, polyneuropathy disability score ≤IIIb	NA	60.5 years, n=47	58.6 years, n=15
Wang et al, 2020	CMT	Clinical examination, NCV, confirmatory genetic testing, Rasch modified CMT Neuropathy and Examination Scores	NA	Group 1: 47.4 years Group 2: 42.1 years, n=32 (total)	Group 1: 47.1 years Group 2: 49.4 years, n= 18 (total cohort)
Wang et al, 2021	CMT	Clinical examination, NCV, confirmatory genetic testing, Rasch modified CMT Neuropathy and Examination Scores	NA	46.7 years, n=61 (total cohort)	46.9 years, n=33
Ziegler et al, 2019	Diabetic neuropathy	ADA criteria, electrophysiological testing, QST, skin biopsies, NCV	13.5 years	68.0 years, n=228	68.9 years, n=313
Morgenstern et al, 2021	Diabetic neuropathy	German Diabetes Association criteria, neuropathy deficit score, neuropathy symptom score, QST, EMG	11.9 years	62.6 years, n=23	58.0 years, n=16
Celikbilek et al, 2014	Pre-diabetic neuropathy	Fasting glucose as defined by ADA, sensory and motor nerve conduction studies, score of ≥4 on the Douleur Neuropathique 4 questionnaire	NA	54.1 years, n=35 (entire cohort)	59.3 years, n=17

Legend: Acute inflammatory demyelinating polyneuropathy (AIDP); Acute motor axonal neuropathy (AMAN); American Diabetes Association (ADA); Cerebrospinal fluid (CSF); Charcot-Marie-Tooth Disease (CMT); Chronic inflammatory demyelinating polyneuropathy (CIPD); Electromyography (EMG); European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS); Guillain-Barre Syndrome (GBS); Michigan Neuropathy Screening Instrument (MNSI); National Institute of Neurological Disorders and Stroke (NINDS); Nerve conduction velocity (NCV); Not available (NA); Polyneuropathy disability score (PND); Quantitative sensory testing (QST); World Health Organization (WHO)

Supplemental Table 3.3. Summary of biomarker results reported in single studies

Biomarker	Diagnosis	Reported results compared to controls
Myelin associated integrin	Guillain-Barre Syndrome	Increased
Piccolo	Guillain-Barre Syndrome	Increased
Anti-neuroblastoma cell antibodies	Rheumatoid arthritis with peripheral neuropathy	Increased
p75 neurotrophin receptor	Acute inflammatory demyelinating polyneuropathy	Increased
	Chronic inflammatory demyelinating polyneuropathy	Increased
	Charcot-Marie-Tooth Disease	No significant difference
Tau	Critical illness polyneuropathy	No significant difference
GAP-43	Diabetic neuropathy	Decreased
BACE1	Acute inflammatory demyelinating polyneuropathy	Downregulated
	Chronic inflammatory demyelinating polyneuropathy	Downregulated
Neurotrophin-3	Diabetic neuropathy	Decreased

Supplemental Table 3.4. Quality Assessment using the Newcastle-Ottawa Scales

Author (year)	Selection	Comparability	Outcome	Total score
Case-control	**(/4)**	**(/2)**	**(/3)**	**(/9)**
Afarideh et al. (2019)	4	2	1	7
Altman et al. (2020)	4	2	2	8
Azoulay et al. (2020)	4	2	2	8
Bischof et al. (2018)	3	0	1	4
Celikbilek et al. (2014)	3	2	1	6
Ghafouri-Fard et al. (2021)	3	2	1	6
Hayashi et al. (2021)	2	1	1	4
Kapoor et al. (2019)	4	0	0	4
Kim et al. (2019)	2	1	0	3
Kortvelyessy et al. (2020)	2	1	1	4
Li et al. (2021)	4	2	2	8
Mariotto et al. (2020)	3	1	1	5
Mateos-Hernandez et al. (2016)	2	0	0	2
Millere et al. (2021)	3	2	1	7
Niezgoda et al. (2017)	2	1	1	4
Rossor et al. (2016)	4	2	1	7
Salih et al. (2000)	3	1	1	5
Sandhu et al. (2008)	2	0	1	3
Qiao et al. (2015)	4	2	2	8
Sessa et al. (1997)	3	1	1	5
Sun et al. (2018)	4	2	2	8
Ticau et al. (2021)	4	2	1	7
Wang et al. (2020)	4	1	1	6
Wang et al. (2021)	4	1	1	6
Cross-sectional	**(/5)**	**(/2)**	**(/3)**	**(/10)**
Li et al. (2013)	4	2	3	9
Xiaowei et al. (2014)	3	1	1	5
Ozuguz et al. (2016)	4	2	2	8
Maia et al. (2020)	4	2	3	9
Sandelius et al. (2018)	3	2	2	8
Ziegler et al. (2019)	4	2	1	7
Celikbilek et al. (2014)	4	2	1	7
Cohort	**(/4)**	**(/2)**	**(/3)**	**(/9)**
Frithiof et al. (2021)	4	1	1	6
Jadhav et al. (2011)	2	0	2	4
Lieverloo et al. (2019)	4	2	3	9
Martin-Aguilar et al. (2021)	4	1	2	7
Morgenstern et al. (2021)	4	2	3	9

Supplemental Table 3.5. Diagnostic criteria and neurofilament light chain concentrations used in the subgroup meta-analysis comparing primarily axonal versus demyelinating peripheral neuropathies.

Source (author, year)	Diagnosis (number)	Diagnostic criteria	Control type (number)	Patient concentration: mean (SD)	Control concentration: mean (SD)
Altmann et al, 2020	**GBS** Demyelinating, (n=17) Axonal, (n=5)	Neurologic examination, Brighton criteria (levels 1 or 2), NCV	Patients without neuropathy, n=22	Demyelinating: 161.64 (124.83) pg/mL* Axonal: 62.96 (50.25) pg/mL*	9.07 (3.24) pg/mL*
Martin-Aguilar et al, 2021	**GBS** AMAN, (n=12) AMSAN, (n=7) AIDP, (n=58)	NINDS classification, Brighton diagnostic criteria, NCV	Healthy, n=53	AMAN: 386.67 (381.74) pg/mL* AMSAN: 589.85 (1274.50) pg/mL* AIDP: 93.57 (183.90) pg/mL*	9.67 (6.42) pg/mL*
Sandelius et al, 2018	**CMT** CMT1 – demyelinating, (n=48) CMT2 – axonal, (n=27)	Genetically confirmed CMT, NCV	Healthy, n=67	CMT1: 25.23 (11.80)* pg/mL CMT2: 25.93 (12.31)* pg/mL	15.57 (7.48) ng/mL*
Lieverloo et al, 2019	**CIDP** Induction, n=29 Maintenance, n=24 Remission, n=27	EFNS/PNS 2010 criteria, neurologic exam, NCV	Healthy, n=30	Induction: 47.33 (38.18) pg/mL* Maintenance: 29.7 (14.06) pg/mL* Remission: 30.67 (18.02) pg/mL*	24.03 (10.80) pg/mL*
Hayashi et al, 2021	**CIDP**, n=11	EFNS/PNS 2010 criteria	Healthy, n=7	166.6 (337.8) pg/mL*	12.2 (6.3) pg/mL*
Wang et al, 2020	**CMT1**, Group 1 n=20 Group 2 n=31	Clinical examination, NCV, confirmatory genetic testing, Rasch modified CMT Neuropathy and Examination Scores	Healthy, Group 1 n=20 Group 2 n=24	(NPX): Group 1: 3.60 (0.38)* Group 2: 3.52 (0.76)*	(NPX) Control 1: 2.91 (0.43)* Control 2: 2.84 (0.44)*

Wang et al, 2021	**CMT1**, n=41	Clinical examination, NCV, confirmatory genetic testing, Rasch modified CMT Neuropathy and Examination Scores	Healthy, n=40	(NPX): 3.5 (0.60)*	(NPX): 2.83 (0.45)*

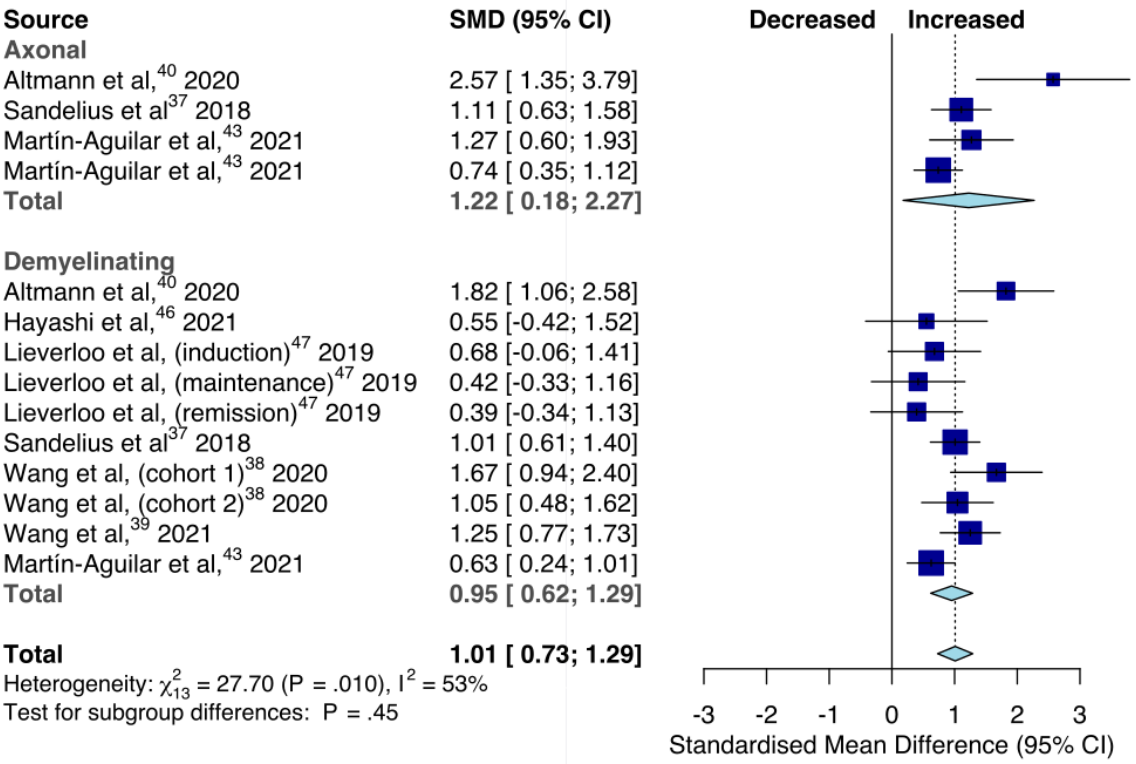
Legend: Legend: Acute inflammatory demyelinating polyneuropathy (AIDP); Acute motor axonal neuropathy (AMAN); Acute motor-sensory axonal neuropathy (AMSAN); Brain derived neurotrophic factor (BDNF); Charcot-Marie-Tooth Disease (CMT); Charcot-Marie-Tooth Disease Type 1 (CMT1); Charcot-Marie-Tooth Disease Type 2 (CMT2); Chronic inflammatory demyelinating polyneuropathy (CIPD); European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS); Guillain-Barre Syndrome (GBS); National Institute of Neurological Disorders and Stroke (NINDS); Nerve conduction velocity (NCV); Neural cellular adhesion molecule (NCAM); Neurofilament light chain (NfL); Normalised protein expression (NPX); *indicates means estimated from graphs/figures

Supplemental Table 3.6. Descriptive synthesis of biomarker diagnostic accuracy.

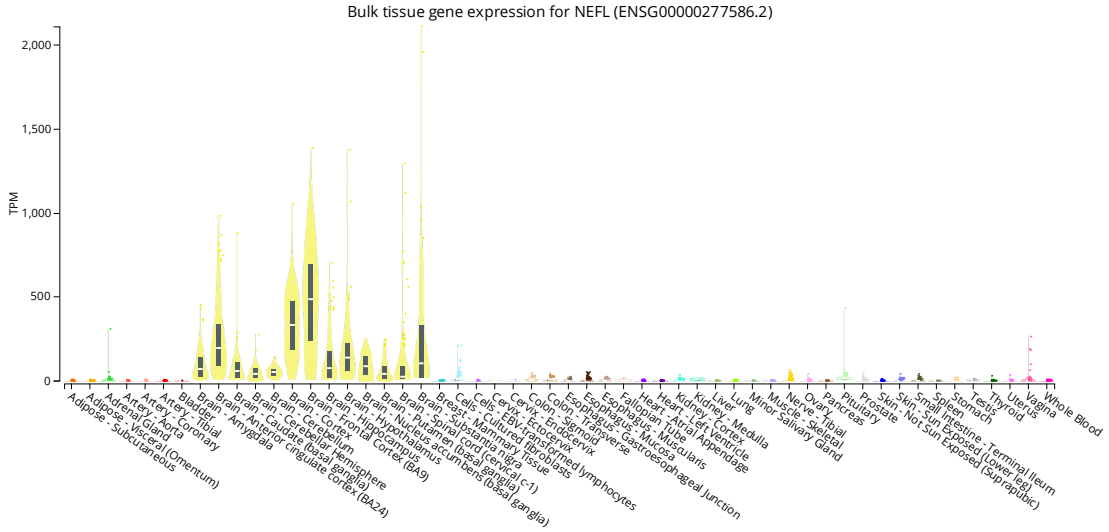
Study	Diagnosis	Comparison	Biomarker	Cut-off	Sensitivity & specificity	Area under curve
Bischof (2018)	Vasculitic neuropathy	Healthy controls	NfL	155 pg/mL	Sensitivity 82%, specificity 100%	0.96
Millere (2021)	Charcot-Marie-Tooth Disease	Healthy controls	NfL	8.9 pg/ml	Sensitivity 74%, specificity 95%	0.88 (95% CI: 0.83–0.93)
Maia (2020)	hATTR amyloidosis with polyneuropathy	Cohort 1: PND 0 vs ≥1	NfL	10.6 pg/mL	Sensitivity 96.2%, specificity 93.8%	0.99
	hATTR amyloidosis with polyneuropathy	Cohort 2: PND 1 ≥ 2	NfL	75.7 pg/mL	Sensitivity 84.6%, specificity 80.0%	0.86
Sandelius (2018)	Charcot-Marie-Tooth Disease	healthy controls	NfL	20 pg/mL	Sensitivity 71%, specificity 75%	0.76
Ticau (2021)	hATTR amyloidosis with polyneuropathy	Healthy controls	NfL	37 pg/mL	false-positive rate 3.6%, true-positive rate 84.9%	0.96 (95% CI 0.96–0.97)
Wang (2020)	Charcot-Marie-Tooth disease	Healthy controls	NfL	NA	NA	0.81 (95% CI 0.72–0.90)
			TMPRSS5	NA	NA	0.91 (95% CI 0.85–0.97)
Wang (2021)	Charcot-Marie-Tooth disease	Healthy controls	NfL	NA	NA	0.80 (95% CI 0.70 - 0.90)
			TMPRSS5	NA	NA	0.87 (95% CI: 0.80-.94)
Ghafouri-Fard (2021)	AIDP	healthy controls	BDNF	NA	Sensitivity 23%, Specificity 95%	0.56
	CIDP				Sensitivity 41%, Specificity 93%	0.68
Sun (2018)	Diabetic neuropathy	Healthy controls	BDNF	1981.05 pg/mL	Sensitivity 98.5%, specificity 74.5%	0.93
			NGF	50.25 pg/mL	Sensitivity 96.9%, specificity 77.3%	0.93
Li (2013)	Diabetic neuropathy	Diabetic without neuropathy	NSE	10.1 μg/L	Sensitivity 66.3%, specificity 72.5%	0.73 (95% CI 0.68–0.77)

Kim (2019)	CIDP	CMT	p75	56.45 pg/mL	Sensitivity 92.1%, specificity 95%	0.97
		AMAN		103.4 pg/mL	Sensitivity 63.2%, specificity 85%	0.78
		AIDP		103.4 pg/mL	Sensitivity 84.6%, specificity 40%	0.37
	Charcot-Marie-Tooth Disease	CIDP	Neural cellular adhesion molecule	7038.7 pg/mL	Sensitivity 39.5%, specificity 100%	0.67
		AIDP		6896.1pg /mL	Sensitivity 42.1%, specificity 100%	0.85
		AMAN		3937.1 pg/mL	Sensitivity 97.4%, specificity 72.2%	0.85

Legend: Acute inflammatory demyelinating polyneuropathy (AIDP); Acute motor axonal neuropathy (AMAN); Brain derived neurotrophic factor (BDNF); Chronic inflammatory demyelinating polyneuropathy (CIDP); Confidence interval (CI); hereditary transthyretin-mediated (hATTR); Not available (NA); Nerve growth factor (NGF); Neurofilament light chain (NfL); Neuron-specific enolase (NSE); Polyneuropathy disability score (PND); Transmembrane protease serine 5 (TMPRSS5)



Supplemental Figure 3.1. Meta-analysis of neurofilament light chain comparing axonal and demyelinating subtypes in patients with peripheral neuropathy compared to controls.



Supplemental Figure 3.2. Bulk tissue gene expression profile for neurofilament light chain (NEFL) according to GTEx Portal (accessed on June 6th, 2022)[60].

4

Protocol & methodology for a longitudinal cohort study in people with WAD2.

4.1. Aims

The primary aims of this longitudinal, multi-centre cohort study are to identify the temporal development of nerve pathology and assess its prognostic role in participants with WAD2.

4.2. Objectives

The specific objectives of this longitudinal cohort study are to:

1. Identify clinical signs of nerve pathology and neuropathic pain using standard clinical assessment and quantitative sensory testing (QST) during the acute and chronic stages.
2. Determine the presence of small nerve fibre pathology during the acute and chronic stages using skin biopsies.
3. Assess the temporal profile of a serum measure of nerve pathology using neurofilament light chain (NfL).
4. Evaluate whether serologic or bedside sensory testing during the acute stage predicts neck-related disability at six months.

5. Identify whether bedside sensory tests can detect functional somatosensory nerve pathology as determined from QST.
6. Identify a molecular signature of persisting moderate-severe symptoms in WAD2 using RNA sequencing from blood samples.

4.3. Background of study development and funding

The study team's breakthrough findings of neuroinflammation in chronic WAD2[117] led to their successfully funded Versus Arthritis Pain Challenge Grant. The aim of this grant is to determine if neuronal and systemic inflammation are present in participants with WAD2. And, if present acutely, assess their ability to predict pain persistence. Nerve inflammation will be measured using advanced MRI techniques (neurography) and a blood draw will examine systemic levels of inflammation. This is done using the combined whiplash cohort collected in Oxford and Sussex. This unique multicentre cohort provides an exciting opportunity to ask further critical questions emerging from the most novel findings of nerve pathology after whiplash injury.

DPhil Project

My DPhil project builds upon the Versus Arthritis Whiplash Cohort but answers novel objectives by comprehensively assessing signs of nerve pathology after whiplash injury. I will assess the use of both clinical and laboratory-based measures of nerve pathology to predict pain persistence. My study utilises a comprehensive set of assessments to evaluate both structural and functional nerve pathology in WAD2.

4.4. Prospective cohort study design and overview

This study used a prospective multicentre study design including a minimum of 69 participants with WAD2. Testing was completed at university research facilities in Oxford and Brighton, United Kingdom. Complete study protocol details have been published[349] and were pre-registered at ClinicalTrials.gov (protocol version is V2 25 September 2020). Ethical approval was received from the South Central – Oxford C Ethics Committee (18/SC/0263) and London-Brighton & Sussex Research Ethics Committee (20/PR/0625). Study data were entered and managed using REDCap electronic database[350].

I organised and led all participant recruitment and assessment for the Oxford cohort. Recruitment and participant assessment from Brighton was completed by a clinical researcher (Dr Colette Ridehalgh [CR]) at the Brighton and Sussex Medical School. Following data collection, I performed the entirety of all experiments and analysis described henceforth (summarised in Figure 4.1).

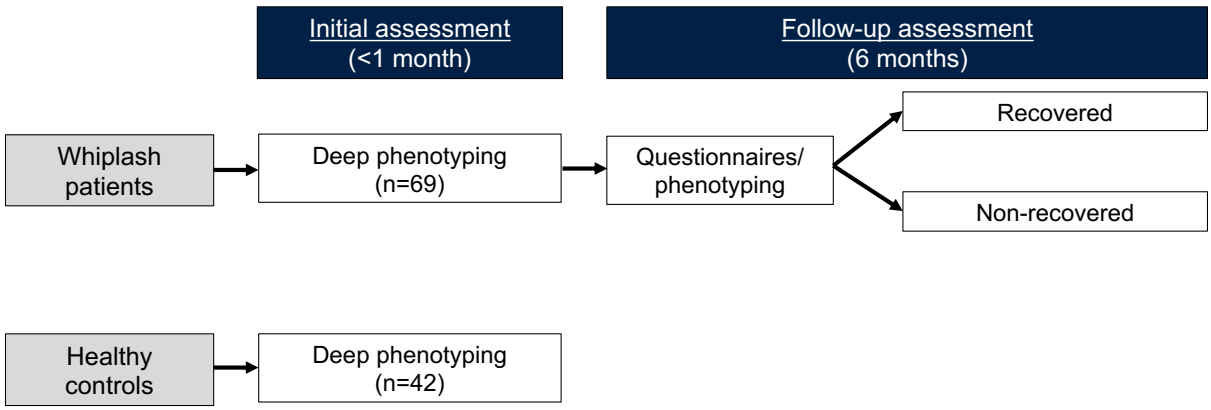


Figure 4.1. Study flowchart for initial and follow-up assessment.

Participants were recruited through the Accident and Emergency Departments from Oxford University Hospitals and University Hospitals Sussex. Participants who sustained a motor vehicle collision were contacted by the corresponding University Hospitals research team regarding permission to be contacted for study participation. The participants were then contacted by me or the clinical researcher in Brighton to assess eligibility. Inclusion criteria was people ages 18 - 85 with a clinical diagnosis of acute WAD2 within 4 weeks of injury. Exclusion criteria for whiplash participants included pregnancy; history of cervical/arm pain lasting >3 months; pain from a previous whiplash injury within the previous 12-months; diagnosis of a peripheral neuropathy; history of systemic illness that is known to cause small fibre pathology or neuropathy (e.g., diabetic neuropathy).

A minimum of forty-two healthy age- and gender-matched control participants were recruited through advertisements and local universities (Oxford and Brighton) to provide normative data. Healthy control participants must not have had any of the above-mentioned exclusion criteria in addition to no previous whiplash injury, and no history of treatment for cervical or thoracic spine, or upper limb pain within the past 3 months. Additional normative healthy control data for QST, skin biopsies, and serum samples were used from a previously published cohort recruited within Oxford and obtained by Professor Annina Schmid (AS)[250]. Previously collected healthy control participant data used in my cohort included 25 participants for QST, 18 participants

for skin biopsies and 11 participants for serum NfL. All other healthy control data was collected as part of the prospective whiplash study between me and CR.

Baseline appointment

Initial baseline assessment for participants with WAD2 included completing a set of self-reported outcome measures, detailed demographic and medical history, clinical assessment, QST, and skin and blood samples. Healthy control participant assessment included the same testing, aside from self-reported questionnaires. Detailed description of each baseline testing component is described below.

Follow-up assessment

All whiplash participants were invited for follow-up testing six months after the date of their whiplash injury. I repeated all baseline testing, including a validated question regarding their perception of recovery[351].

Determining 'recovery'

The concept of recovery is inherently subjective and difficult to objectively quantify. Furthermore, previous longitudinal whiplash cohorts have used various methods of defining recovery (e.g., pain scales, NDI, recovery questionnaires)[352-354]. All outcome measures were kept continuous for linear regression analyses, in keeping with current guidelines[186]. My differential gene expression analysis however required dichotomising participant samples into two groups based on levels of recovery six-months after injury.

To create this dichotomisation, I evaluated different previously used definitions of recovery. This included measures of participant reports of recovery using the aforementioned recovery question, persistent neck-related disability using the NDI, continued pain using the painDETECT average pain question (numeric pain rating scale 0-10); and persistent whiplash-related symptoms reported at follow-up appointment (VAS 0-100). I carried out a sensitivity analysis to compare different outcome definitions. I assessed the robustness of the impact of definitions based on the coefficient estimates of logistic regressions. The log odds of each are shown in Figure 4.2. Overall, the estimates had large confidence intervals but also showed similar coefficient estimates between outcome definitions for most variables.

Overall, I assessed five different definitions of recovery. These included 1) global recovery question indicating 'all better'; 2) global recovery question including 'all better' or 'quite a bit of improvement'; 3) NDI scores < 5; 4) average pain scores $<$ 3/10 (from painDETECT questionnaire); and 5) whiplash-related symptoms $< 30/100$ (VAS). Since the coefficient estimates were similar across definitions, I based my classification of recovery on whiplash-related symptoms. This choice was informed by a recent comparative RNA-sequencing study of persistent pain in WAD[354], which also utilised whiplash-related symptoms. Dichotomising participants using whiplash-related symptoms also yielded small variance and similar point estimates compared to the other definitions (Figure 4.2). As a result, six-month follow-up participants with

whiplash-related symptoms < 30/100 (VAS) were classified as 'minimal pain', and participants with scores of 30 or higher were classed as 'moderate/severe pain'.

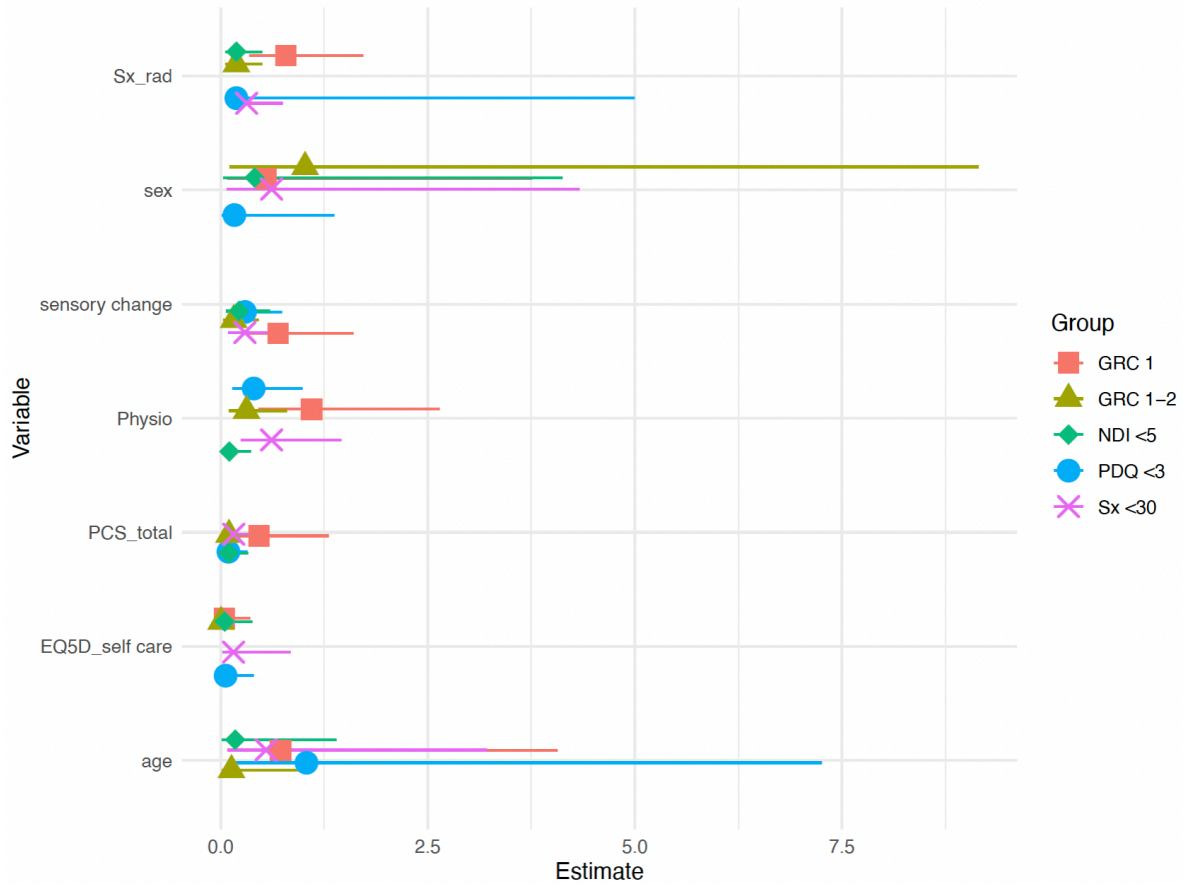


Figure 4.2. Coefficient estimates from logistic regression models evaluating different definitions of recovery and selected clinical variables.
Abbreviations: GRC: Global rating of recovery question (1= 'all better [cured]'; 2= 'There has been quite a bit of improvement'); NDI: Neck Disability Index (scores < 5/ 50, PDQ: painDETECT questionnaire (average pain < 3/10), Sx: whiplash-related symptoms (visual analogue scale < 30/100 used); sx_rad: whiplash-related radiating symptoms; sensory change: subjectively reported change in sensation due to whiplash injury (e.g., numbness, tingling); physio: reports of physiotherapy use for whiplash-related symptoms; PCS_total: Pain Catastrophisation Scale total score; EQ5D_self care: self-care question within the EQ-5D-5L Questionnaire.

4.5. Clinical phenotyping

Self-reported outcome measures

All outcome measures were selected through consensus with the collaborating research team in Brighton. The range of outcome measures cover questions relating to the six

core domains recommended for tracking outcomes following whiplash injury[355]. These domains include physical functioning, perceived recovery, work and social functioning, psychological functioning, quality of life and pain.

Self-reported neck disability was evaluated using the Neck Disability Index (NDI, zero is no disability; 50 is complete disability)[47]. I used previously described NDI disability scoring of mild <5, moderate 5-14, and severe ≥15[212]. The NDI has shown to be a reliable, valid, and responsive measure for self-reported disability following whiplash injury[356]. The painDETECT questionnaire was used to screen for neuropathic pain components (rated 0 - 38, scores >18 categorised as likely neuropathic pain[357]) and has shown to be a reliable screening tool for neuropathic pain[358].

Psychological functioning was evaluated using a broad set of measures. Trauma-related distress was evaluated using the Impact of Events Scale-Revised (IES-R) and short posttraumatic stress inventory (PTSD-8). IES-R scores ≥ 33/88 suggests that PTSD is probable[359]. The PTSD-8 suggested a score of ≥18/32 had the best predictive value to be symptomatic for PTSD[360]. Pain-related worrying was assessed using the Pain Catastrophisation Scale (PCS, cut-off score ≥30/52 for significant pain-related worrying)[361]. The level of negative emotional states was measured using the Depression, Anxiety and Stress Scale (DASS42). Each of the three DASS42 subscales is scored out of 42 with mild scores <6, moderate 7-12, an severe ≥13[362]. Of which, all psychological functioning measures have shown good psychometric properties after trauma[360,363,364] and whiplash injuries[365].

Quality of life was evaluated using the EQ-5D-5L questionnaire[366] which has been shown to be a responsive and reliable measure in numerous musculoskeletal conditions[367]. Per recommended guidelines[366], I calculated the EQ-5D-5L health-related index value (0: as bad as being dead, 1: full health) using the most recently published value set for England[368] using the R package eq5d[369].

Perceived recovery was evaluated using a single question asking, "How do you feel you are recovering from your injury?" This question was shown to be associated with pain, functional limitations, and depression in a large cohort of people following whiplash injury[351].

Background information

I collected detailed demographic and medical histories of each participant, including age, sex, height/weight, occupation, dominant handedness, past medical history, current medication use, and current or previous nerve injury or involvement. A copy of the clinical research form developed for WAD2 participants in this study is included in Appendix B. Participants after whiplash injury were asked details regarding the presence and distribution of pain and/or symptoms, and subjective reports of radiating symptoms and sensory changes (e.g., paraesthesia or weakness).

The distribution of symptoms was recorded via a participant-completed pain body diagram as well as VAS of current whiplash-related symptoms (0 – 100 mm). Participants after whiplash were also asked about the motor vehicle crash characteristics, crash-related litigation, current work status, imaging or other medical investigations, and any current management for symptoms.

Neuropathic pain grading

The certainty of neuropathic pain was classified for each WAD2 participant according to the Neuropathic Pain Grading System published by the Neuropathic Pain Special Interest Group of the International Association for the Study of Pain (Figure 4.3).[194] In brief, grading of possible neuropathic pain includes pain descriptors related to neuropathic pain (e.g., burning, tingling, shooting) and a neuroanatomically plausible pain distribution. Probable neuropathic pain includes negative sensory signs in the primary pain location identified through bedside neurological assessment. A grading of the next higher category can only be reached if the previous categories were met. Participants were characterised as 'unlikely' neuropathic pain if no neuropathic pain descriptors were reported.

The likelihood of neuropathic pain was also graded using the painDETECT questionnaire with the previously published grading criteria with scores $> 18/38$ categorised as 'a neuropathic pain component is likely'[357].

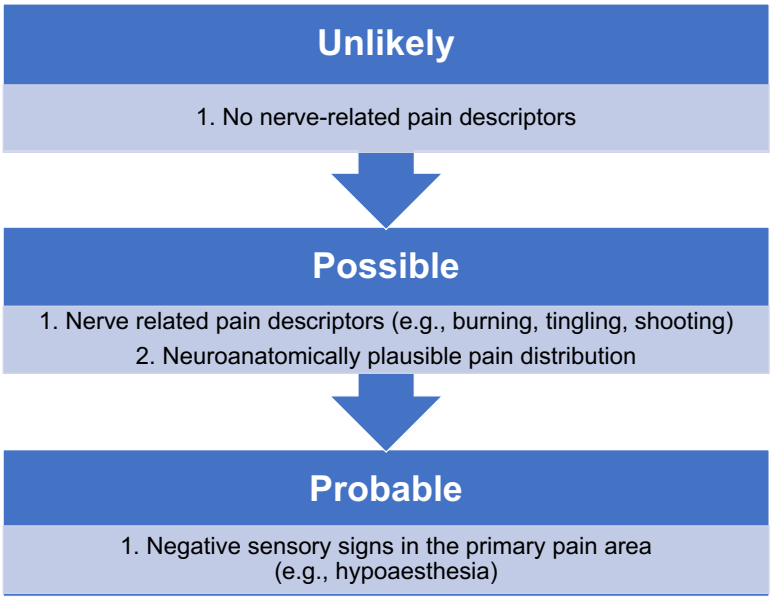


Figure 4.3. Neuropathic Pain Grading System published by the Neuropathic Pain Special Interest Group of the International Association for the Study of Pain.

Clinical assessment

A summary of the locations and grading for all clinical tests are summarised in Table 4.1. A brief musculoskeletal assessment including active range of motion of the cervical spine and bilateral upper extremities was performed. Active movement was graded on a four-point categorical scale: 0: full movement; 1: full movement with symptoms; 2: reduced movement without symptoms; 3: reduced movement with symptoms.

I performed a neurological assessment of the bilateral upper extremities within the C5 - T1 innervation territories. I chose to perform a bedside neurological assessment to investigate signs of neurological dysfunction using standard clinical tools and to compare with reference standards for assessing somatosensory dysfunction (QST). The neurological assessment included cutaneous sensation using cotton wool and pin prick (using Neurotips). Myotomal isometric muscle strength was graded using the six-point

ordinal MRC grading scale (0: no muscle contraction, 5: normal power)[370]. Deep tendon reflexes of the biceps and triceps muscles were tested and graded using the National Institute of Neurological Disorders and Stroke five-point ordinal scale (0: absent reflex, 4: enhanced reflex, including clonus)[371]. Pin prick (using Neurotips) and thermal sensation using warm and cool coins were assessed at the index finger and self-reported main pain area, as previously described and validated[372]. In brief, both the Neurotip and thermal coins were tested bilaterally. Each test was first performed on the side contralateral to their most symptomatic upper extremity. Both pinprick and thermal tests were graded on a four-point ordinal scale (0: Absent; 1: Reduced; 2: Normal; 3: Enhanced). If participants reported equal laterality to their symptoms, familiarisation to the Neurotip and thermal coins was performed near the superior aspect of their chest and clavicle.

The Neurotip was applied perpendicular to the skin with adequate pressure to blanch but not penetrate the skin. Participants were asked if the sharp sensation was comparable to the site of familiarisation (e.g., less affected contralateral upper extremity or chest). Thermal sensation was then tested using two metal coins (50 pence pieces). One coin was left at room temperature (cool coin) and one coin was left in the pocket of the clinical researcher for at least 30 minutes (warm coin). Each coin was placed on the proximal phalanx of each index finger and in the participants' self-reported main pain area. Participants were asked to compare the perceived temperature of the coin to the initially tested less symptomatic side. If a participant

reported a sensation as abnormal, the test was then repeated a maximum of two times to confirm the initially reported finding. I selected the index finger as it has previously shown somatosensory dysfunction in chronic WAD using QST[16,40,120]. The main pain area was selected to measure sensory signs for the Neuropathic Pain Grading System (Figure 4.3).

I categorised neurological assessment results as normal, loss of function (reduced sensation, strength, or reflexes), gain of function (increased response to sensation or reflex testing), or mixed (signs of both loss and gain of function).

I also classified neurological loss of function for each corresponding test according to the following criteria: subjectively reported reduced cutaneous sensation to light touch or pin prick[373]; Isometric muscle strength values less than five from the MRC grading scale[370]; biceps or triceps muscle deep tendon reflex values less than two out of four[371]; subjectively reported decrease in warm or cold temperature sensation compared to the less affected side using previously validated coins[374].

Neurological gain of function was categorised for each corresponding test according to the following criteria: subjectively reported increase in cutaneous sensation to light touch (e.g., reports of paraesthesia or allodynia) or pin prick (e.g., increased painful sensation); biceps or triceps muscle deep tendon reflex values greater than two out of four[371].

Table 4.1. Summary of clinical assessment measures and corresponding grading scales.

Site	Test	Grading
C5-T1 innervation territory	ROM	0: full movement; 1: full movement with symptoms; 2: reduced movement without symptoms; 3 reduced movement with symptoms.
	Muscle strength	0: No contraction; 1: Flicker or trace of contraction; 2: Active movement, with gravity eliminated; 3: Active movement against gravity; 4: Active movement against gravity and resistance; 5: Normal power.
	Deep tendon reflexes	0: no response; 1: slight response; 2: brisk, normal response; 3: very brisk response.
	Cutaneous sensation (cotton wool, pin prick, thermal coins)	0: Absent; 1: Reduced; 2: Normal; 3: Enhanced
Main pain area	Cutaneous sensation (pin prick & thermal coins)	0: Absent; 1: Reduced; 2: Normal; 3: Enhanced.
Index finger	Cutaneous sensation (pin prick & thermal coins)	0: Absent; 1: Reduced; 2: Normal; 3: Enhanced.

Quantitative sensory testing

I performed QST according to the German Network for Neuropathic Pain protocol[375]. The full QST protocol was performed on the ventral aspect of the proximal phalanx of the index finger. The index finger was chosen as has previously shown changes in chronic WAD[16,40,120]. Participants with WAD2 in our study were tested and on the most symptomatic side; healthy controls were tested on their non-dominant side.

Warm and cold detection thresholds (WDT, CDT), cold and heat pain thresholds (CPT, HPT), and thermal sensory limen (TSL) were measured using a Thermotester (Somedic, Sweden). I measured mechanical (MDT) and vibration detection thresholds (VDT) using von Frey filaments (Optihair 2 MRC Systems, Germany), and a rydel Seiffer tuning fork (Wagner, USA), respectively. Pressure pain thresholds (PPT) using

a handheld algometer (Wagner Force dial, USA) and mechanical pain sensitivity (MPS) using weighted pinprick stimulators (MRC Systems, Germany) were measured. I also quantified the number of paradoxical heat sensations during TSL and the presence of dynamic mechanical allodynia.

CDT, WDT, and PPT were performed over the contralateral lower limb (upper anterolateral aspect of the tibia) to assess for potential widespread sensory changes. A recent systematic review of temporal QST measures in WAD showed heightened PPT at the tibialis only in those who developed persistent symptoms[158]. No previous studies in this review assessed lower extremity somatosensory loss of function. As such, I chose to assess both loss and gain of function over the skin of the tibialis anterior muscle to assess these differences in my longitudinal cohort.

Psychometric properties of clinical assessments

Clinical assessments of neurological function, including reflexes, myotomal strength, and cutaneous light touch sensation have previously been shown to have moderate to substantial reliability (sensory testing: kappa = 0.53; manual muscle testing: kappa = 0.68)[376]. The pinprick and thermal coin testing have been shown to be a valid method to detect somatosensory dysfunction[374] and small-fibre degeneration[372]. The QST protocol has shown to have high intra- and good inter-tester reliability[377-379]. In addition, our QST protocol has shown good long-term stability over time[379].

Standardisation across sites

All clinical assessments were initially piloted with the clinical researcher at the Brighton and Sussex Medical School (CR). Both sites used identical testing equipment, as described above.

Histological analysis

I performed two 3mm diameter skin biopsies taken from the ventrolateral aspect of the proximal phalanx of the index finger and 10cm above the contralateral lateral malleolus in sterile conditions using local anaesthesia (1% lidocaine, 1 – 2.0 mL).

The index finger skin was selected because of its innervation by the potentially affected nerves in the neck and has previously shown small fibre pathology in chronic WAD2[40].

The biopsies were fixed in periodate-lysine-paraformaldehyde for 30 minutes followed by washing samples in 0.1 M phosphate buffer. The tissue was then embedded in OCT compound and stored at -80° Celsius.

I cut 50µm sections and performed immunohistochemistry, as previously described [279]. In brief, I blocked skin sections in 5% fish gelatine solution for one hour. Next, I added the primary antibodies for protein gene product 9.5 (PGP9.5, Zytomed 1:200) and myelin basic protein (MBP, Abcam 1:500) for overnight incubation. Following three one-hour wash cycles in PBS, secondary antibodies were added to free floating tissue sections and incubated overnight (Alexa 488, 1:1000, Abcam; Cy3, Stratech, 1:500). See full protocol in the attached Appendix C.

I evaluated the integrity of intraepidermal and dermal nerve fibres as previously detailed[250,279,380] and blinded to participant allocation. In brief, I calculated intraepidermal nerve fibre density from three samples per participant with the average expressed as epidermal fibres/mm, per recommended guidelines[380]. I also counted the number of Meissner corpuscles and expressed per millimetre of epidermis at the index finger.[279] All skin samples were collected and stored in accordance with the Human Tissue Act.

As reported above, 16 healthy control skin samples were used from a previous study investigating carpal tunnel syndrome[250]. To ensure rating was accurate between previously counted samples, both assessors (AS and JF) counted the same nine sections of stained skin samples. Overall, we had excellent inter-rater reliability (ICC = 0.92). In addition, eight samples from the carpal tunnel syndrome healthy control cohort were proportionally age-matched for a direct comparison of intraepidermal nerve fibre density (fibres/mm). There was no significant difference in the counts between the carpal tunnel syndrome and whiplash cohort ($p = 0.33$).

<u>Serum marker of axonal injury</u>

I analysed serum levels of the neuronal protein neurofilament light chain (NfL) using the Simoa SR-X System (Quanterix, Billerica, MA, USA). Blood measures of NfL have never been measured in participants after whiplash injury. Thus, there was no estimate of NfL protein concentrations for this population. Because of this, I chose the Simoa

platform for its superior detection threshold capacity (limit of detection: 0.0552 pg/mL vs MSD R-PLEX lower limit of detection 5.5 pg/mL). Recent studies have also shown it to be more sensitive than ELISA or other electrochemiluminescence platforms[381].

To perform, blood was first collected from the cubital fossa into a BD Vacutainer SST tube (BD Vacutainer Tube SST Advance) and was processed by centrifuge at 3,000 rpm at 4°C for 10 minutes. Serum was then extracted and stored at -80°C in compliance with the Human Tissue Act 2004.

I analysed protein concentrations of NfL using the Simoa SR-X and NF-Light v2 Advantage Kit (Quanterix, Billerica, MA, USA), included in Appendix D. Accordingly, I thawed all serum samples at room temperature and spun at 10,000g for 5 minutes. I prepared the Quanterix canonical plate, including the addition of prepared calibrators, controls, and samples. I then aliquoted serum samples in duplicate followed by adding the capture bead and NfL antibody detector solutions with 30-minute incubation. I then added streptavidin-ß-galactosidase and Rersorufin ß-Dgalactopyranoside reagents and washed twice. Following a 10-minute plate drying period, I loaded all samples into the Quanterix SR-X system for analysis.

RNA extraction

To further understand the biological processes of persistent pain after whiplash injury, I performed RNA sequencing followed by differential gene expression analysis from

blood samples of WAD2 participants to identify transcriptional changes in the minimal vs moderate-severe persistent pain. Persisting moderate-severe pain was determined using participant-reported whiplash symptom visual analogue scale (VAS) ≥ 30/100, as previously used in WAD[354] and described above in detail. The minimal pain and moderate/severe pain groups were sex-matched (n = 7 females per group) with no significant differences in ages between groups ($p = 0.86$). The moderate/severe persistent pain group had a mean whiplash-related symptoms of 44.7/100 (SD 14.7) using the VAS. The mean whiplash-related symptoms for the minimal persistent pain group was 4.8/100 (SD 5.7). Per selection for RNA extraction, there was a statistically significant difference in whiplash-related symptoms at follow-up using an independent two-tailed t-test ($p < 0.001$).

Blood for RNA-sequencing was collected from the cubital fossa in participants using Tempus™ Blood RNA Tubes (ThermoFisher, num. 4342792, Massachusetts, USA). Blood samples were initially shaken for approximately 10 seconds after collection to stabilise RNA. Samples were stored at minus 20° Celsius until processing. I extracted RNA using the Tempus Spin RNA Isolation Kit (ThermoFisher, num. 4380204, United Kingdom). Blood samples were thawed and diluted with 1x PBS, vortexed and centrifuged. I then removed the supernatant and resuspended the RNA pellet. The resuspended RNA was then purified and eluted using a microcentrifuge. Extracted RNA was stored at minus 80°C in Nucleic Acid Purification Solution until sequencing. A full RNA extraction protocol is included in Appendix E.

4.6. Statistical analysis

A summary of the primary objectives, analysis, and estimated samples sizes are provided in Table 4.1. All reporting followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for observational cohort studies[382]. Data was analysed using R software (version 4.0.3). Data normality was assessed using the Kolmogorov-Smirnov test and visual inspection. The distribution of clinical phenotypic data was calculated for parametric and non-parametric data as mean/standard deviation (SD) and median/interquartile range (IQR), respectively. The fraction of missing data was <5%, so missing values were discarded and imputation procedures were not required[383,384]. Statistical significance is set at $P<0.05$.

Table 4.2. Summary of the sample size estimations and statistical analysis plan for each primary study objective.

Objective	Sample size	Statistical analysis
1. Clinical assessment		Descriptive statistics (mean/SD, median/IQR)
2. Intraepidermal nerve fibre density	68 WAD2 participants and 34 healthy control participants Power 0.80, α=0.05 effect size=0.53	Independent/paired t-tests or non-parametric alternatives
3. Serum neurofilament light chain	15 WAD2 participants and 15 healthy control participants Effect size=1.4, 95% power =0.95. α=0.05	Two-tailed independent/paired t-tests or Mann Whitney/Wilcoxon rank-sum.
4. Prognostic role of nerve pathology	69 acute WAD2 participants Power 0.80, effect size f^2=0.15, 13% squared partial correlation of	Regression models with four predictor variables (neurofilament light chain, age, sex, initial NDI score)
5. Clinical tests of small fibre nerve pathology		Sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio

6. RNA-sequencing of blood samples	n=15 participants per group (recovered and non-recovered) minimum fold change log2 = 2.0, FDR 5%, 100 reads per prognostic gene, 0.16 dispersion.	

Objective 1 – Detailed clinical assessment

Clinical neurological assessment: Neurological assessment results were descriptively reported and presented as the percentage of individuals with normal, loss, or gain of function.

QST: Data transformation and analysis for all QST parameters followed previously recommended methods for clinical testing[375,385]. All testing parameters, excluding CPT, HPT, and VDT, were log transformed to achieve normal distribution. MPS values included the addition of a constant 0.1 to avoid losing zero-rated values. QST z-scores were calculated using at least n = 7 healthy controls per age decade[386]. A lack of variance for VDT within the 20-year-old age decade did not allow for z-score calculation due to ceiling effect in VDT measure. Therefore, VDT z-scores for 20- and 30-year-olds were derived from a combined total mean and standard deviation incorporating both decades into one calculation. The remaining VDT z-scores were calculated for each decade.

I compared each QST parameter for acute WAD2 and healthy control participants using two-sided independent t-tests. Longitudinal changes in QST parameters

comparing acute, follow-up, and control participants were assessed using ANOVA with post hoc testing.

I clustered QST parameters into one of four previously established deterministic sensory phenotypic profiles: 'normal', 'sensory loss', 'thermal hyperalgesia', and 'mechanical hyperalgesia'[387]. In brief, the probability value for each QST parameter was calculated and averaged for each of the aforementioned sensory phenotypes. The final phenotypic cluster allocation was determined by allocating each participant into their highest probability phenotype.

Objective 2 – Small fibre nerve pathology using skin biopsies.

To assess whether nerve pathology occurs acutely after injury, I evaluated epidermal nerve fibre density as a reflection of small fibre pathology. I completed two comparisons: 1) comparing skin innervation between acute WAD2 participants and age- and gender-matched healthy control participants and 2) comparing epidermal nerve fibre densities within WAD2 participants comparing the acute to chronic stages. Mann-Whitney U tests were used to compare acute WAD2 and control intraepidermal nerve fibre densities. Changes in nerve fibre density between acute, follow-up, and control participants were assessed using aligned ranks transformation ANOVA with post hoc testing.

Objective 3 - Serum NfL

To identify a blood-based measure of nerve pathology, I compared serum concentrations of NfL 1) between acute WAD2 and age-matched healthy control participants and 2) between acute and chronic WAD2 participants. I used a Mann Whitney U test to compare median serum concentrations of NfL for acute WAD2 and controls. Changes in NfL concentrations of comparing acute, follow-up, and control participants was done using aligned ranks transformation ANOVA with post hoc testing.

Z-scores for serum NfL were calculated for all participants as previously described[388]. In brief, serum NfL z-scores were calculated by adjusting serum NfL concentrations for age and BMI. Normative data was derived from a large general population cohort without diagnosis of central nervous system disease or other major diseases (N = 4,532 control participants)[388]. Similar serological analytical approaches were used between participants in the current and previous healthy control study; however, two versions of the Simoa NfL assays (NF-light assay v1 and v2) were used resulting in slight divergence in variability. This was adjusted for by multiplying NF-light v2 concentrations by a factor of 0.89, based on technical recommendations from the supplier (Simoa, Quanterix, USA).

I performed three main exploratory analyses to better understand the role of acute NfL levels in my WAD2 cohort.

1) To identify a blood-based measure of nerve pathology, I compared serum NfL z-scores 1) between people with acute WAD2 and age-matched healthy control participants using an independent two-sided t-test and 2) within WAD2 participants (acute vs follow-up stage) and healthy controls using ANOVA with post hoc testing.
2) I compared clinical variables associated with high vs low acute NfL z-score values. To do so, I dichotomised NfL z-scores in WAD2 participants using the following cut-offs, as previously identified[388]. All z-scores ≥1.5 (corresponding with the top 10%) were classified as having 'elevated NfL'. Z-scores <1.5 were classified as 'baseline NfL'. I then used kernel density estimation plots to assess the distributions between elevated and baseline NfL levels. I chose kernel density estimation as a nonparametric method to assess exploratory trends in my data.
3) I assessed the ability of clinical neurological assessments to predict NfL z-scores in the acute stage. I used linear regression to analyse clinical nerve pathology measures (bedside sensory detection thresholds) predicting acute NfL z-score values for participants with WAD2. I calculated the coefficient estimate, standard error, and p-value for each parameter.

Objective 4 – Prognostic role of nerve pathology

I used the NDI as my clinical endpoint to determine the prognosis of participants with WAD2. The NDI has been extensively used for prognosis in WAD[13,58,59] and is recommended as a core outcome measure in current guidelines for whiplash-related

outcome selection[355]. NDI scores were kept as a continuous variable for analysis, per current guidelines.[186] Statistical methods followed international guidance for prognostic factor study design and analysis (PROGRESS framework)[186].

I assessed the prognostic role of NfL as well as bedside sensory tests of nerve pathology. I chose to use NfL as a quantitative biological measure of neural pathology. Blood-based measures of NfL have been shown to be highly prognostic in a range of pathological conditions, including TBI[389], Alzheimer's Disease[390], and Multiple Sclerosis[388]. As NfL is not readily available in the clinic, I selected thermal and pin prick sensation at the index finger as surrogate bedside measures of nerve pathology that is easily measured in the clinic. My systematic review in WAD (Chapter 2) showed the index finger to be the most affected area of nerve pathology and included QST measures for loss of thermal and mechanical sensation.

The first model assessed neurofilament light chain as a prognostic factor controlling for age, sex, and initial NDI score. I analysed the prognostic role of NfL using both absolute serum concentrations of NfL (pg/mL) as well as serum NfL z-scores. As discussed in Chapter 1, previous preliminary evidence has highlighted the potential influence of age, sex, and initial disability levels on recovery in WAD[36,50]. As such, each were included as covariates in the linear regression models. Predictive ability using the bedside sensory tests at the index finger (pin prick, warm, and cold sensation) were analysed

using multiple linear regression including the previously listed confounders of age, sex, and initial NDI score.

In addition to strict linear regression, I performed exploratory analyses of acute factors that may be related to persistent whiplash-related symptoms six-months after injury. To do this, I dichotomised participants using my previously described cut-off of symptom VAS ≥ 30/100 as moderate/severe pain and <30/100 as minimal pain. I used kernel density estimations to visually assessment acute factors that may distinguish group differences between minimal pain and moderate/severe pain. Kernel density estimations were only used for exploratory analyses and not part of my primary prognostic objective. Thus, these exploratory assessments did not have adequate sample sizes to run strict statistical comparisons (e.g., t-tests or Mann-Whitney U tests).

Objective 5 – assessment of bedside tests to detect somatosensory nerve pathology.

I assessed the performance of clinical bedside tests for somatosensory hypoaesthesia at the index finger in participants with acute WAD2. I selected somatosensory hypoaesthesia as it is a hallmark of nerve pathology[194].

I used cold, warm, and mechanical detection thresholds from the previously described QST protocol as the current best standard for detecting somatosensory dysfunction[375]. I then compared these QST parameters to the previously detailed cool and warm coins

as bedside tests for thermal hypoaesthesia at the index finger. I also compared QST mechanical detection thresholds with cottonwool at the index finger. I did not perform any other bedside clinical tests at the index finger that could be directly compared to the QST measures taken at the same location.

I defined somatosensory hypoaesthesia as QST z-score values $<$ -1.96, per recommended guidelines[375,385] and in line with a previous validation study of bedside neurological testing[374]. I defined sensory hypoaesthesia for bedside tests using the aforementioned criteria (subjectively reported reduced cutaneous sensation to light touch or thermal coins).

I compared the bedside tests for thermal and mechanical hypoaesthesia in two primary ways: 1) concurrent validity and 2) testing accuracy:

1) Bedside test concurrent validity

I calculated the concurrent validity of each bedside test (cool coin, warm coin, and cotton wool) by creating a two-by-two contingency table and calculating the percent agreement between each bedside test and its corresponding QST variable. I used Fisher's exact test to measure the correlations between the bedside and QST hypoaesthesia parameters. Lastly, to analyse the strength of correlation between each parameter, I calculated the Phi correlation coefficients for each test.

2) Bedside test accuracy

I assessed the accuracy of each bedside test (cool coin, warm coin, and cotton wool) compared to its corresponding QST parameter. I calculated the sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio for each. I also calculated the positive likelihood rations using the formula sensitivity/ (1 – specificity), and negative likelihood ratios with the formula (1 – sensitivity)/specificity.

Objective 6 – RNA-sequencing

RNA sequencing was completed by Azenta Life Sciences (Essex, United Kingdom). Library preparation of mRNA used Poly(A) selection. Multiplexed sequencing was performed using Illumina NovaSeq with 2x150 bp sequencing, 20M read pairs. Initial quality control of RNA-sequencing was done using FASTQC[391] and Samtools[392]. Initial control metrics included sequence counts, mean sequence quality, per base sequence content, per sequence GC content, sequence length distribution, adapter content, and duplication levels. All samples passed quality control with high quality base content, but all samples included high levels of adapter content. To address this, I performed an additional trimming step to remove adapter sequences from my raw sequencing reads using Trimmomatic[393]. Following trimming, quality control was re-assessed confirming that adapter contamination had been rectified. Then, we proceeded to mapping the reads to the Grch38 human genome using the STAR aligner[394] and

standard ENCODE parameters. Read mapping quality control was assessed by the fraction of uniquely mapped reads (Figure 4.4).

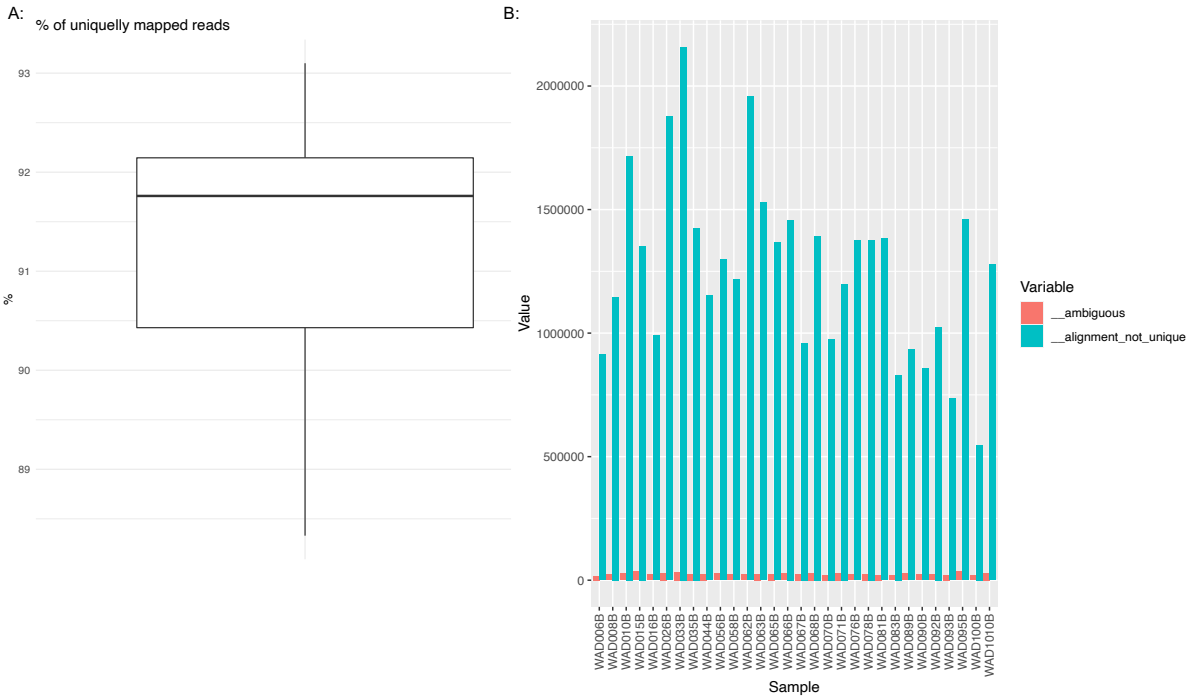


Figure 4.4. Quality control of RNA-sequencing after trimming reads. (A) percentage of uniquely mapped reads to genome. (B) Number of mapped reads that can be allocated uniquely and ambiguously to genes for all RNA-sequencing samples. N=30 WAD2 blood samples total.

Gene expression was quantified by counting the overlaps between mapped reads[394] and the ENSEMBL gene set annotation GRC.h.38.88 using HTSeq. All analyses were carried out in R. Raw sequencing counts were normalised for library size using DESeq2[395]. A negative binomial distribution was fitted to normalised read counts and a Wald test determined differential expression between conditions, controlling for gender and age strata. Normalised counts were log2 transformed for visualisation and correlation analysis purposes. Following completion of the study, data will be made publicly available under managed access through GEO/dbGAP.

Genes that were significantly differentially expressed were assessed for correlation with clinical variables of whiplash-related symptoms and nerve pathology. I used Pearson correlation coefficients to assess for the strength and significance in correlation between the identified dysregulated genes and measures of pain and nerve pathology at six-month follow-up.

4.7. Sample size estimations

Sample size calculations for objectives 2 - 4 were performed using G*Power software (version 3.1.9.6)[396]. The RNA-sequencing sample size for Objective 6 was calculated using RnaSeqSampleSize[397]. The primary sample size for this longitudinal cohort study is based on the multiple linear regression model used to answer my main hypothesis in Objective 4. Samples sizes for objectives 1 and 5 were not calculated, as these are secondary exploratory objectives in this study.

Objective 2 - Acute skin biopsy: 68 WAD2 participants and 34 healthy control participants (80% power, 5% significance, 0.53 effect size) would allow the detection of a 20% smaller difference in intraepidermal nerve fibre density in the acute stage compared to healthy controls based on our previous cohort of participants with chronic WAD2[40].

Objective 3 - Serum marker of nerve pathology: Based on a previous study analysing plasma neurofilament light chain concentrations in participants after acute traumatic

non-brain injury trauma compared to healthy controls,[291] n = 15 participants are required in each group to detect a significant difference (d = 1.4, power = 0.95. α = 0.05) using a two-sided independent t-test.

Objective 4 - Measures of nerve pathology as a prognostic factor: A sample size of 69 provides 80% power to detect a squared partial correlation of 13% between 6-month disability scores (NDI) and NfL measures (mean concentration and z-scores). This corresponds to a medium effect size f^2=0.15, in a linear regression model at a 5% significance level, controlling for the known factors age, sex and initial NDI allowing for 20% drop-out (N = 55 paired WAD2 participants are required for final model).

Objective 6 - RNA-sequencing: Based on previous RNA-sequencing in blood for participants after whiplash injury[354], N = 15 participants per group (moderate-severe pain vs minimal pain) will yield 80% power to detect 56 prognostic genes out of 10,000 (assuming minimum fold change log2 = 2.0, FDR 5%, 100 reads per prognostic gene, and dispersion of 0.16).

5

Characterising acute nerve pathology in WAD2

5.1. Aims

The aim of this chapter is to evaluate the presence of nerve pathology and neuropathic pain in participants with acute WAD2. To do this, I will investigate:

1) The clinical certainty of neuropathic pain.
2) Somatosensory neurological assessments using clinical and quantitative sensory testing (QST).
3) Serological measure of nerve pathology using neurofilament light chain.
4) Histological analysis of small fibre nerve pathology using immunohistochemistry in skin biopsies.
5) Validity and accuracy of bedside clinical tests to detect somatosensory loss of pathology in acute WAD2.

5.2. Methods

Chapter 4 includes detailed descriptions of all methodologies and statistical analyses used for this chapter.

5.3. Results

5.3.1. Participant characteristics

Figure 5.1 shows the study flow diagram for acute WAD2 and healthy control participants included in the study. One-hundred and thirty participants with acute WAD2 (median age: 35.5 years, 57.7% female), and 67 healthy controls (median age: 43.0, 55% female) completed baseline assessment (Table 1). The combined healthy control cohort is derived from three testers (JF, CR, and AS). The ages between each collected healthy control cohort are significantly different between each tester ($P < 0.0001$), which limits the ability to assess inter-rater reliability between assessors (Supplemental Table 1). The median BMI for acute WAD2 is 26.5 (SD 7.9) and 24.5 (SD 5.7) for controls. Most people with acute WAD2 are back at work since the accident (76%). Rear impact is the most common collision direction (36.9%) occurring at speeds greater than 10 mph (55.4%). Nearly one-third of participants (31.5%) report anticipating the impact occurring before it happened. Lastly, a minority of participants (28.4%) report being involved in litigation due to the collision.

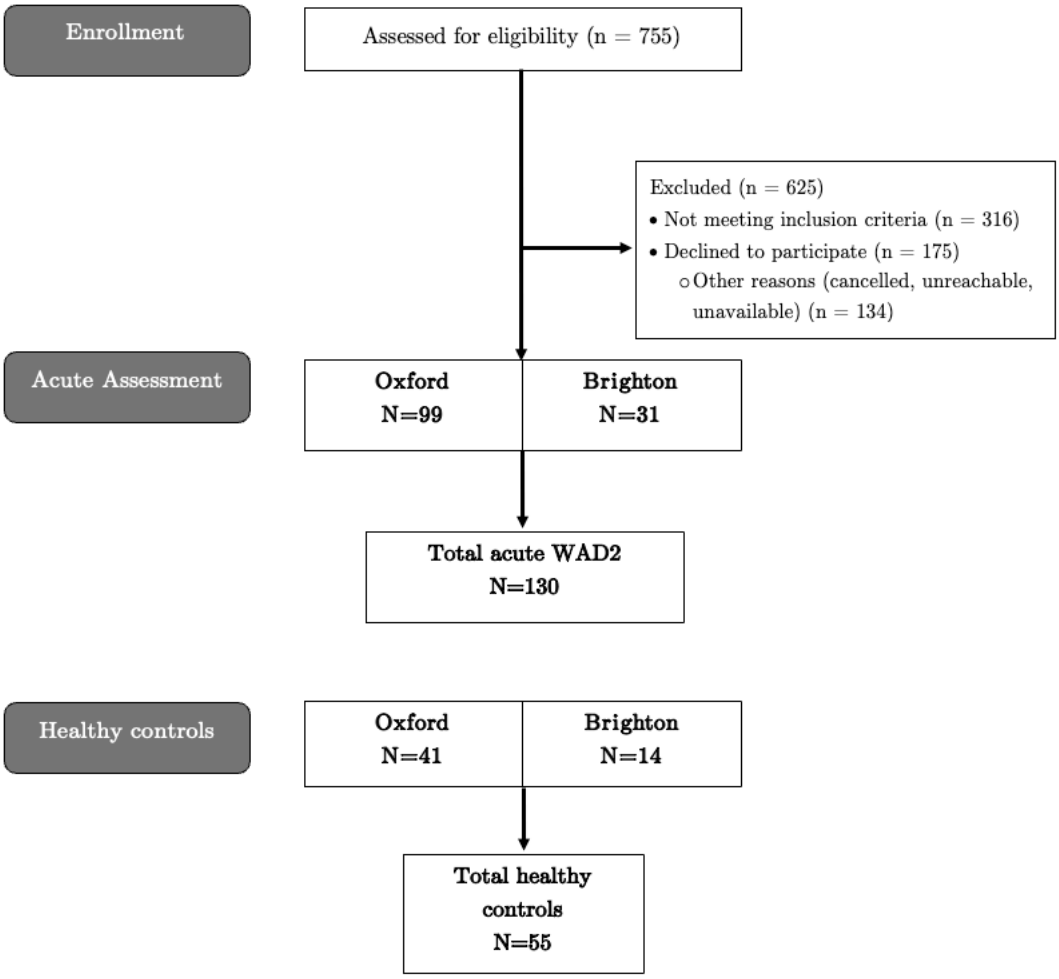


Figure 5.1. Flow diagram of included study participants for acute WAD2 and healthy controls.

Approximately 75% of acute WAD2 participants report using medication due to the accident. This includes the use of paracetamol (59%), nonsteroidal anti-inflammatories (56%), and nearly one-third of participants using codeine. The use of diagnostic investigations varies, including 35% receiving radiographs, 14% computerised tomography scans, 4% MRI, and 1.5% report nerve conduction studies acutely following the collision. Participants report the use of nonpharmacological treatment including 25% attending physiotherapy and 5% having osteopathic/chiropractic treatment. Participant medication use is predominantly from paracetamol (59% [77/130]) and NSAID use (56.2 % [73/130]). Weak opioids (tramadol, codeine, co-

codamol, co-dydramol, or co-proxamol) are reportedly used by 32.3 % (42/130) of WAD2 participants. Additional reported medications include diazepam (3% [4/130]), deep heat (1.5% [2/130]), diclofenac (0.8% [1/130]), amitriptyline (0.8% [1/130]), and etoricoxib (0.8% [1/130]).

Supplemental Table 3 shows acute WAD2 participants range of motion limitations. Most participants present with acute limitations in active cervical movement (range 51% - 66%). Shoulder movement is also impaired in approximately one-third of participants with greater movement limitations identified on participants' symptomatic side.

	Acute WAD2	Healthy controls
Number of participants	130	67
Age (med/IQR)	35.5 (21.8)	43.0 (31)
Gender (med/IQR)	57.7% (75/130)	55% (37/67)
BMI (med/IQR)	26.5 (7.9)	24.5 (5.7)
Returned to work	76% (99/130)	
Collision direction (%)		
rear	36.9% (48/130)	
forward	27.7% (36/130)	
side	35.4% (46/130)	
Personal speed at collision (%)		
slow (<10 mph)	14.6% (19/130)	
fast (>10 mph)	55.4% (72/130)	
stopped	29.2% (38/130)	
unable to recall	0.8% (1/130)	
Anticipated collision %	31.5% (41/130)	
Currently in litigation	28.4% (37/130)	
Conservative management		
Physiotherapy	25.4% (33/130)	
Osteopathy/Chiropractic	5.4% (7/130)	
Currently using medication due to whiplash	74.6% (97/130)	
Paracetamol	59% (77/130)	
NSAIDs	56.2 % (73/130)	
Weak opioids	32.3 % (42/130)	
Others:		
Diazepam	3% (4/130)	
Deep heat (patch, rub)	1.5% (2/130)	
Diclofenac	0.8% (1/130)	
Amitriptyline	0.8% (1/130)	
Etoricoxib	0.8% (1/130)	
Investigations		
MRI	3.8% (5/130)	
Radiographs	34.6% (45/130)	
Nerve conduction study	1.5% (2/130)	
CT scan	13.8% (18/130)	

Table 5.1. Baseline demographic data for acute WAD2 participants and controls. Data are presented as median/interquartile range (IQR) or as percentage (%). All parameters in Table 5.1 were subjectively reported; BMI (kg/m^2) was calculated from subjective reports of height and weight. NSAID medications included any of the following medications not requiring prescription within the United Kingdom: ibuprofen or naproxen. Weak opioids included: tramadol (n=2), codeine, co-codamol, co-dydramol, or co-proxamol.
Abbreviations: BMI: body mass index; MRI: magnetic resonance imaging; CT: computerised tomography, NSAID: nonsteroidal anti-inflammatory.

5.3.2. Acute symptom profiles

Nearly all acute WAD2 participants report having current symptoms due to the whiplash injury (97% [126/130]; Table 5.2). The cervical spine is the primary site of pain (93% [121/130]). Participant reports of the side of primary symptoms was predominantly on the right (60% [77/130]; Figure 5.2A and Table 5.2). The median severity of whiplash symptoms using VAS is 35/100 (IQR 33; Figure 5.2B and Table 5.2). A significant portion of participants also report radiating symptoms past their shoulders (58% [75/129]) and subjective sensory changes (42% [54/129]).

Participant reported outcome measures indicate moderate levels of neck-related disability using the NDI with a median score of 15 (IQR 10, Table 5.2). Measures of emotional state using several self-reported measures indicate moderate levels of stress and posttraumatic stress symptoms. This is calculated from the DASS-42 anxiety subscale (median 12, IQR 12), IES-R scale (median 26.5, IQR 28.3) and PTSD-8 (median 17, IQR 10). Symptoms suggestive of posttraumatic stress disorder (PTSD) are present for 51% and 40% using the PTSD-8 and IES-R questionnaires, respectively.

Levels of general depression and anxiety do not reach cut-off thresholds of significance using DASS subscale scores (depression median 5.5, IQR 11.3; anxiety 6, IQR 9). There are no signs of elevated pain-related worrying at group level using the PCS (median 13, IQR 17). Pain-related worrying is present in 14% of acute

WAD2 participants using PCS cut-off scores. The mean EQ-5D-5L health index value is 0.73 (SD 0.19).

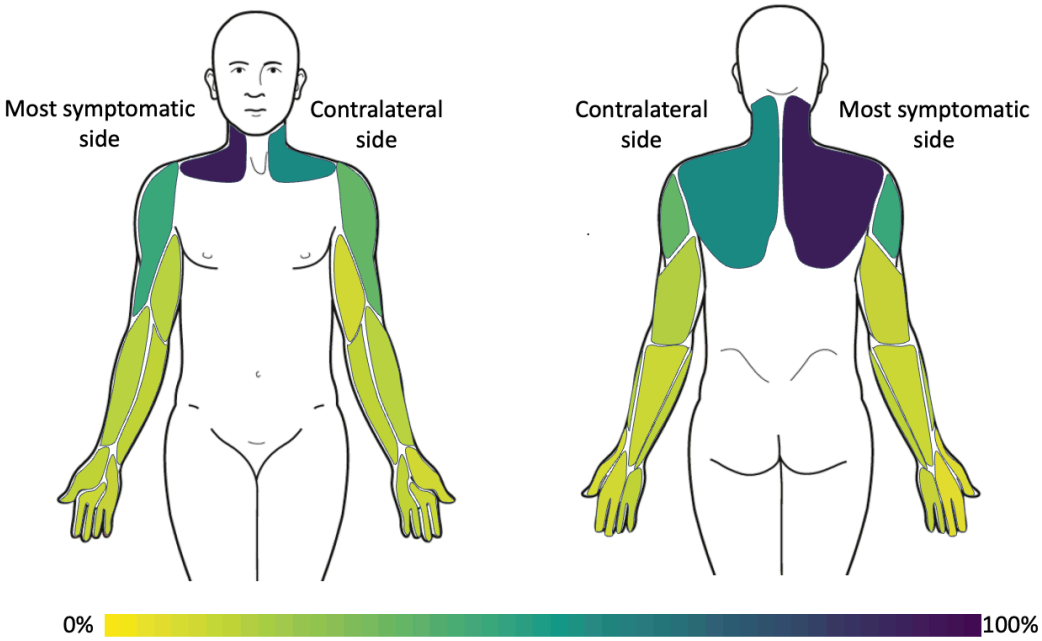


Figure 5.1. The cervical spine and shoulders were the most prevalent symptomatic areas. The body diagram shows the percentage and location of whiplash-related symptoms for participants with acute WAD2. Areas in the body diagram locations without colour were not recorded. Each side was defined according to participants reported most symptomatic side and corresponding contralateral side.

5.3.3. Neuropathic Pain

The likelihood of neuropathic pain using the NeuPSIG grading system for individual participants indicates 36.9% (48/130) are classified as unlikely, 27.7% (36/130) as possible, and 35.4% (46/130) as probable neuropathic pain. Median painDETECT scores of 9.5 (IQR 9) do not indicate the WAD2 group are likely having neuropathic pain at group level. Ten percent of acute WAD2 participants (13/130) are graded as likely having a neuropathic component using the painDETECT.

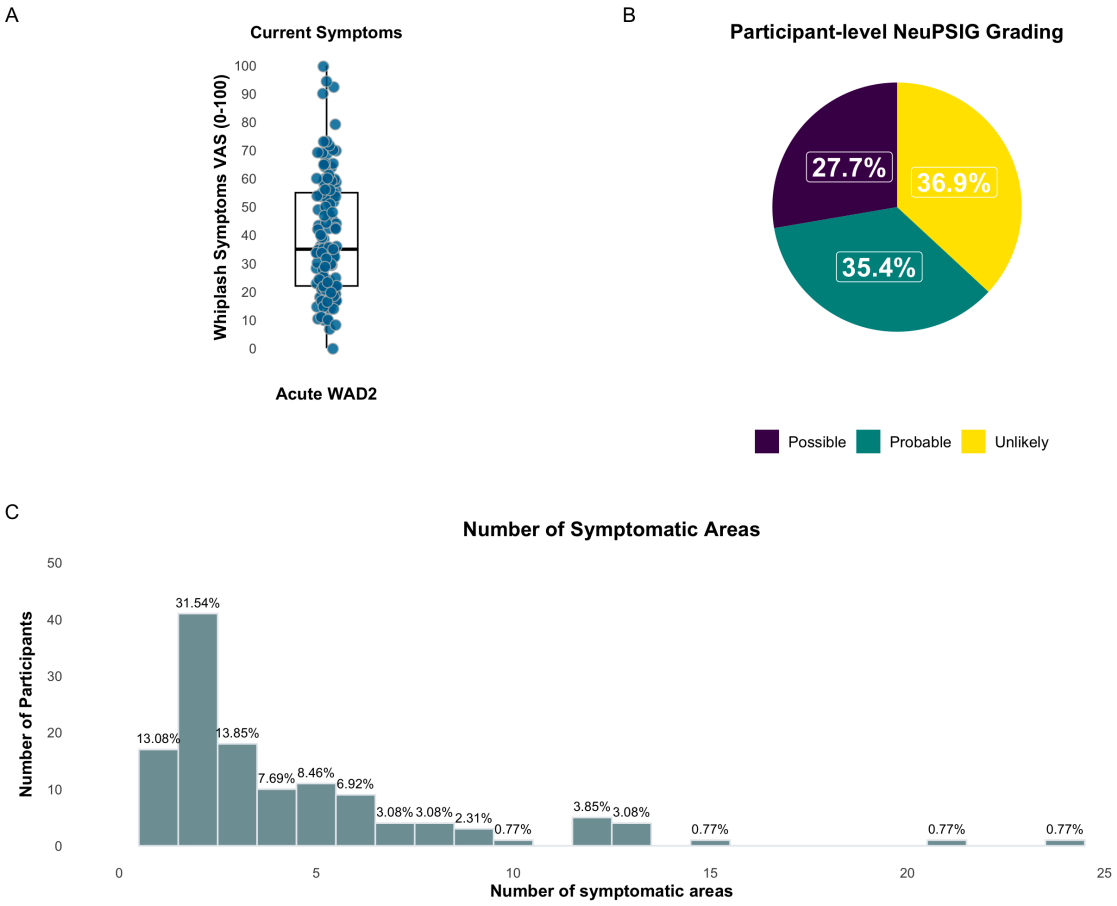


Figure 5.2. Acute WAD2 pain characteristics. (A) Visual analogue scale (VAS) of participant-reported severity of whiplash symptoms at baseline testing (0-100). (B) Distribution of the certainty of neuropathic pain for individual acute WAD2 participants using the Neuropathic Pain Special Interest Group (NeuPSIG) Grading System. (C) Distribution of the total number of symptomatic areas per acute WAD2 participant as selected from the above body diagram.

Table 5.2. Baseline participant reported outcome measures.

	Count	Acute WAD2
Current symptoms	126/130	97%
Side of primary symptoms (right, %)	77/130	59%
Current whiplash-related symptom VAS (med/IQR)	130	35 (33)
Current pain (med/IQR)	120	4 (3.3)
Strongest pain (med/IQR)	120	8 (2)
Average pain (med/IQR)	120	6 (1.2)
Radiating symptoms (past the shoulder)	75/129	58%
Subjective sensory change (e.g., numbness, tingling)	54/129	42%
Neck Disability Index (med/IQR)	127	15 (10)
Mild	5/127	4%
Moderate	53/127	42%
Severe	69/127	54%
PainDETECT (med/IQR)	120	9.5 (9)
Likely neuropathic	13/120	11%
Uncertain	39/120	33%
DASS-42: depression (med/IQR)	124	5.5 (11.3)
DASS-42: anxiety (med/IQR)	124	6 (9)
DASS-42: stress (med/IQR)	124	12 (12)
EQ-5D-5L (Index value; mean/SD)	122	0.73 (0.2)
PTSD-8 total (med/IQR)	122	17 (10)
Likely PTSD	62/122	51%
Impact of Events Scale - Revised (med/IQR)	108	26.5 (28.3)
Probable PTSD	43/108	40%
Pain Catastrophizing Scale (med/IQR)	117	13 (17)
Likely catastrophizing	16/117	14%

Data are presented as median (IQR), mean (SD), or percentage (%). Neck Disability Index scoring: mild <5, moderate 5-14, and severe ≥15. Current, strongest, and average pain levels were taken from the corresponding questions within the painDETECT questionnaire. PainDETECT scoring: likely neuropathic (>18/38), uncertain (>12/38). PTSD-8 scoring: likely PTSD (≥18/32). Impact of Events Scale scoring: probable PTSD (≥33/88). Healthy control participants did not complete self-reported outcome measures and, per inclusion criteria, did not have any neck or arm symptoms. Abbreviations: DASS: Depression Anxiety and Stress Scale; IQR: interquartile range; med: median, PTSD: posttraumatic stress disorder; PCS: Pain Catastrophizing Scale; SD: standard deviation; VAS: visual analogue scale.

5.3.4. Neurological assessment

Neurological dysfunction is identified throughout the upper extremity in participants with acute WAD2 (Figure 5.3). Per inclusion criteria, all healthy control participants present with normal bedside neurological assessments. Overall, 37.7% of acute WAD2 participants are classified as having normal neurological bedside assessments. The type of neurologic dysfunction includes 40% with only loss of function, 8.5% with only gain of function, and 13.8% that have a mixed presentation including both loss and gain of function (Figure 5.3A). Similarly, pinprick is the most dysfunctional loss of function parameter (37.7%), followed by reduced light touch (20%), reduced thermal detection (19.2%), weakness (10.8%), and hyporeflexia (0.8%) (Figure 5.3B). Heightened pinprick sensation is the predominant clinical test indicating gain of function (20.8%) followed by light touch gain of function (6.2%).

Neurological dysfunction is predominantly identified as loss of function on participants' most symptomatic side (Figures 5.3C - 5.F). C6 is the most affected innervation territory (26.2% loss of function, 10.8% gain of function; Figure 5.3C) followed by T1 (20% loss of function, 8.5% gain of function). There are minimal signs of upper extremity neurological dysfunction present contralateral to the primary symptom side (Figures 5.3D and 5.3F). Nerve dysfunction on the contralateral side is below 7% for both gain and loss of function measures (Figure 5.3D). Participants' main pain area shows the greatest amount of nerve dysfunction

(Figures 5.3E - 5.3F). The main pain dysfunction is primarily loss of function on the symptomatic side (48%).

Participants with acute WAD2 present with an anatomical spread of nerve dysfunction (Figures 5.3G - 5.H). Over one-third of participants have loss of function in two or more segmental innervation territories (36.25%, Figure 5.3G). Approximately 19% have loss of function within only one segmental area. Nine percent have gain of function in only one segmental area and approximately 14% present with gain of function at two or more levels (Figure 5.3H).

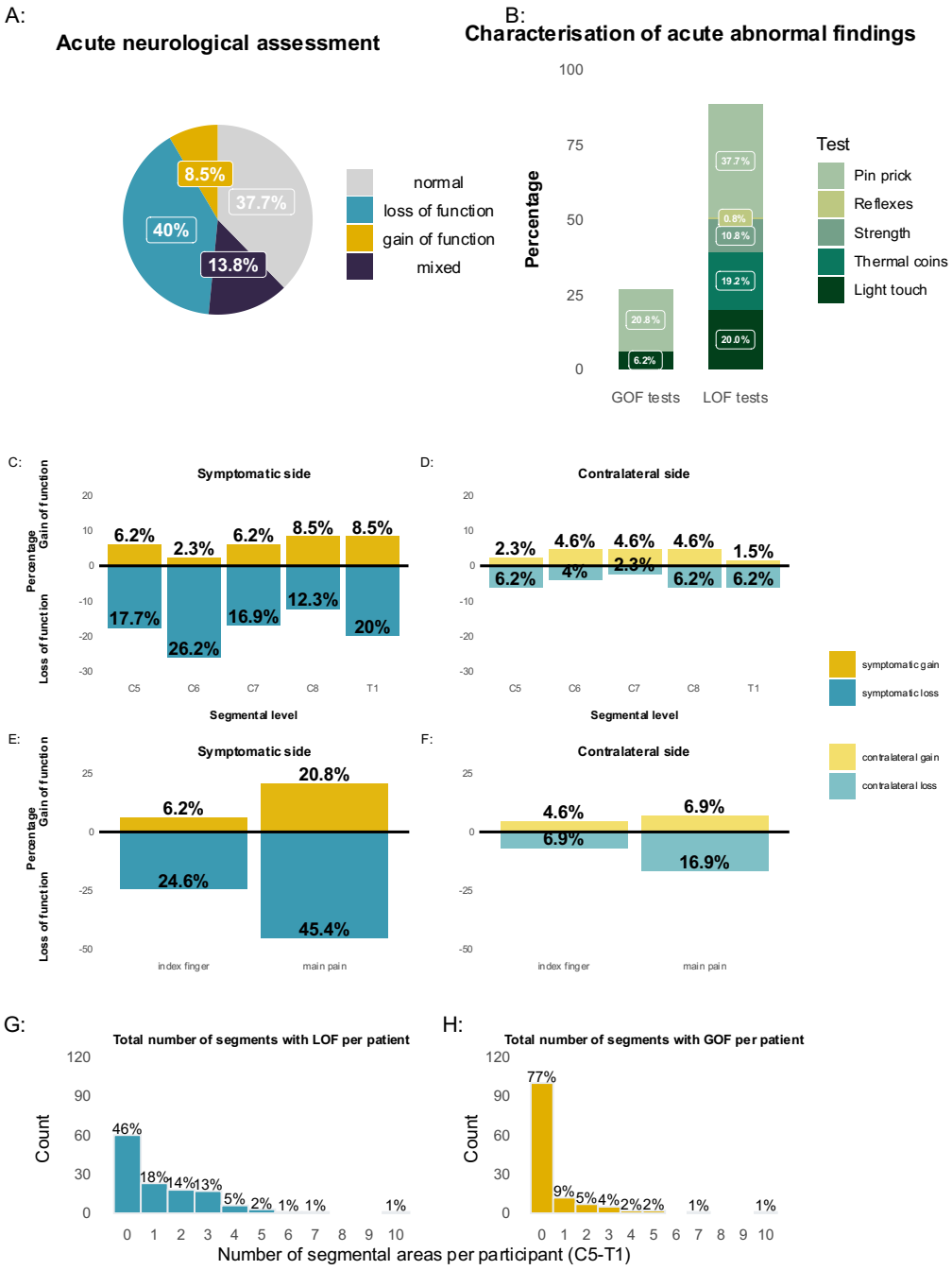


Figure 5.3. Neurological assessment demonstrates upper extremity hypoaesthesia in a subgroup of people with acute WAD2. (A) Categorisation of upper extremity neurological assessment for acute WAD2, including measures for strength, reflexes, light touch, pinprick, and thermal coins. (B) Type and percentage (%) of tests contributing to measures of neurological dysfunction. (C-D) Percentage of neurological loss and gain of function in the C5-T1 innervation territories, including strength, reflexes, light touch, pinprick, and thermal coins. Panel C shows most symptomatic side and panel D shows the contralateral side. (E-F) Percentage of bedside small fibre testing for neurological loss and gain of function within the symptomatic index finger and main pain area. Tests included pinprick, warm and cool coins. Panel E shows the most symptomatic side and panel F shows contralateral side. (G) Total number of upper extremity dermatomal segments with loss of function (LOF) per

participant. (H) Total number of upper extremity dermatomal segments with gain of function (GOF) per participant.

5.3.5. QST shows subgroup of acute WAD2 with sensory hypoaesthesia.

At group level, sensory detection thresholds are significantly reduced over the index finger for cold (Z = -2.1, P<0.001), warm (Z = -1.0, P<0.001), sensory thermal limen (Z = -1.0, P<0.001), mechanical detection (Z = -1.4, P<0.001) and vibration detection (Z = -4.2, p<0.001) in acute WAD2 compared to controls (Figure 5.4A). There are no significant differences in cold, heat, or mechanical pain thresholds, or mechanical pain sensitivity in acute WAD2 compared to controls. Additionally, there are no significant differences in windup ratio or pressure pain thresholds for acute WAD2 compared to controls. As these are exploratory analyses, I only correct p-values for the joint null hypothesis that none of the 11 QST measurements are different from baseline.

Deterministic phenotypic clusters of acute index finger QST results designate 66.9% as healthy, 20% as mechanical hyperalgesia, 6,9% as thermal hyperalgesia, and 6.2% as sensory loss. Deterministic phenotyping excluding the healthy sensory phenotype designate 48.9% as thermal hyperalgesia, 30.9% as mechanical hyperalgesia, and 20.2% as sensory loss.

There is a significant decrease in cold detection thresholds at the tibialis anterior (Z = -1.3, P<0.01) in acute WAD2 compared to controls (Figure 5.4B). No significant

differences are identified in warm detection or pressure pain thresholds at the tibialis anterior in acute WAD2 compared to controls.

Supplemental Figure 5.3 shows the number of abnormal QST parameters per acute WAD2 participant (defined as z-score ± 1.96). Overall QST loss of function is present for 66% of participants, with 42% of participants demonstrating loss in two or more QST parameters (e.g., cold, warm, mechanical detection). Overall signs of gain of function are present in 48% of participants, with 32% of participants having an increase in only one QST parameter.

Supplemental Figure 5.2 shows the complete details for the percentage of abnormal acute WAD2 QST parameters at the index finger (defined by z-scores = ± 1.96). Overall, there is a larger subgroup presenting with abnormal loss of function parameters compared to gain of function parameters. The most frequent abnormal QST parameters in acute WAD2 include warm detection (33.1%), mechanical detection (28.6%), thermal sensory limen (23.6%), and warm detection (22.8%). The most frequent gain of function QST parameters for acute WAD2 include mechanical pain sensitivity and thresholds (15% and 13.5%, respectively), and pressure pain thresholds (11.9%). There is also a greater percentage of acute WAD2 participants with loss compared to gain of function at the leg with 18.1% having abnormal cold detection, 11% with abnormal warm detection, and 6.3% with abnormal pressure pain thresholds.

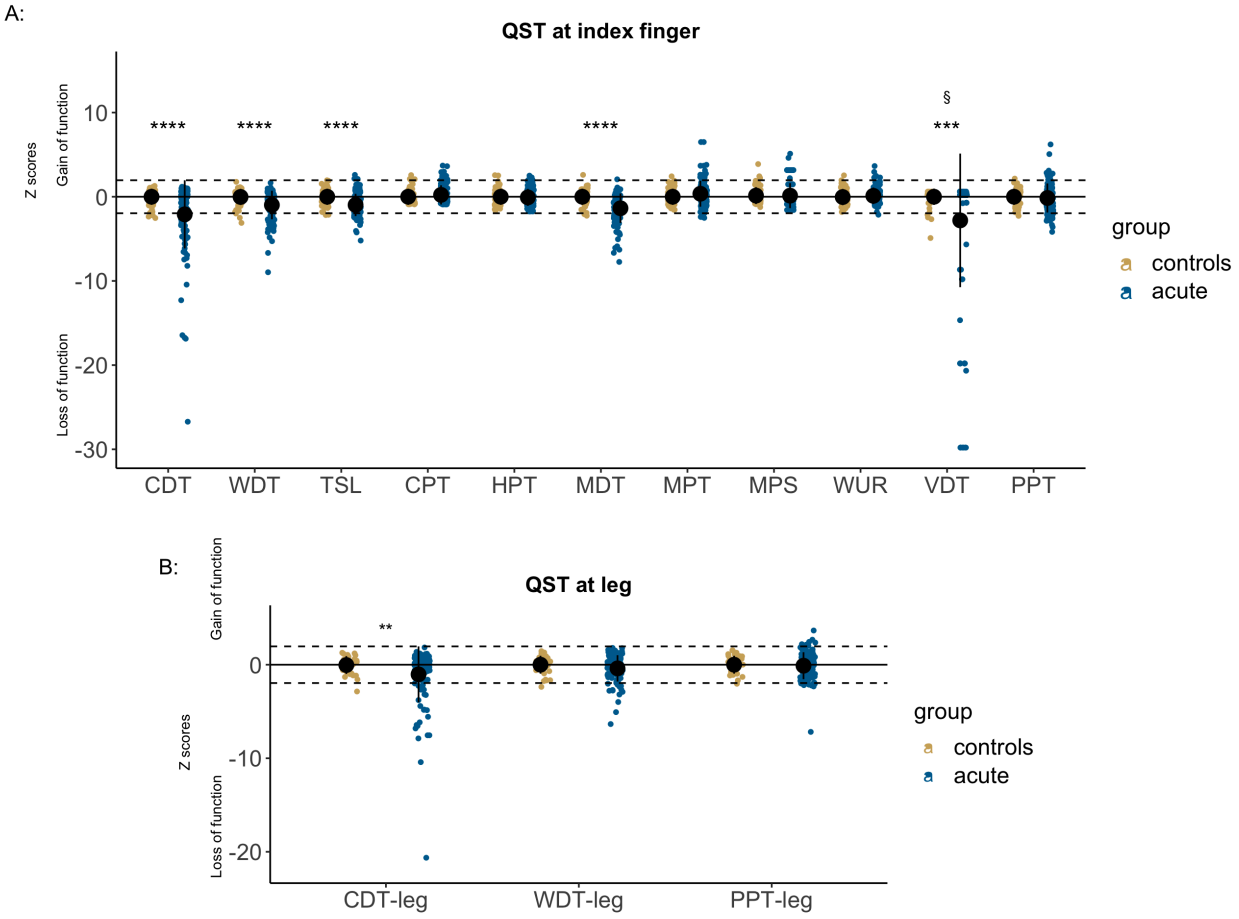


Figure 5.4. Reduced sensory detection thresholds at the index finger and tibialis anterior in acute WAD2 compared to healthy controls. (A) QST measured at the index finger in acute WAD2 and healthy controls. Due to ceiling effects of VDT, there are three VDT z-score values less than -30, which are out of figure range (indicated as §). The three missing values include z-score = -39.76, -79.7, -59.79. (B) QST measured over the tibialis anterior in acute WAD2 and healthy controls. Index finger data includes n=124 acute WAD2 and n=61 controls. Leg data includes n=111 acute WAD2 and n=33 controls. An independent two-sided t-test was used for each comparison. *P $<$ 0.05; **P $<$ 0.01; ***P $<$ 0.001; ****P $<$ 0.0001.
Abbreviations: CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio

5.3.6. Serum marker of nerve pathology

Based on a previous study demonstrating acutely elevated plasma NfL levels in non-TBI traumatic injury[291], I hypothesised there would be an acute increase in serum NfL in WAD2 compared to healthy controls. There are significant increases in both absolute serum NfL concentrations ($p < 0.05$) and serum NfL z-scores ($p < 0.01$) in participants with acute WAD2 compared to age and sex-matched controls (Figure 5.5, Table 5.3). Exploratory analysis of dichotomised serum NfL z-scores (high NfL = z-score ≥ 1.5[388]) shows a trend toward increased whiplash symptom scale and IES-R scores, as well as decreased cold and warm detection thresholds in the group with elevated NfL (Supplemental Figures 5.1-5.2).

There are no significant correlations for absolute or z-score serum NfL values and clinical measures of nerve pathology (Table 5.5).

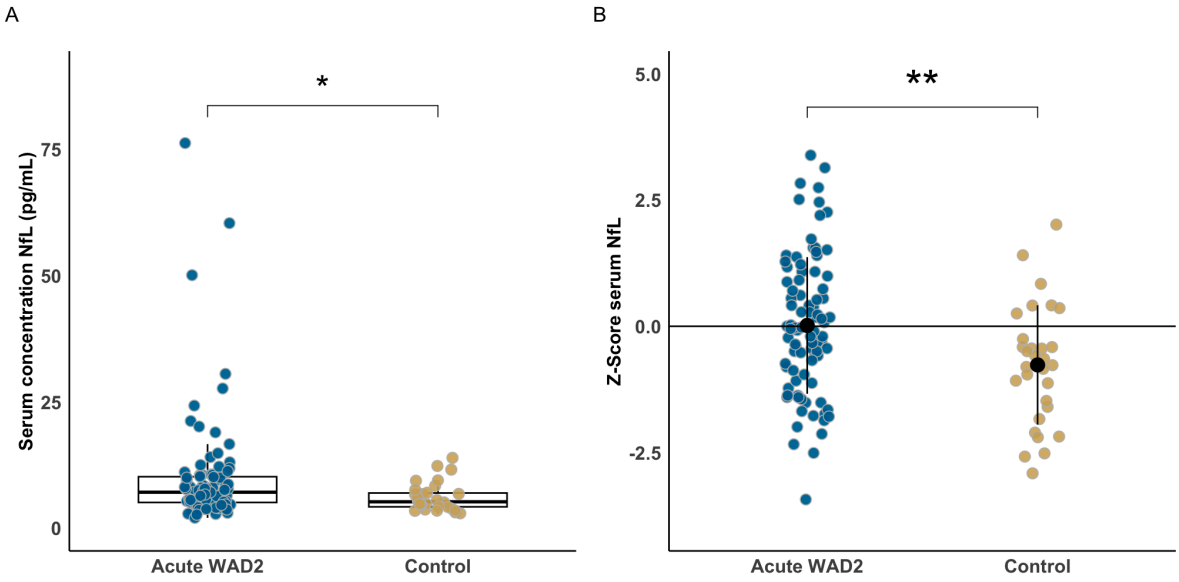


Figure 5.5. Elevated serum NfL levels in acute WAD2 compared to healthy controls. (A) Increased absolute serum NfL concentrations in acute WAD2 compared to controls. Mann-Whitney U test was used for comparison. (B) Increased serum NfL-age and BMI adjusted z-score values in

acute WAD2 compared to controls. An independent two-sided t-test was used for comparison. Data from N=91 acute WAD2 and n=30 controls. *P $<$ 0.05; **P $<$ 0.01.
Abbreviations: NfL: neurofilament light chain.

Table 5.3. Serum NfL levels in acute WAD2 and controls.

	Acute WAD2	Controls	P-value
Serum NfL concentration (pg/mL) - median (IQR)	7.13 (5.31)	5.24 (3.53)	0.016
Serum NfL z-scores (mean/SD)	0.016 (1.35)	-0.766 (1.18)	0.004

Comparison of absolute serum concentrations was done using a Mann-Whitney U test. Comparison of NfL z-scores was performed using an independent two-sided t-test.
Abbreviations: NfL: neurofilament light chain, SD: standard deviation, IQR: interquartile range. (IQR).

Histological analysis of skin biopsies

There is no significant difference in intraepidermal nerve fibre densities at the index finger (p = 0.17) or distal leg (p = 0.78) between acute WAD2 and controls (Figures 5.6A, 5.6C, and Table 5.4). Similarly, there is no significant difference in the number of Meissner corpuscles measured at the index finger in acute WAD2 compared to controls (P = 0.62; Figure 5.6B). There is also no significant correlations between the intraepidermal nerve fibre density of acute WAD2 participants at the index finger and the clinical measures of nerve pathology (Table 5.5).

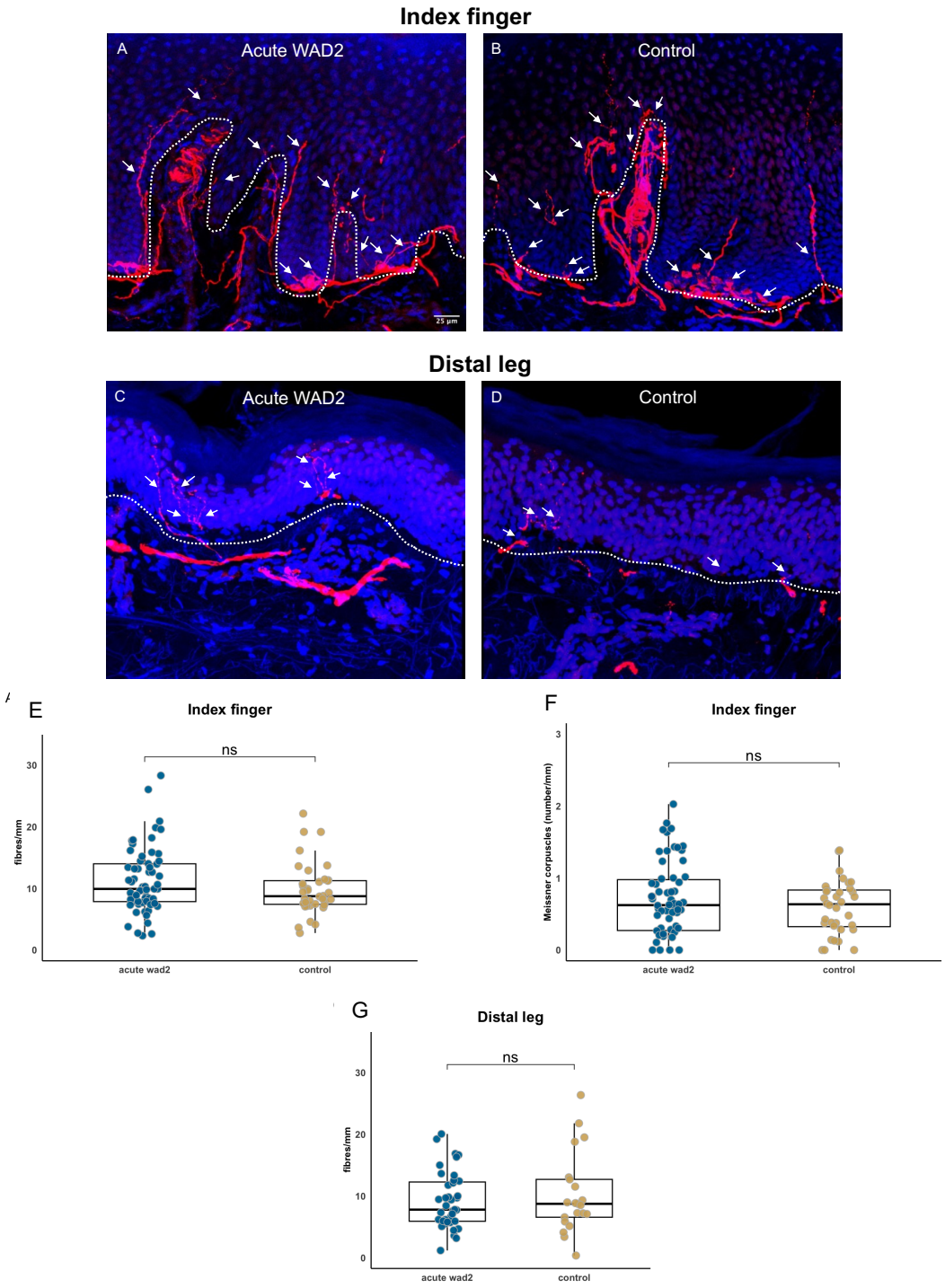


Figure 5.6. No change in intraepidermal nerve fibre density at the index finger or distal leg in acute WAD2 compared to controls. (A-D) representative images of skin immunohistochemistry taken at the index finger and distal leg in a participant with acute WAD2 and age- and sex-matched healthy controls. Arrows represent epidermal nerve fibres and the dashed line indicates the dermal-epidermal border. Dotted line indicates dermal-epidermal border. (E-F) Preserved intraepidermal nerve fibre densities and Meissner corpuscles at the index finger of participants with acute WAD2 compared to controls. (G) preserved intraepidermal nerve fibre densities at the distal leg in participants with acute WAD2 compared to controls. Mann-Whitney U tests were used for each comparison.

Table 5.4 Intraepidermal nerve fibre densities at the index finger and distal leg were preserved acutely after injury.

	Acute WAD2	Controls	p-value
Index finger			
Fibres/mm	9.9 (6.1)	8.72 (3.8)	0.17
Meissner's corpuscles (number/mm)	0.62 (0.7)	0.64 (0.5)	0.62
Distal leg			
Fibres/mm	7.8 (6.3)	8.7 (6.1)	0.78

Data presented as median (IQR). Index finger skin biopsies were taken from the volar aspect of the proximal phalanx of the index finger for all participants. Index finger skin biopsies for acute WAD2 were taken from the most symptomatic side and from the non-dominant side for controls. Mann-Whitney U test was used for comparison. Index finger data includes N=62 acute WAD2 and N=38 controls. Distal leg data include N=39 acute WAD2 and N=21 controls.
Abbreviations: IQR: interquartile range.

Table 5.5. Associations of measures of nerve pathology with reported outcomes.

	Avg pain	Subj sensory change	Sensory loss	Headache	CDT	WDT	MDT
Absolute NfL concentration	-0.09 (1.0)	0.014 (1.0)	-0.04 (1.0)	-0.12 (1.0)	-0.06 (1.0)	-0.04 (1.0)	-0.11 (1.0)
NfL z-score	0.04 (1.0)	0.03 (1.0)	0.01 (1.0)	-0.04 (1.0)	-0.21 (1.0)	-0.20 (1.0)	-0.21 (1.0)
Index IENFD	-0.14 (1.0)	0.00 (1.0)	0.00 (1.0)	0.01 (1.0)	0.21 (1.0)	0.15 (1.0)	0.11 (1.0)

Data are presented as Spearman ranks coefficients and corresponding adjusted p-values using Holm's method. 'Sensory loss' was taken from subjective participant reports, included symptoms of numbness, tingling, burning, weakness, etc. 'Headache' (taken from question 5 of the NDI) was used a surrogate marker of potential concussive-like symptoms.
Abbreviations: Avg: average; Subj: subjective; NfL: Neurofilament light chain; CDT: cold detection thresholds; WDT: warm detection thresholds; MDT: mechanical detection thresholds; Abs: absolute; IENFD: intraepidermal nerve fibre density.

5.3.7. Bedside clinical tests show moderate agreement but limited validity to detect somatosensory loss of pathology in acute WAD2.

There is moderate agreement between QST and clinical bedside tests for cold, warm, and mechanical detection thresholds (Table 5.6). No significant correlation is identified between QST parameters for cold, warm, or mechanical detection thresholds and corresponding bedside tests. Phi correlation coefficients show negligible (cold and warm detection) to weak correlations (mechanical detection thresholds). This indicates moderate agreement but minimal validity for the use of coins and cotton wool in detecting cool, warm, and mechanical detection thresholds as compared to QST.

Table 5.6. Validity of clinical sensory to assess somatosensory loss of function compared to QST parameters for cold, warm, and mechanical detection.

Parameters	Agreement (%)	Fisher's exact test (p-value)	Phi Coefficient
CDT – cool coin	61.2%	0.54	-0.07
WDT – warm coin	72.7%	0.70	0.04
MDT – cotton wool	71.9%	0.08	0.18

Data only included acute WAD2 participants measured at the index finger; no healthy control data was included. Criteria for QST z-score loss of function cut-off values: z< -1.96 for loss of function. Abbreviations: CDT: cold detection threshold; WDT: warm detection threshold; MDT: mechanical detection thresholds; QST: quantitative sensory testing.

Table 5.7 shows the accuracy of thermal coins and cotton wool for detecting somatosensory loss of function compared to a corresponding QST z-scores (cut-off: -1.96). All three clinical bedside tests have low sensitivity (range 0.08 – 0.10) but high specificity (range 0.88 – 0.99). Mechanical detection using cotton wool has the highest positive predictive value (0.75; 95% CI: 0.33 - 1.17). Thermal coins have

similarly low positive predictive values compared to QST (cool: 0.23; 95% CI 0.05 – 0.54; warm: 0.30; 95% CI 0.07 – 0.65). Negative predictive values are similar for cool (0.66; 95% CI 0.56 – 0.75), warm (0.77; 95% CI 0.68 – 0.84), and cotton wool (0.72; 95% CI 0.63 – 0.80). The highest positive likelihood ratio is for cotton wool (7.08; 95% CI 0.76 – 65.83), followed by warm (1.36, 05% CI 0.038 – 4.92), and cool testing (0.61, 95% CI 0.18 – 2.09). Negative likelihood ratios are similar for all three tests, including cool (1.06; 95% CI 0.94 – 1.19), warm (0.97, 95% CI 0.85 – 1.19), and touch testing (0.93; 95% CI 0.84 – 1.03).

Table 5.7. Accuracy of clinical bedside to test somatosensory loss of function compared to QST.

Parameters	Sensitivity	Specificity	PPV	NPV	+ LR	-LR
CDT – cool coin	0.07 (0.02 – 0.20)	0.88 (0.78 – 0.94)	0.23 (0.05 – 0.54)	0.66 (0.56 – 0.75)	0.61 (0.18 – 2.09)	1.06 (0.94 – 1.19)
WDT – warm coin	0.10 (0.02 – 0.27)	0.92 (0.85 – 0.97)	0.30 (0.07 – 0.65)	0.77 (0.68 – 0.84)	1.36 (0.38 – 4.92)	0.97 (0.85 – 1.11)
MDT – cotton wool	0.08 (0.02 – 0.22)	0.99 (0.94 – 1.0)	0.75 (0.19 – 0.99)	0.72 (0.63 – 0.80)	7.08 (0.76 – 65.83)	0.93 (0.84 – 1.03)

Data only included acute WAD2 participants measured at the index finger and are presented as: value (95% confidence intervals); no healthy control data was included.
Abbreviations: CDT: cold detection threshold; WDT: warm detection threshold; MDT: mechanical detection thresholds; QST: quantitative sensory testing, PPV: positive predictive value; NPV: negative predictive value; + LR: positive likelihood ratio; - LR: negative likelihood ratio.

5.3.8. Ability of bedside clinical tests or clinical variables to predict acute NfL z-scores.

Clinical bedside tests for loss of function at the index finger, including cool, warm, and pinprick sensation, do not predict acute serum NfL z-scores in acute WAD2 (p

> 0.05; Table 5.8). I identify a similar trend using density plots of participants with high levels of NfL and lower WDT thresholds (Supplemental Figure 5.2).

Table 5.8. Clinical factors did not predict acute absolute serum NfL concentrations.

Parameter	Coefficient estimate	Standard error	P-value
Cool coin	-2.76	3.70	0.46
Pinprick	-1.07	3.17	0.74
Total number loss of function	0.50	0.81	0.54
Cool + warm + pinprick + total loss of function	-1.41	3.44	0.68

Data only included acute WAD2 participants; no healthy control data was included. Bedside clinical tests for cool coin, warm coin, and pinprick were performed at the index finger and dichotomised into loss of function or normal. Linear regression included age as covariate for each model.

5.4. Discussion

The primary aim of this chapter was to investigate the extent and location of nerve pathology and neuropathic pain in acute WAD2. While nerve pathology has been identified in chronic WAD, the acute presence is inadequately understood. My findings suggest that a subgroup of people with acute WAD2 present with signs of nerve pathology and neuropathic pain. The likelihood of neuropathic pain in my acute WAD2 cohort was 35% using the NeuPSIG grading scale and 11% using the painDETECT questionnaire scores. Functional nerve pathology was common with a prevalence of 53.8% determined by clinical assessment and 66% using QST. The prevalence of structural nerve pathology derived from serum NfL z-scores was 13% and 1.6% from skin biopsies.

Demographic variables were comparable to other WAD2 cohorts.

The baseline characteristics of my cohort, including age, sex, BMI, and acute neck-related disability, were similar to other prospective WAD2 cohorts[16,34,59,398]. The frequency of returning to work and litigation status were also comparable to previously published findings. Approximately 76% of WAD2 participants in my cohort had already returned to work at baseline testing (within one month after injury). This finding is consistent with a systemic review showing a median of 95% of people after whiplash returning to work[399]. Only 25% of participants in my cohort were involved in litigation due their injury. While data on litigation trends in the UK are limited, a study from the largest whiplash cohort in England revealed that 66% - 81% of participants filed claims[35].

In addition to demographic similarities, treatment patterns in my cohort were similar to current recommended guidelines. Self-reported management for my WAD2 cohort align with the UK National Institute of Health and Care Clinical Knowledge Summary[283]. This includes participant use of analgesic medication for symptom relief (75% of our cohort) and physiotherapy referral (approximately 25% of participants). Although medication is commonly recommended following whiplash injuries, there is no randomised controlled trial looking at its effectiveness[75]. The use of weak opioids acutely in my cohort was 32.3%, which is nearly identical to a large US cohort of participants after trauma (predominantly motor vehicle collision) that reported 29% received acute opioids[80]. Importantly, this study also identified a

significant association between acute opioid exposure and increased odds of subsequent opioid use three months following injury[80]. Acute opioid use may be an important consideration for subsequent recovery and continued medication use after whiplash injuries.

Posttraumatic stress was a common psychological sequela in acute WAD2.

A significant subgroup of acute WAD2 participants, ranging from 40% to 51%, exhibited symptoms of posttraumatic stress from questionnaire data. Predictably, the questionnaire-based measures of posttraumatic stress in my cohort are slightly higher compared to a recent systematic review that measured the clinical diagnosis of PTSD three months after whiplash injury (range 9% - 22%)[45]. Measures of other psychological sequelae were not significant at group level. Median scores did not indicate signs of state levels of anxiety, depression, or stress. Similarly, pain-related worrying was only present in 14% of my cohort. Posttraumatic stress is a common sequela, thought to influence persistent pain, and is associated with poor recovery in WAD[45,46,175,400]. As such, the influence of posttraumatic stress is an important element to consider in my cohort that I will discuss in later sections.

Moderate levels of neck and arm pain were present acutely.

The neck was the primary location of pain in my cohort, which aligns with previous findings[37]. However, reports of pain outside of the neck was also common in participants with acute WAD2. Fifty-eight percent of my cohort reported radiating

symptoms past their shoulder and 42% reported feeling changes in sensation (e.g., numbness, tingling, weakness). These values are broadly aligned with my systematic review in WAD (Chapter 2), which identified 10 studies reporting arm and shoulder pain and five studies reporting signs of paraesthesia after whiplash injuries[401]. My cohort findings indicated these painful areas to be more frequent on the primary symptomatic side and rarely extending below the elbow. I did not collect pain data for other anatomical areas (e.g., lumbar spine or lower extremities). Previous studies of whiplash injury have reported symptoms outside of the upper extremities[38,39,402], but this was not the focus of my study.

A subgroup presented with signs of neuropathic pain.

Over one-third of participants with acute WAD2 presented with signs of probable neuropathic pain using the NeuPSIG grading system. Importantly, the grading of probable neuropathic pain included not only nerve-related symptoms (e.g., burning, tingling, electric shocks) but also loss of sensory function within the main pain area[194]. Over 60% of my acute WAD2 cohort was classified as having at least possible neuropathic pain. My systematic review did not identify any other studies that used the NeuPSIG grading on data from individual participants. However, I retrospectively applied the NeuPSIG grading criteria at study level in my systematic review (Chapter 2), which identified 38% of studies suggesting possible neuropathic pain and another 38% as probable.[401] These findings are strongly aligned with our

current cohort indicating that a subgroup of participants classified as WAD2 do present with neuropathic pain characteristics.

I also classified the likelihood of neuropathic pain using painDETECT scores. Only 11% of WAD2 participants were classified as likely having neuropathic pain using the painDETECT. Although the painDETECT hasn't previously been used in WAD, it has shown to be a valid tool in similar conditions, like cervical radiculopathy and non-specific neck and arm pain. It is important to note however that questionnaire-based classification to detect neuropathic pain has previously been suggested to miss up to 30% of participants who do have clinically diagnosed neuropathic pain[403]. Questionnaire-based classification likely does not provide the sensitivity that can be achieved using detailed clinical examination that is included in the NeuPSIG classification.

The identification of neuropathic pain in a majority of my acute WAD2 cohort is clinically relevant. Neuropathic pain has been shown to have greater psychosocial burdens and requires different treatment strategies compared to non-neuropathic conditions[195]. Although approximately one-third of WAD2 participants presented with signs of neuropathic pain, treatment for neuropathic pain was limited. Only one participant (0.8%) received a first-line neuropathic pain medication (amitriptyline) and two (1.5%) reported use of a second-line medication (tramadol). Similar to my findings after acute whiplash, epidemiological studies have reported dissonance between the presence of neuropathic pain and matching treatment[193,404].

International treatment guidelines for WAD currently do not include recommendations for using neuropathic pain medications. This is an important consideration, as the treatment of neuropathic pain is suggested to be distinct from other pain mechanisms[84,195]. Chapter 7 includes a detailed discussion of the clinical implications of neuropathic pain in WAD.

My study, which is the first to use the NeuPSIG grading system in WAD, shows nearly one-third of acute WAD2 present with neuropathic pain. This is a substantial subgroup that is potentially not receiving effective treatment. Though there currently are no randomised controlled trials assessing the efficacy of neuropathic pain medications in WAD, recent feasibility and pilot studies suggest it is a feasible and tolerated approach for WAD treatment[73,74]. However, high quality randomised controlled trials are needed to evaluate the effectiveness of first-line neuropathic pain medications in WAD.

A subgroup of WAD2 exhibited a spectrum of functional and structural changes indicative of nerve pathology.

It is well accepted that the severity of whiplash injuries can include a large spectrum from no signs of injury (grade 0) to complex traumatic fractures (grade 4). It is conceivable that a similar spectrum occurs for nerve pathology. The mean prevalence of frank nerve pathology from studies using electrodiagnostic testing was 32% in my systematic review (Chapter 2)[401]. Caution on the interpretation of this prevalence is

warranted, however, as the mean was derived from 16 studies that primarily only included participants with positive findings and was not a representative sampling of WAD. Frank lesions to the central nervous system can also occur, as evidence by participants with traumatic brain injury[405] and frank spinal cord injuries following motor vehicle collisions[406]. Yet, participants with frank nerve lesions to the peripheral or central nervous constitute a minority, with most graded as WAD2, indicating the absence of such pathology[401,407].

There is however increasing evidence that suggests a subgroup of WAD2 present with more subtle signs of nerve pathology[401]. The findings in this chapter corroborate the presence of nerve pathology in a subgroup of my cohort, as shown using a range of modalities. Signs of acute nerve pathology include the presence of neuropathic pain, widespread sensory hypoaesthesia, and elevated levels of NfL with preserved nerve fibre densities at the index finger. Collectively, these findings suggest signs indicative of central nervous system pathology rather than a predominantly peripheral source. Importantly, these findings were only present in a subgroup of my WAD2 cohort and in a heterogenous manner. In the following sections, I will describe each finding in detail.

<u>Peripheral spinal nerve injury is unlikely for most WAD2 participants.</u>

A hallmark of postganglionic nerve lesions includes is a reduction in intraepidermal nerve fibre densities. The preservation of nerve fibre densities identified in my study limits the likelihood of somatosensory hypoaesthesia arising from postganglionic

nerve injury. Importantly, the median timeframe of my acute assessment was 24 days after injury, which would theoretically allow for Wallerian degeneration to occur if present[408,409]. The findings of preserved nerve fibre densities, in part, may be explained by the anatomy of peripheral nerves. Compared to the dorsal roots, the epineurium in postganglionic peripheral nerves is more robust and protective of injury compared to the dorsal roots[410-412]. Preclinical models of whiplash injury emphasise the susceptibility of cervical nerve root injury due to their decreased ability to withstand pressure gradient changes compared spinal nerves[136]. The dorsal root ganglia are also unlikely to be the main source of the identified pathology as evidenced by previous studies in humans with ganglionopathy demonstrating reduced intraepidermal nerve fibre densities from skin biopsies compared to controls[413,414]. In addition to nerve fibre loss, preclinical models of dorsal root ganglia injury also typically present with signs of sensory hypersensitivity after the injury. Both findings contradict the findings in my acute WAD2 cohort. Accordingly, analysis of group-level data suggests that the signs of nerve pathology in my WAD2 cohort are unlikely to occur from peripheral spinal nerve or dorsal root ganglia injuries.

Injury to multiple cervical dorsal nerve roots may be possible for a subgroup of WAD2 participants.

Multiple anatomical explanations are hypothesised to make nerve roots more susceptible to injury compared to spinal nerves. Nerve roots lack an epineurium and

perineurium, have less endoneural collagen, and include more intricate vasculature[410,415]. Injury to multiple cervical nerve roots is one potential explanation for the widespread upper extremity hypoaesthesia seen in my cohort. A porcine model of whiplash injury provides support for nerve root injury by demonstrating altered pressure gradients within the spinal canal due to rapid head and neck movements[136]. Other animal models of nerve root injury also suggest that rapid compression of the spinal canal may induce changes to multiple vertebral levels compared to a slower onset[415,416]. In this rapid compression injury model, intraneural oedema formation was observed and believed to result from heightened microvascular permeability in the endoneural capillaries[415,417]. Theoretically, intraneural oedema resulting from nerve displacement may alter the nutritional supply of the nerve[412,415] potentially impacting multiple nerve roots surrounding the injury. Previous research has shown associations between signs of oedema and neuroinflammation to nerve pathology and neuropathic pain[160]. This hypothesis may help explain the more widespread signs of sensory hypoaesthesia observed in my cohort.

Preclinical models of nerve root compression injuries suggested to also cause changes in the corresponding dorsal root ganglia include signs of neural oedema and reduced blood flow[418]. Compared to spinal nerve crush models, preclinical nerve root injuries also demonstrate less neuronal apoptosis in the dorsal root ganglia and less mechanical hypersensitivity[419,420]. This is in alignment with my findings of preserved

intraepidermal nerve fibre densities and sensory hypoaesthesia. Collectively, subsequent oedema to the cervical nerve roots and dorsal root ganglia may be one explanation for my findings of neuropathic pain and nerve pathology. This injury however may not only be isolated to the nerve roots but may include changes at the central nervous system level. Spinal nerve root injury has also shown spinal cord dysfunction[421]. Preclinical models suggest that oedema from spinal nerve root injuries may cause alterations in blood flow affecting the spinal cord[412].

Central nervous system pathology may be another explanation for the findings in my cohort.

A subtle injury to the spinal cord may result in widespread upper and lower extremity hypoaesthesia, as identified in a subgroup of my cohort. Alterations to the spinal cord in my cohort may come from direct cord compression or secondary effects of oedema within the spinal canal. Theoretical models have suggested that spinal cord compression can occur with whiplash injuries when canal stenosis is present. Acutely elevated NfL levels with preserved nerve fibre densities also support potential for central nervous system dysfunction.

Previous findings from a small cohort of WAD2 have shown worse outcomes for participants with increased radiological findings, which included the presence of stenosis for 63% (5/8)[422]. Signs of spinal cord pathology have also been shown in previous WAD2 cohorts. Though debate exists on the severity of its presence, signs

of spinal cord pathology have previously been reported in WAD2, including changes to spinal cord white matter tracts in non-recovered participants using MRI[115,423,424] and impaired voluntary plantar flexor activation[423,424].

When considering additional injury mechanisms, a mild TBI/concussion is also plausible after whiplash injury and shares many similar symptoms as WAD (e.g., headache, dizziness, fogginess). Mild TBI however typically presents with higher levels of NfL, which may limit this possibility in my cohort and discussed in detail below. The temporal patterns of nerve pathology within my cohort will also help elucidate potential origins of my identified pathology (Chapter 6). Summarising the potential injury from the rapid mechanism of whiplash is unlikely to result in isolated damage. As such, injury to both the dorsal nerve roots and central nervous system may occur. I will now discuss each aspect of my findings in greater detail.

Elevated serum NfL levels identified structural nerve pathology in a subgroup of acute WAD2 participants.

The significant increase in serum NfL levels in acute WAD2 suggests that a subgroup exhibit signs of axonal injury. This increase was not only identified from absolute NfL concentrations in age and sex-matched controls, but also when comparing age- and BMI-adjusted z-scores. Although similar serological methodologies were used to derive the z-score values[388], the NfL z-score values in my acute and control cohort are relatively low. This finding may be due to inter-assay differences in NfL testing.

However, my values do normalise at the six-month follow-up (see Chapter 6). This difference may also be from stricter inclusion criteria used for my healthy control cohort compared to the cohort used to derive the z-score values[388]. For example, I screened each healthy control participant with an in-depth neurological assessment, which wasn't included in the healthy control cohort from the z-score derivation study[388].

Data from my deep phenotyping in acute WAD2 suggests that signs of nerve pathology may arise from the cervical nerve roots or spinal cord. The following sections will provide further context on how NfL plays a role in pinpointing the source of nerve pathology.

Elevated NfL is unlikely to arise from peripheral nerve injury.

One possibility for increased acute NfL levels is from a direct peripheral nerve injury. The findings from my systematic review of blood-based biomarkers in Chapter 3 demonstrate the possibility for elevated NfL levels from the peripheral nervous system. Only one previous study assessed levels of NfL in WAD using cerebrospinal fluid from seventeen participants with WAD grade 3 (with a frank nerve lesion)[425]. This study found three WAD participants with increased acute NfL levels compared to healthy controls[425]. Another study demonstrated an acute increase in plasma NfL within ten days after sustaining a non-TBI traumatic injury[291], but did not provide corresponding neurological assessment data. However, contextualising my NfL values

with my acute clinical factors does not suggest NfL is from a purely peripheral source.

Clear signs of traumatic brain injury are not present in acute WAD2.

Another hypothesis for increased NfL in acute whiplash is due to a traumatic brain injury. NfL after traumatic brain injury (TBI) has been more extensively studied than in the peripheral nervous system. Studies measuring acute levels of NFL after TBI show much greater acute NfL concentrations compared to my findings (e.g., 500 pg/mL in TBI vs 7 pg/mL in WAD2)[291]. My study inclusion criteria deliberately limited the possibility of participants who experienced a TBI; I excluded participants who experienced loss of consciousness, diplopia, drop attacks, dysarthria, or dysphagia. Overall, it is unlikely that my findings of increased NfL arose from a severe TBI but may arise from a milder brain injury.

Concussion, also referred to as mild TBI, does share more commonalities to whiplash injuries than severe TBI. These similarities include symptoms of headache, dizziness, and cognitive impairment[110,426-429]. Using similar serological analysis, Shahim et al. report higher but comparable median serum NfL concentrations in participants one month after mild TBI compared to my WAD2 cohort (30 pg/mL in mild TBI vs 7 pg/mL in WAD2)[430]. However, both mild and severe TBI typically show increased NfL levels that persist for months after injury[291,430-433]. My six-month follow-up data

could help elucidate the similarities or differences in nerve pathology in WAD2 compared to mild TBI (see Chapter 6).

Spinal cord pathology may explain acutely elevated NfL levels in WAD2.

The final possibility for raised NfL levels could be from trauma to the spinal cord. Small cohort and case studies have suggested possible spinal cord injury in participants with WAD2[423,424]. Although I did not identify any studies measuring NfL in spinal cord injured participants following whiplash, there is increasing literature on acute NfL levels following spinal cord injury. The trends of peak NfL concentrations in the acute phase of spinal cord injury are comparable to the high levels following severe TBI (200 - 400 pg/mL seven days after injury)[434,435]. Participants in my study were not likely to be included with frank spinal cord injury as any participants who presented with any signs of hyperreflexia, ataxia, or paralysis would have been excluded. Though it is less likely that my elevated NfL levels occurred from a frank spinal cord injury, subtle damage to the tracks of the spinal cord or cervical nerve roots following whiplash is theoretically possible and has been suggested as a mechanism in some patients[115,423,424].

Histological signs of small nerve fibre pathology were not present at the index finger or distal leg.

There were no significant differences in acute intraepidermal nerve fibre densities at the index finger or leg in participants with acute WAD2 compared to age- and sex-

matched controls. Although my bedside assessments and QST identified functional small nerve fibre changes, these findings suggest there are not structural signs of acute small nerve fibre pathology at the index finger. I obtained acute skin biopsies approximately 3 weeks after injury. This timeline would theoretically allow for the process of Wallerian degeneration to occur postganglionically (at approximately two weeks[408,409]). As this is a group-level comparison, it is possible that some participants with acute WAD2 may still have small fibre nerve pathology.

Although there is no published normative nerve fibre density data for the index finger, a previous study in carpal tunnel syndrome found a reliable cut-off for detecting small fibre pathology was 7.1 fibres/mm[279]. However, this cut-off does not account for age, which is known to inversely correlate to nerve fibre densities. To adjust for this discrepancy, I created normative nerve fibre densities from my healthy control data for each age decade (Supplemental Tables 5.4 - 5.5, Supplemental Figure 5.5). Using this criteria, 1.6% (1/62 with acute skin biopsies) of my acute WAD2 cohort would be classified as having small fibre nerve pathology from skin biopsies.

The findings of preserved intraepidermal nerve fibre densities and Meissner corpuscles at the index finger suggest a postganglionic nerve injury did not occur in most participants in my cohort. Previous studies of unilateral peripheral nerve injuries commonly show signs of reduced intraepidermal nerve fibre densities with accompanying sensory hypoaesthesia. This has been seen in carpal tunnel

syndrome[250,279], complex regional pain syndrome (type 1)[436], and peripheral nerve injury of the lower limb[437]. My systematic review of nerve pathology in WAD (Chapter 2) did not identify any studies assessing acute nerve fibre density in WAD. One study identified reductions in nerve fibre densities in participants with chronic WAD2 at the index finger and no changes at the distal leg[40]. This small nerve fibre pathology however was from a group of 24 participants with chronic symptoms persisting for a median 5 years after their injury[40]. The identified reduction in nerve fibres in chronic WAD2 could therefore be caused by a range of different factors besides a frank nerve lesion (e.g., systemic inflammation[164], reduced activity levels[438]).

Another consideration for the preservation of nerve fibres in my acute WAD2 cohort may be due to the location of the skin biopsy. I chose to take skin biopsies from the index finger as this has previously shown changes in the chronic stage and represents the area of the most affected innervation territory from my systematic review (Chapter 2). My acute clinical assessment also identified C6 as the most affected innervation territory. However, I identified widespread changes that occurred throughout the upper extremity. Thus, I would not have identified small fibre nerve pathology if present outside a C6 distribution.

Sensory hypoaesthesia was present in a subgroup of acute WAD2.

I identified widespread sensory hypoaesthesia throughout the upper extremity. As discussed above, my phenotypic data suggest the origins of nerve pathology likely stem from the cervical nerve roots or spinal cord in my acute WAD2 cohort. I will contextualise the literature regarding sensory hypoaesthesia.

Clinical neurological assessment

I identified signs of nerve dysfunction throughout the upper extremity (C5 - T1). My clinical neurological assessment indicated a predominant hypoaesthesia phenotype. Of note, sensory hypoaesthesia occurred in a range of modalities, but predominantly affected the small nerve fibres (56.9% small fibre loss of function, 31.6% large fibre loss of function). These findings are supported by previous reports in participants with acute whiplash injury. Previous studies reporting detailed description of acute nerve pathology are limited, however two studies assessed signs of loss of function a mean seven days after whiplash injury. These indicated 10% - 14% had acute signs of cervical radiculopathy (reduced reflexes, strength, and cutaneous light touch)[233,235]. I found a similar mean prevalence 13% neurological loss of function from clinical assessments taken from 16 studies included in my systematic review in Chapter 2 in both acute and chronic WAD. One possibility for the slightly higher occurrence in my cohort could result from the more comprehensive battery of neurological assessment that I used compared to previous studies only using the typical bedside tests (strength, reflexes, light touch).

Most participants experienced sensory loss in their primary pain area and within the C6 distribution. Biomechanical studies of whiplash injury also demonstrate C6 to be the most consistently affected innervation territory after whiplash injury[9,439]. Nerve dysfunction within the C6 distribution is consistent with previous findings in both acute and chronic WAD cohorts[401]. This dysfunction was predominantly identified within participants' most symptomatic side. This is akin to previous studies assessing QST profiles in participants with chronic cervical radiculopathy. Both studies showed bilateral reductions in cold, warm, and vibration detection thresholds near the affected innervation territories[189,440]. The identified contralateral deficits in this study were however mild and less severe compared to the primary symptomatic side.

Intriguingly, a previous study of acute WAD showed no side-to-side differences within individuals for cold and warm detection at the index and little finger and vibration detection at the thumb and index finger using QST[16]. Though the clinical assessment identified more subtle changes bilaterally, I did not measure QST bilaterally. This finding, taken from the only other study measuring acute loss of function in WAD, suggests that the more sensitive detection using QST may indicate bilateral neural injury (e.g., cervical nerve roots) or pathology of the central nervous system. However, mild bilateral findings have also been demonstrated in other unilateral peripheral nerve injuries.

Per definition of WAD2, participants were excluded if two or more bedside neurologic findings were present within the same distribution (e.g., C5 strength loss paired with C5 sensory hypoaesthesia). Thus, no participants in my cohort had characteristic signs of a single level sensory and motor cervical radiculopathy (classified as WAD grade 3). However, I cannot rule out the possibility that a pure sensory radiculopathy was present for some WAD2 participants. To further interrogate this possibility, I analysed the number of anatomical areas with loss of function per participant. I identified only 18% of participants having a single level loss of function finding potentially reflecting a single level sensory radiculopathy. Furthermore, over 36% of participants presented with loss of function in two or more anatomical areas. These findings suggest a more common widespread sensory hypoaesthesia phenotype compared to a single-level sign of nerve pathology. My detailed QST results provide greater understanding to the sensory phenotype in my cohort.

QST at the index finger

In line with my clinical neurological assessment, QST results showed a significant loss of function in both large and small nerve fibres at the index finger (C6 distribution) in participants with acute WAD2 compared to controls. A recent systematic review of longitudinal QST profiles in WAD only identified one paper which assessed sensory loss of function in acute WAD. This study, led by Chien et al., showed a reduction in cold, vibration, and electrical detection at the index finger compared to controls[16]. Compared to other conditions with similar clinical

presentations, cervical radiculopathy presented with similar QST profiles compared to my acute WAD2 cohort. Participants with chronic cervical radiculopathy showed comparable reductions in thermal and vibration detection thresholds in the most affected dermatome[189,440]. Both the chronic radiculopathy and acute WAD2 cohorts also did not present with significant increases in gain of function measures[189,440]. The converse QST profiles have been seen in non-specific neck and arm pain. These studies indicate participants with non-specific neck and arm pain have increased gain of function to thermal hyperalgesia and a lack of hypoaesthesia measured at the hand or related dermatomal level of pain[17,440,441].

Another possibility for acute sensory hypoaesthesia is from secondary mechanisms that develop after the accident. This includes altered central descending control mechanisms influencing somatosensory perception, even in the absence of a nerve lesion[257,258]. I identified moderate levels of posttraumatic stress symptoms in my cohort. Biological processes related to stress have been suggested to modulate both nociceptive and anti-nociceptive stimulation along pathways in the neuraxis[132,442-444]. Changes related to posttraumatic stress after the collision are unlikely to explain the current QST somatosensory profiles in my cohort. Previous studies in people with chronic combat-related PTSD have shown signs of somatosensory hypoaesthesia, but also show consistent gain of function to thermal pain thresholds[445,446]. These findings are counter to my QST profiles in WAD2, which do not show elevated pain thresholds for any measure at the baseline or six-month

follow-up testing. A previous study of sensory profiles in WAD2 also shows acute hypoaesthesia occurred independently to measures of psychological distress[16]. In summary, these findings suggest that pathological changes from posttraumatic stress do not explain the group-level somatosensory profiles in my WAD2 cohort.

I stratified my index finger QST parameters into phenotypic clusters to gain mechanistic insight from sensory profiling to achieve greater detail of my findings[387]. This deterministic clustering assigns participants to one of four groups: normal, thermal hyperalgesia, mechanical hyperalgesia, and sensory loss[387]. I found that a majority of my cohort was classified as a healthy sensory profile (67%) or with mechanical hyperalgesia (20%). This is likely because sensory loss parameters are still included in the mechanical hyperalgesia phenotype[387]. Numerous studies have identified similar challenges when interpreting the results of the QST clusters to their overall profiles when considering the full details of their sensory profiles[185,447,448]. The deterministic nature of this analysis forces a single classification for each participant that may not comprehensively illustrate their QST phenotype. I also repeated this deterministic clustering without the healthy sensory profile, which did not improve the explanation of my group-level QST findings. As such, the phenotypic clusters in my cohort do not provide additional mechanistic understanding to my cohort.

QST at the tibialis anterior

The QST findings for acute WAD2 at the tibialis anterior identified reduced cold detection, but no differences in warm detection or pressure pain thresholds. My systematic review (Chapter 2) only identified one other study assessing thermal detection at the tibialis anterior. This study did not find any significant differences in a small cohort with chronic WAD[208]. My findings of acute cold hypoaesthesia at the leg could result from numerous possibilities, which may not be mutually exclusive. Acute spinal cord pathology resulting from the whiplash injury may explain the cold hypoaesthesia present in a subset of participants. Both an occult injury or secondary mechanisms (e.g., neuroinflammation) may result in reduced cold detection thresholds[449]. Second, cold hypoaesthesia could result from altered central processing following whiplash injury. Widespread sensory hypoaesthesia has been suggested to occur in the absence of apparent nerve lesion[258,260] and may occur after whiplash injury. The third possibility is that a subgroup of acute WAD2 participants have reduced cold detection due to the presence of low back pain or sciatica. It is well-known that low back pain commonly occurs after whiplash injuries (in up to 63% of participants[450]). I however did not collect comprehensive data regarding the presence of low back pain or sciatica data in my cohort.

It is important to note that, while the finger QST data was properly powered to compare to controls (n = 7 controls per age decade[386]), I had limited healthy control

data for the tibialis anterior. These may all be factors to consider when interpreting the acute reduction in cold detection at the leg.

The absence of mechanical hyperalgesia at the tibialis anterior aligns with numerous studies of acute and chronic WAD. This is summarised in a recent systematic review suggesting strong evidence that widespread mechanical hyperalgesia does not occur at the acute or chronic stages of WAD[158]. The acute presence of widespread mechanical hyperalgesia in participants with WAD has been suggested to only occur in those who do not recover[158].

Implications of clinical neurological assessments to detect nerve pathology.

The collective results of the beside testing accuracy indicate moderate confidence when ruling out findings negative for thermal and mechanical hypoaesthesia and limitations in interpreting positive test results.

Validity of bedside tests for detecting sensory hypoaesthesia

The concurrent validity of cotton wool and thermal coins showed moderate to strong agreement in detecting sensory hypoaesthesia compared with their corresponding QST parameters. Yet, none of the bedside tests showed significant correlations to QST. To my knowledge, there are no previously published studies assessing the validity of neurological assessments in cohorts with WAD. My results align with previous validity in participants with non-specific neck arm pain. This study, which

used the same bedside neurological testing parameters, indicated similar moderate agreement with cold, warm, and mechanical detection[374]. There was a small but significant correlation to cold detection and cool coins within this study, but all three parameters showed similar trends to my cohort.

It is important to note that I defined QST hypoaesthesia using the suggested methodology of z-scores $<$ -1.96[375]. Although QST is currently the most sensitive measure for detecting somatosensory dysfunction, this cut-off may be too strict and miss some participants who may have more subtle signs of somatosensory hypoaesthesia. Although my findings suggest moderate levels of agreement, care must be taken when interpreting the concurrent validity of bedside testing for thermal and mechanical hypoaesthesia in my cohort of acute WAD2.

Accuracy of bedside tests in detecting sensory hypoaesthesia.

I further examined the accuracy of the clinical bedside tests for the detection of thermal and mechanical hypoaesthesia compared to QST. I identified high specificity and low sensitivity for all three parameters (cold, warm, and mechanical detection) in my acute WAD2 cohort. This suggests higher confidence to detect a true positive finding (confirming hypoaesthesia in those with positive tests) and lower confidence in detecting a true negative finding (ruling out hypoaesthesia in those with normal sensation). The limited sensitivity values may also contribute to the lack of

correlations between bedside neurological assessments and other measures of nerve pathology.

While comparative studies on the accuracy of bedside neurological testing are scarce, my results demonstrate similarly high specificity values, but lower sensitivity compared to two studies with similar methodologies[372,451]. The first study, assessing the accuracy of the same thermal coin methodology, compared its efficacy in detecting small fibre nerve degeneration from skin biopsies in participants with carpal tunnel syndrome[372]. In contrast to this cohort, I identified higher specificity and lower sensitivity for thermal detection thresholds compared to QST. This divergence might be attributed to the distinct signs of small nerve fibre degeneration present in carpal tunnel syndrome, which was not present in my cohort. This implies that the accuracy of thermal detection using coins may vary based on the type of nerve pathology.

Another study using slightly different bedside tests (cold, warm, and mechanical detection) in participants with neuropathic pain from a range of aetiologies showed similarly high specificity but higher sensitivity[451]. The higher specificity in this study may be attributed to the more specific thermal parameters, involving larger metal pieces cooled in a fridge or kept at room temperature[451]. Collectively, these findings suggest moderate confidence in identifying participants who have positive tests for loss of function in thermal detection thresholds using coins and mechanical detection

with cotton wool in acute WAD2. The lack of positive predictive value limit this, but still presents with high test specificity. The low levels of sensitivity and negative predictive values indicate that we cannot be confident a negative test is truly negative within my WAD2 cohort.

Association of clinical variables with NfL levels

There were no significant correlations between bedside clinical tests and measures of serum NfL values in participants with acute WAD2. To my knowledge, there is no previous study assessing NfL correlations with clinical variables in WAD. In addition, my systematic review and meta-analysis of NfL in peripheral neuropathies did not show any previous research comparing NfL to QST the associations of NfL and clinical measures of nerve pathology in WAD cohorts with frank nerve injury (grade 3) may help improve our understanding of its clinical correlations.

In contrast, I did not identify any other variables significantly correlated with NfL levels. This included a range of participant reported outcomes (pain, sensory loss, headache severity, severity of posttraumatic stress symptoms). There was also no correlation with functional or structural measures of nerve pathology (presence of loss of function using upper extremity clinical assessment, cold and mechanical detection, and intraepidermal nerve fibre densities at the index finger). Although less research has been done assessing NfL in painful conditions, NfL is commonly correlated with clinical outcomes in other neurological conditions. This includes

strong correlations with neurological symptoms and disease severity[389,452], disease progression[341,390], and structural neurological changes from MRI[291,390]. The limited association of NfL and clinical variables in my findings may stem from more subtle signs of nerve pathology that are more difficult to detect when compared to more frank neurologic injuries (e.g., TBI, multiple sclerosis, Alzheimer's Disease).

Limitations

There are limitations to consider when interpreting the results of this chapter. First, I did not reach my target a priori sample size calculation for determining differences in acute intraepidermal nerve fibre densities in acute WA2 compared to controls. My original power calculation included n = 68 acute WAD2 and n = 34 controls (2:1 allocation, 80% power, 5% significance, 0.53 effect size). My final analysis included n = 62 acute WAD2 and N = 38 controls. The small reduction in acute WAD2 biopsies was primarily due to errors in obtaining skin biopsies and participants not consenting to provide a biopsy. Although my final analysis provides post hoc power for a 1.8:1 ratio using the same parameters, I did not reach my initial sample size estimate. Although unknown, the similarities and distribution of nerve fibre densities between groups would unlikely be influenced by the addition of 6 acute WAD2 participants.

My study attained full statistical power for the primary nerve fibre density and QST analysis at the index finger but was not fully powered for the leg. Due to limited

nerve fibre density and QST leg data, I did not achieve full statistical power. The inclusion of additional age-matched healthy control leg data may influence the hypoaesthesia identified for leg cold detection thresholds. I aim to achieve adequately powered leg QST analysis in my future work.

The generalisability of my cohort is another limitation to consider. Although we conducted a multi-centre study in two different geographical areas within England, I recruited and assessed the majority of acute WAD2 participants (76%). My participants were all from the Oxfordshire area, which may not be representative of the wider UK population regarding ethnic and sociodemographic features. We did not collect demographic data related to race or socioeconomic status, which would have been helpful in better understanding the interpretability of our cohort to broader populations.

The multicentre nature of our cohort study includes potential variability of testing parameters. We attempted to mitigate this problem through pilot testing between myself and the other researcher assessing participants at the Brighton and Sussex Medical School (CR). As discussed in my Methods (Chapter 4), all clinical measures used had good inter-rater reliability[376-378]. Differences however may have still occurred.

5.5. Conclusions

Nerve pathology and neuropathic pain were present in a subgroup of participants with acute WAD2. Acute neuropathic pain was present for over one-third of WAD2 participants using the NeuPSIG grading scale. Functional nerve pathology ranged from 53.8% to 66% using neurological assessment and QST, respectively. Structural nerve pathology was present in 13% of WAD2 participants using serum NfL z-scores and 1.6% from skin biopsies. Summarising the acute phenotypic data suggests dysfunction to the cervical nerve roots or central nervous system is more likely than a purely peripheral nerve injury.

Chapter 6 will discuss the results of longitudinal measures of nerve pathology and neuropathic pain at six-month follow-up timepoint. This will help provide more detailed understanding of the role nerve injury and neuropathic pain in the transition to persistent symptoms in participants with WAD2.

Supplemental Table 5.1. Age is significantly different between the three cohorts of healthy control participants taken from different researchers (JF, CR, AS).

Factor	P-value
Age	< 0.0001

One-way ANOVA was performed to assess differences in ages between healthy control cohorts tested by the three researchers of control participants (JF, CR, AS).

Supplemental Table 5.2. Prevalence of neuropathic pain and nerve pathology in participants with acute WAD2.

	Neuropathic pain
NeuPSIG grading scale	35.4% (46/130)
painDETECT	11% (13/120)
	Nerve pathology
Clinical neurological assessment	53.8% (70/130)
QST loss of function	66% (82/124)
Serum NfL	13.2% (12/91)
Intraepidermal nerve fibre densities (skin biopsies)	1.6% (1/62)

Neuropathic pain was defined for the NeuPSIG grading scale as 'probable' and painDETECT cut-off scores >18/38. Nerve pathology was defined in the following categories: the presence of loss of function upon clinical neurological assessment (strength, reflexes, light touch, pin prick, or temperature); QST z-scores < -1.96 (including CDT, WDT, TSL, MDT, MPT, VDT, CDT-leg, WDT-leg), serum NfL z-score > 1.5; intraepidermal nerve fibre densities <7.1 fibres/mm at the index finger.

Abbreviations: NeuPSIG: Neuropathic pain Special Interest Group; NfL: neurofilament light chain; QST: quantitative sensory testing; CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio.

Supplemental Table 5.3. Findings for range-of-motion limitations for participants with acute WAD2.

	Symptomatic	Contralateral
Cervical spine		
Cervical flexion	67/130 (51.5%)	
Cervical extension	83/130 (63.8%)	
Cervical rotation	79/130 (60.8%)	68/130 (52.3%)
Cervical lateral flexion	71/130 (54.6%)	87/130 (66.9%)
Upper extremity		
Shoulder flexion	38/130 (29.2 %)	22/130 (16.9%)
Shoulder abduction	41/130 (31.5 %)	20/130 (15.4 %)
Elbow flexion	2/130 (1.5%)	1/130 (0.8%)
Elbow extension	2/130 (1.5%)	1/130 (0.8%)
Elbow supination	4/130 (3.1%)	4/130 (3.1%)
Elbow pronation	1/130 (0.8%)	1/130 0.8%))
Wrist extension	15/130 (11.5%)	8/130 (6.2%)
Wrist flexion	13/130 (10%)	7/130 (5.4%)

Data are presented as number (percentage) of acute WAD2 participants with limitations in the corresponding anatomical area. A total of N=78/131 (59.5%) reported primary symptoms on their right side

Supplemental Table 5.4. Intraepidermal nerve fibre density cut-off thresholds at the index finger per age decade within my healthy control cohort.

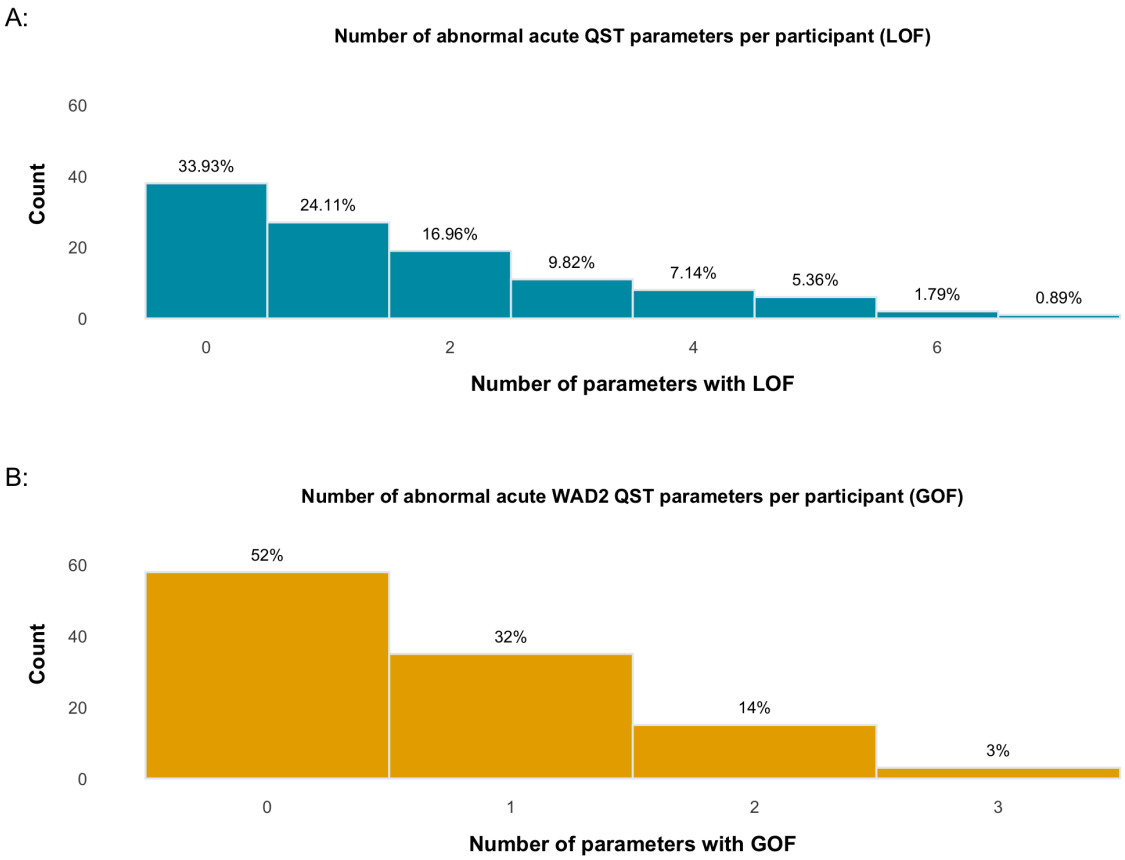
Estimated normative nerve fibre density (index finger)			
Age decade	Mean fibres/mm	SD (of mean fibre count)	Cut-off threshold - 2 SD (fibres/mm)
20 (n=13)	10.2793746	3.80	2.67
30 (n=7)	13.19	5.51	2.17
40 (n=5)	9.23	1.53	6.17
50 (n=4)	8.56	4.54	-0.52
60 (n=5)	5.54	2.03	1.48
70 (n=2)	9.23	2.11	5.02

Intraepidermal nerve fibre density cut-off thresholds (index finger) were derived from two standard deviations below the mean for each age decade. N= 36 healthy controls were included.

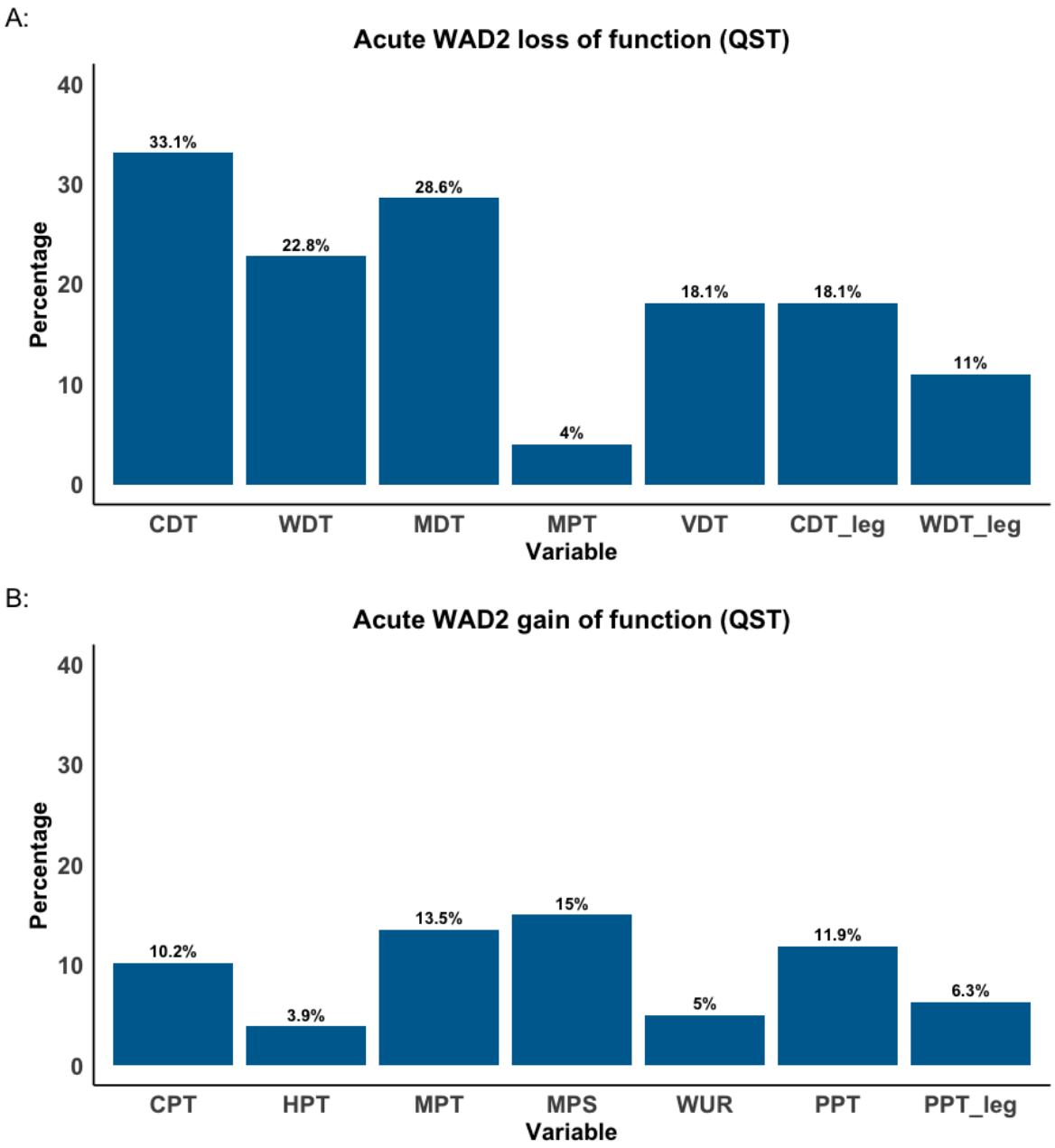
Supplemental Table 5.5. Number of acute WAD2 participants with abnormal intraepidermal nerve fibre densities at the index finger based on healthy control estimates per age decade.

Prevalence of small fibre pathology from skin biopsies at index finger	
	Acute
TOTAL	1.6% (1/62)

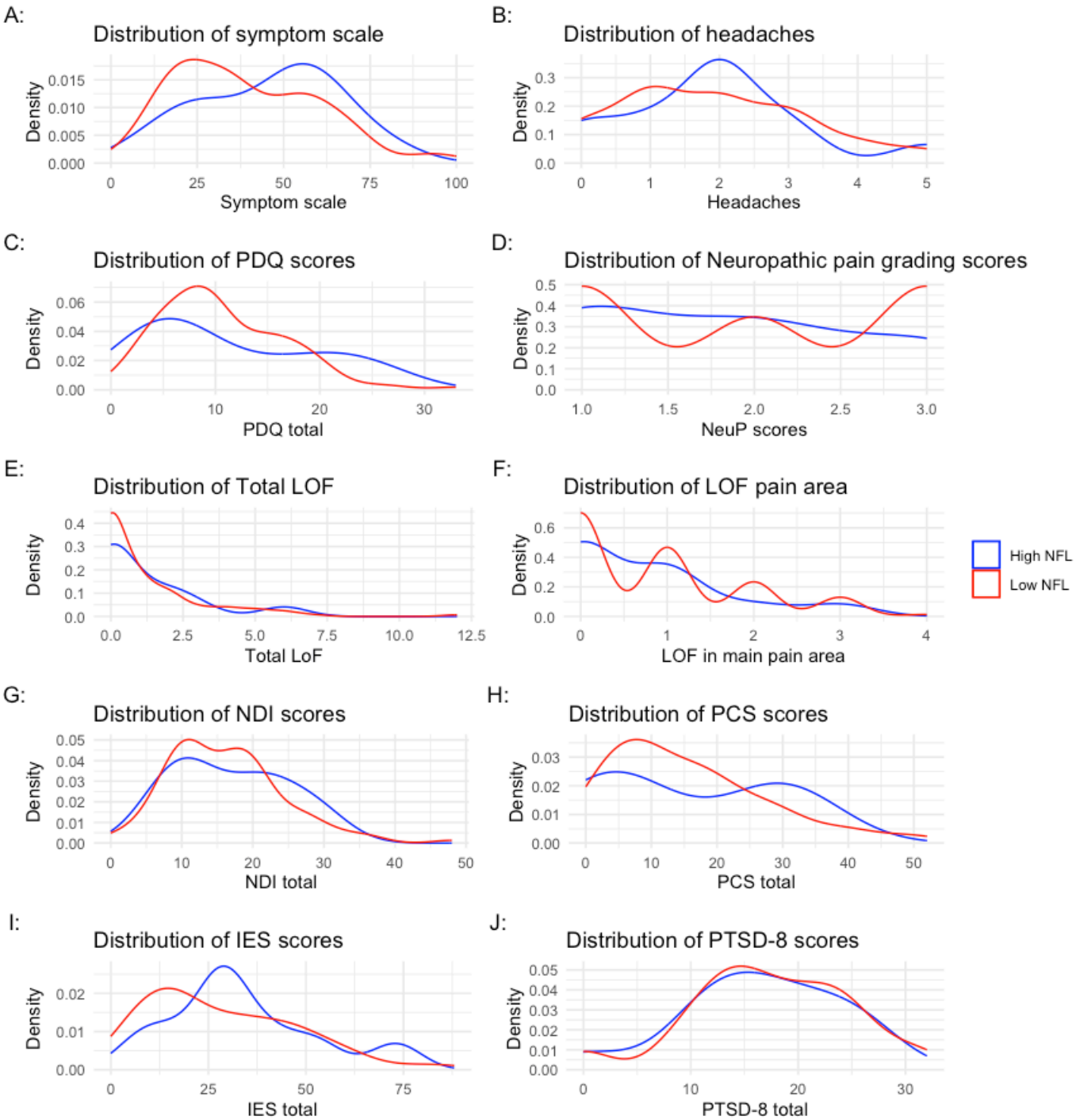
Linear regression including healthy control participants' age and index finger intraepidermal nerve fibre densities was calculated. Corresponding linear regression in Supplemental Figure 5.5 (regression equation: Y = -0.1191*X + 14.72). Derived from N= 36 healthy control participants.



Supplemental Figure 5.1. Number of acute WAD2 QST parameters outside of z-scores ± 1.96 for (A) loss of function and (B) gain of function. QST loss of function parameters (z-scores < -1.96) included: CDT, WDT, TSL, MDT, MPT, VDT, CDT-leg, WDT-leg). QST gain of function parameters (z-scores > 1.96) included: CPT, HPT, MPT, MPS, WUR, PPT, PPT-leg.
Abbreviations: LOF: loss of function; GOF: gain of function; CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio

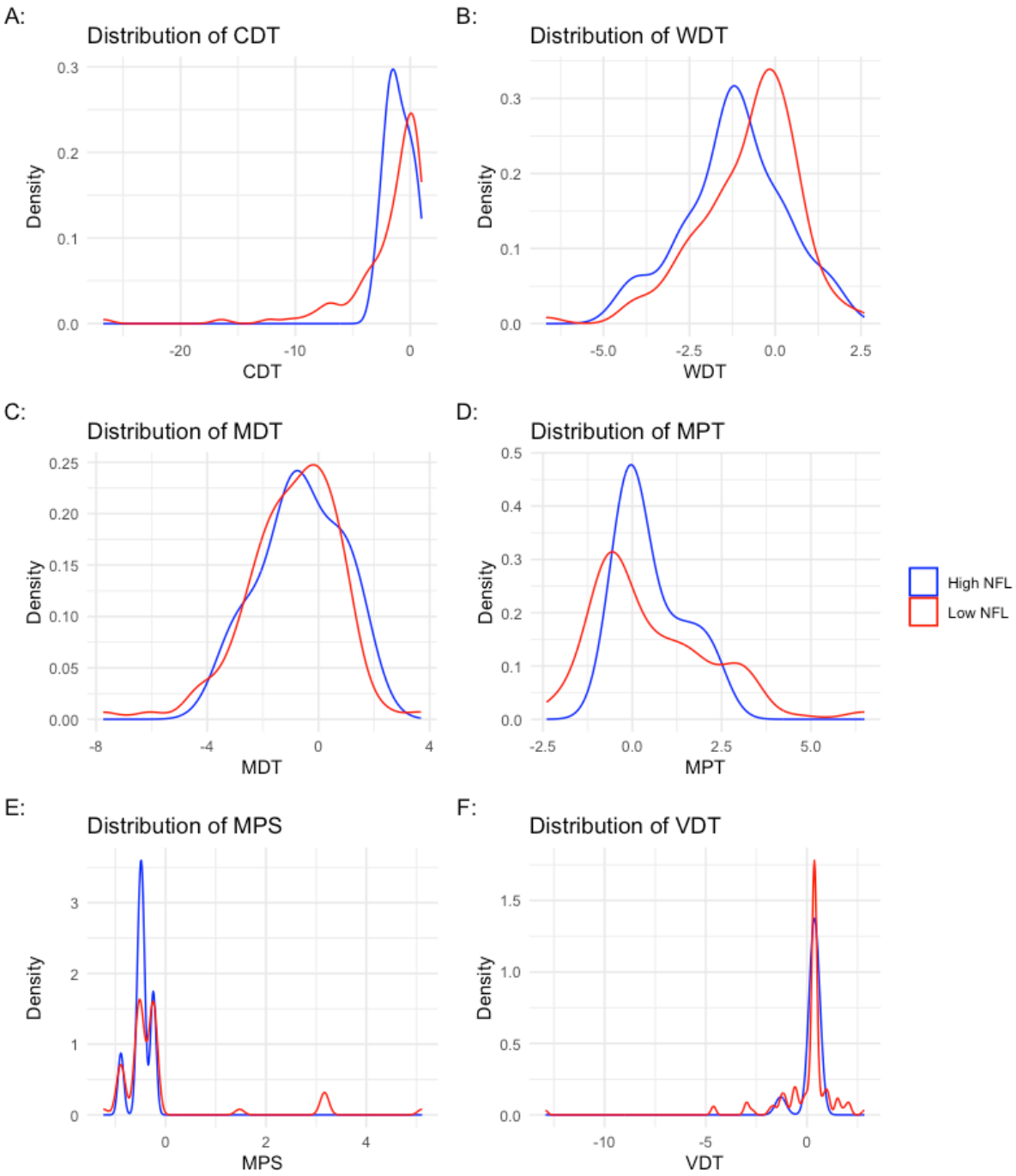


Supplemental Figure 5.2. Percentage of WAD2 participants with QST z-scores outside of standard range (z-score = ± 1.96). (A) Indicates acute changes in loss of function measures. (B) Indicates acute changes in gain of function measures.
Abbreviations: CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio.

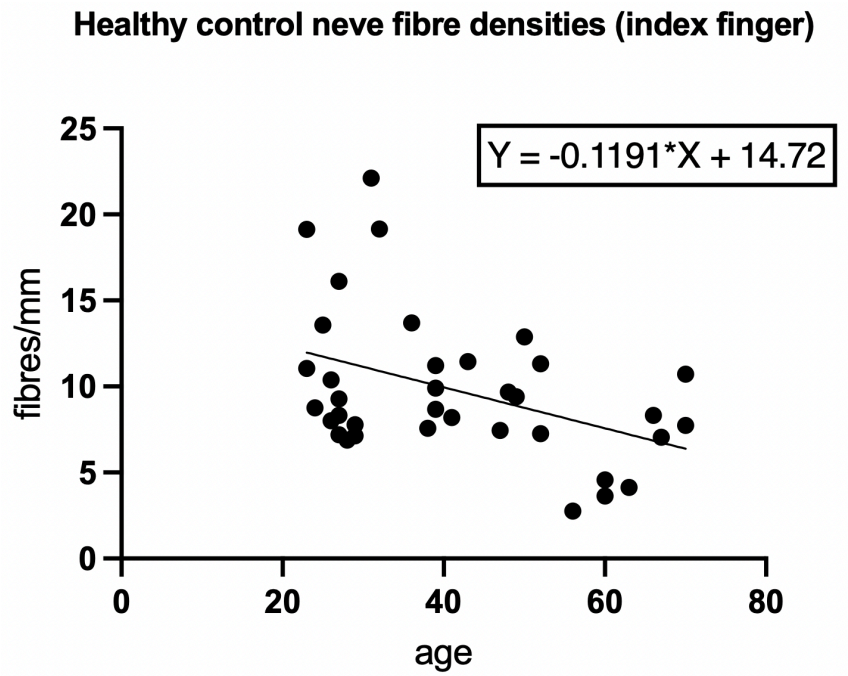


Supplemental Figure 5.3. Density plots of the distribution of demographic and clinical variables for participants with Acute WAD2 dichotomised into high and low NfL z-score values (high z-score cut-off = 1.5). Symptom scale included whiplash-related symptoms using 0-100 VAS at baseline. Distribution of headaches (as determined by the NDI) was selected as a measure to assess potential elevations in NfL from participants with potential concussive-like symptoms.

Abbreviations: PCS: Pain Catastrophisation Scale, PTSD: posttraumatic stress disorder, IES: Impact of Events Scale-Revised. NDI: Neck Disability Index; LOF: loss of function; NP: neuropathic pain; PDQ: painDETECT.



Supplemental Figure 5.4. Density plots of the distribution of QST parameters for participants with Acute WAD2 dichotomised into high and low NfL z-score values (high z-score cut-off = 1.5). Abbreviations: CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio.



Supplemental Figure 5.5. Linear regression of healthy control intraepidermal nerve fibre densities at the index finger with corresponding age (years).

6

Temporal patterns of nerve pathology and factors related to recovery in WAD2.

6.1. Aims:

This chapter is focused on the longitudinal results from the six-month follow-up of the WAD2 cohort. The overall aims of this chapter are to evaluate the temporal patterns of nerve pathology and assess if such measures are prognostic. I also used RNA-sequencing of follow-up blood samples to investigate potential molecular mechanisms related to persistent whiplash symptoms.

My specific objectives in this chapter include evaluating:

1) Differences in WAD2 pain phenotypes from acute to six-month follow-up.
2) The temporal patterns of nerve pathology, including clinical assessment, QST, intraepidermal nerve fibre density, and serum neurofilament light chain.
3) The prognostic role of functional and structural measures of nerve pathology for neck-related disability and whiplash-related symptoms.
4) The molecular signature of persisting moderate-severe symptoms in WAD2 using RNA sequencing of blood samples.

6.2. Methods

Chapter 4 includes detailed descriptions of all methodologies and statistical analyses used for this chapter.

6.3. Results

6.3.1. Symptoms persist in a substantial subgroup of WAD2 participants six months after injury.

Figure 6.1 shows the study flow diagram and Table 6.1 shows a summary of acute and follow-up WAD2 participant-reported outcome measures. Eighty-two participants with WAD2 completed follow-up questionnaires at six months. Of these 82 WAD2 follow-up participants, 56 also attended an in-person appointment that included a repeat of all deep phenotypic data collected acutely (clinical assessment, QST, skin biopsy, and blood sample). Challenges related to participants' absence at in-person follow-up assessment include the distance to travel to assessment sites, limited availability of reimbursements for follow-up appointments, and difficulty contacting participants. The six-month follow-up results show an overall presentation of recovery for participants with WAD2. Reports of recovery, as determined by a six-point Likert scale, indicate 28% (23/82) are 'all better', 43% (35/82) have 'quite a bit of improvement', 21% (17/82) are 'some better', 2% report 'no improvement', 5% (4/82) are a 'little worse', and 1% (1/82) are 'much worse'.

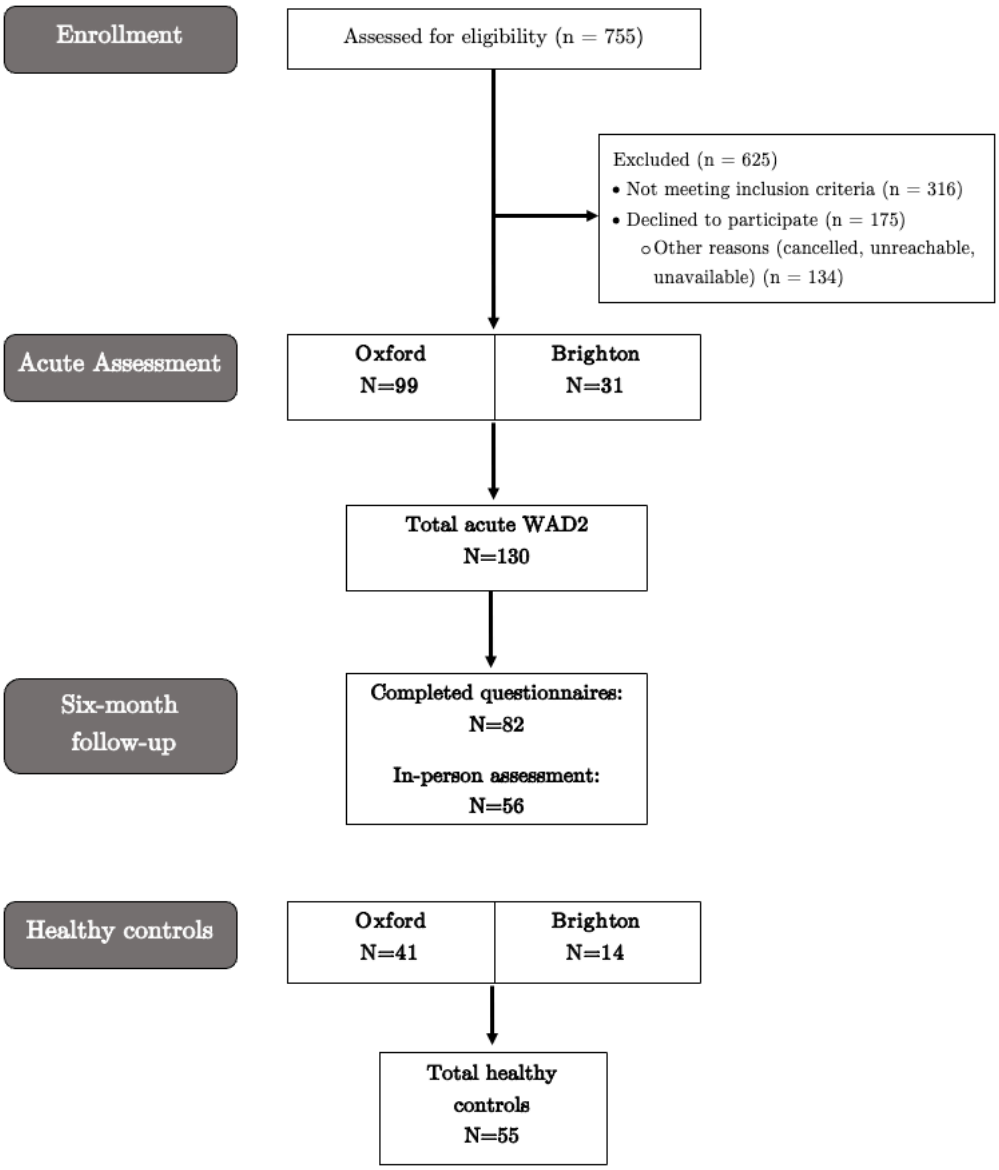


Figure 6.1. Study flow diagram.

TABLE 6.1. WAD2 participant-reported outcomes show improvements six-months after injury.

	Acute WAD2	Follow-up WAD2	Count follow-up WAD2	p-value
Count	130		82 completed questionnaires 56 attended in-person assessment	
Current symptoms (reported yes)	59%	65% (36/55)	55	NA
Current whiplash symptom VAS (med/IQR)	35 (33)	17.5 (30)	56	**< 0.001**
Mild (<30/100)	38.5%	62.5%	35	
Moderate-severe (≥30/100)	61.5%	37.5%	21	
Current pain (med/IQR)	4 (3.3)	2 (3)	78	**0.03**
Strongest pain (med/IQR)	8 (2)	4 (4.8)	78	**< 0.001**
Average pain (med/IQR)	6 (1.2)	3 (2)	78	**< 0.001**
Radiating symptoms	58%	27% (14/51)	51	
Subjective sensory change	42%	30% (15/50)	50	
Neck Disability Index (median/IQR)	15 (10)	7 (10)	81	**< 0.001**
Mild	4%	32%	26	
Moderate	42%	48%	39	
Severe	54%	20%	16	
PainDETECT (med/IQR)	9.5 (9)	6 (8.8)	78	**0.001**
likely neuropathic (>18/38)	11%	3%		
uncertain (>12/38)	33%	13%		
DASS – depression (med/IQR)	5.5 (11.3)	3 (12)	78	0.85
DASS – anxiety (med/IQR)	6 (9)	4 (8)	78	0.41
DASS – stress (med/IQR)	12 (12)	10 (15)	78	0.84
EQ-5D-5L (Index value; mean/SD)	0.73 (0.2)	0.818 (0.1)	79	**< 0.001**
PTSD-8 total (med/IQR)	17 (10)	14 (10)	77	**0.03**
Likely PTSD	51%	36%		
Impact of Events Scale – Revised (med/IQR)	26.5 (28.3)	15 (27.5)	75	0.26
Probable PTSD	40%	23%		
PCS (med/IQR)	13 (17)	8 (14.3)	78	0.22
Likely catastrophizing (n/%)	14%	3.8%		
Treatment at follow-up			50	
Any treatment (at any time)		58%	29/50	
Physiotherapy (at any time)		46%	23/50	
		6%	3/50	

Osteopathy or chiropractic (at any time)		32%	16/50	
Medication (currently taking)		28%	14/50	
Paracetamol		16%	8/50	
NSAID		2%	1/50	
Amitriptyline		2%	1/50	
Citalopram				
Other		2%	1/50	
TENS		2%	1/50	
Topical methyl salicylate (Icy Hot)				
Recovery question	NA		82	
All better		28%	23	
Quite a bit of improvement		43%	35	
Some improvement		21%	17	
No improvement		2%	2	
A little worse		5%	4	
Much worse		1%	1	

Numerical data are presented as median (IQR) when non-parametric, mean (SD) when normally distributed, or percentage (%) in the case of categorical data. Neck Disability Index scoring: mild <5, moderate 5-14, and severe ≥15. Current, strongest, and average pain levels were taken from the corresponding questions within the painDETECT questionnaire. PainDETECT scoring: likely neuropathic (>18/38), uncertain (>12/38). PTSD-8 scoring: likely PTSD (≥18/32). Impact of Events Scale scoring: probable PTSD (≥33/88). PCSNSAID use included both pill and topical applications. Healthy control participants did not complete self-reported outcome measures and, by definition of inclusion criteria, did not have any neck or arm symptoms. Wilcoxon signed-rank tests were used for all non-parametric comparisons. Paired t-test was used for normally distributed variables (EQ-5D-5L index value). Bolded text indicated statistical significance (set at $p < 0.05$).
Abbreviations: DASS: Depression Anxiety and Stress Scale; IQR: interquartile range; med: median; PTSD: posttraumatic stress disorder; PCS: Pain Catastrophizing Scale; NSAID: nonsteroid anti-inflammatory; TENS: Transcutaneous electrical nerve stimulation.

Neck-related disability including all follow-up questionnaires significantly decreased for WAD2 participants at six-months (median = 7, IQR = 10) compared to acute timepoint (median = 15, IQR = 10) ($V = 421$, $p < 0.0001$). Paired comparison in only those participants who had paired acute and follow-up data also indicate a reduction in neck-related disability for most participants at six-months (Figure 6.2A). Trends for whiplash-related symptoms as measured using VAS during in-person follow-up assessment are also significantly reduced at six-moths ($p < 0.0001$).

Paired WAD2 participants with acute (median = 35.5, IQR = 36) and follow-up data display more heterogeneity regarding recovery of whiplash symptoms at follow-up (median = 18.5, IQR = 34.5, Figure 6.2B).

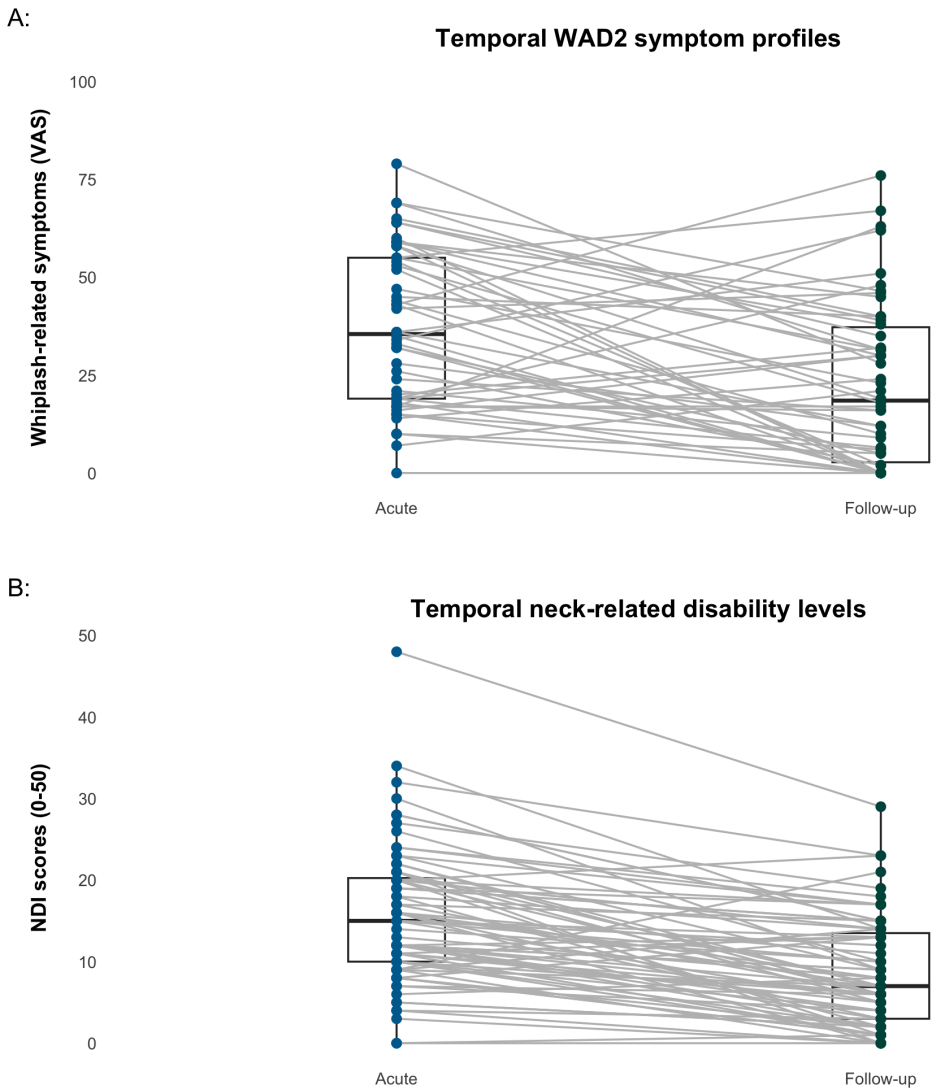


Figure 6.2. Neck-related disability and whiplash symptoms improve but do not resolve six-months after injury. (A) Paired participant acute and follow-up WAD2 whiplash related symptom values. (B) Paired participant acute and follow-up WAD2 NDI scores for neck-related disability. Complete (unpaired) acute and follow-up analysis is displayed in Table 6.1.
Abbreviations: NDI: Neck Disability Index; VAS: visual analogue scale.

Overall reports of the current, strongest, and average pain (PainDETECT) are all significantly reduced at six-months compared to acute timepoints. However, 65% of follow-up participants report continued whiplash-related symptoms. Additionally, over one-third report moderate-severe symptoms six-months after injury. The neck continues to be the primary location of pain (Figure 6.3A). The median current pain level is 2/10 (IQR 3), median average pain of 3/10 (IQR 2), and strongest median pain of 4/10 (IQR 4.75) using a numeric rating scale at six-month follow-up. Sixty-four percent (36/56) report current whiplash-related symptoms when attending the in-person follow-up appointment. Follow-up reports of radiating symptoms and sensory changes are present for 27% (14/51) and 30% (15/50), respectively.

6.3.2. Neuropathic pain scores improve at follow-up.

A summary of neuropathic pain grading measures for acute and follow-up WAD2 are presented in Table 6.2. Neuropathic pain determined using the painDETECT questionnaire shows a significant reduction at follow-up (median = 6, IQR =8.8) compared to acute WAD2 (median = 9.5, IQR =9 [V = 325.5, p = 0.001]). Only three percent were graded as likely neuropathic pain at follow-up and 13% with uncertain levels of neuropathic pain using painDETECT.

The likelihood of neuropathic pain using the NeuPSIG grading also shows improvement. Figure 6.3B shows paired acute and follow-up WAD2 participant data. Seventy-one percent of follow-up WAD2 participants (37/52) present with unlikely,

6% (3/52) with possible, and 23% (12/52) with probable neuropathic pain. Only WAD2 participants who were initially graded as possible, or probable go on to have some level of neuropathic pain six-months after injury.

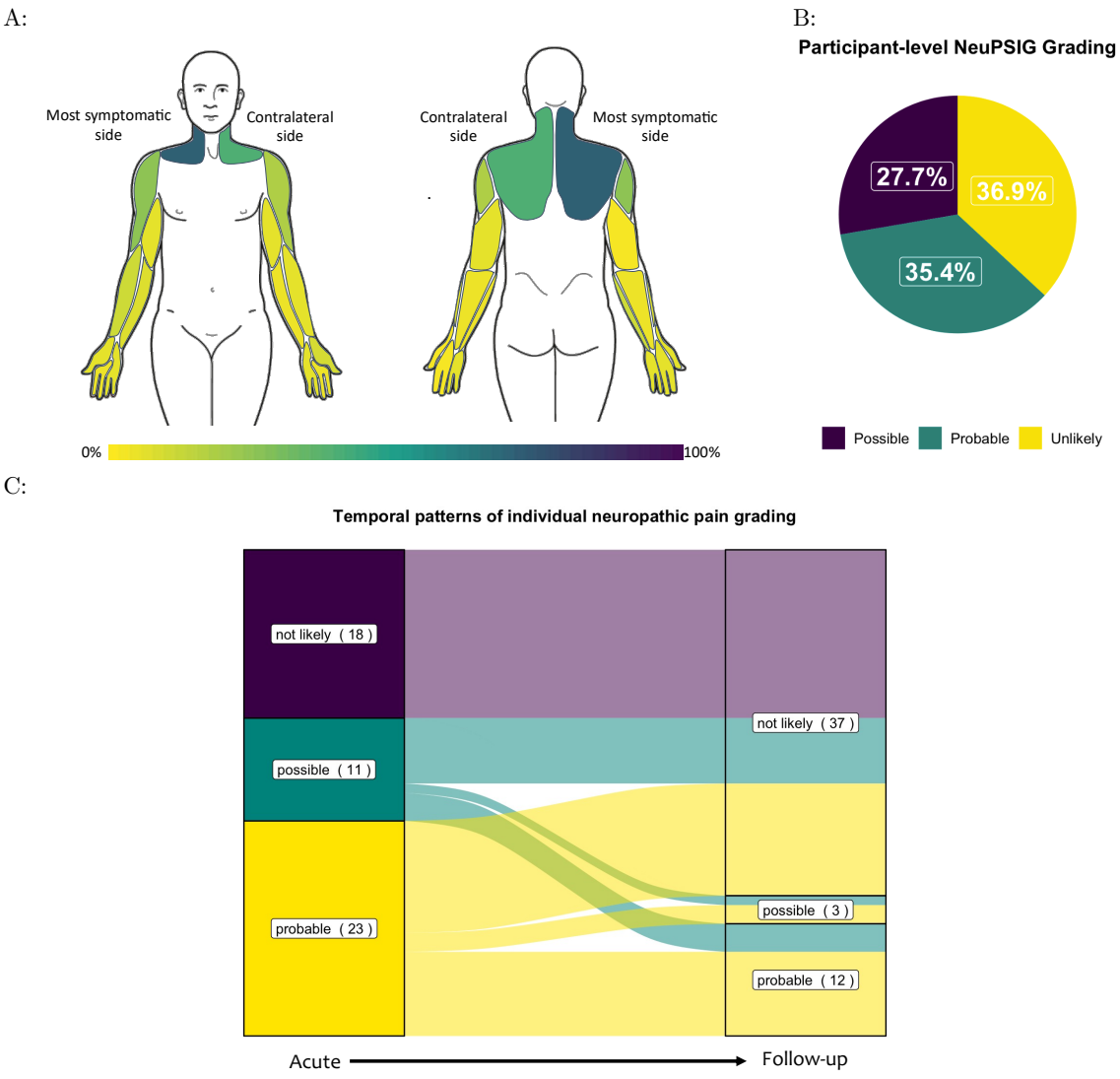


Figure 6.3. Prevalence of neuropathic pain reduces over time in WAD2 participants. (A) The body diagram shows the percentage and location of whiplash-related symptoms for WAD2 participants at six-month follow-up. B) Distribution of the certainty of acute neuropathic pain for individual acute WAD2 participants using the Neuropathic Pain Special Interest Group (NeuPSIG) Grading System. (C) Temporal patterns of the certainty of neuropathic pain in paired acute and follow-up participants using the NeuPSIG Grading System. N = 56 follow-up WAD2 completed body diagram. N = 52 have paired NeuPSIG Grading System data.

Table 6.2. Signs of neuropathic pain and nerve pathology are present acutely and six-months after whiplash injury for a subgroup of WAD2 participants.

	Acute WAD2	Follow-up WAD2
	Neuropathic pain	
NeuPSIG grading scale	35.4% (46/130)	24% (12/52)
painDETECT	11% (13/120)	3% (2/78)
	Nerve pathology	
Clinical neurological assessment	53.8% (70/130)	24% (13/56)
QST loss of function	66% (82/124)	64% (32/50)
Serum NfL	13.2% (12/91)	2% (1/41)
Intraepidermal nerve fibre densities (skin biopsies)	1.6% (1/62)	8.7% (2/23)

Neuropathic pain was defined using the NeuPSIG grading scale as 'probable' and painDETECT cut-off scores >18/38. Nerve pathology was defined in the following categories: the presence of loss of function upon clinical neurological assessment (strength, reflexes, light touch, pin prick, or temperature); QST z-scores < -1.96 (including CDT, WDT, TSL, MDT, MPT, VDT, CDT-leg, WDT-leg), serum NfL z-score > 1.5; intraepidermal nerve fibre densities cut-offs were calculated per age decades based on my healthy control cohort; full description of calculation is included in Chapter 5, Supplemental Table 6.4.

Abbreviations: NeuPSIG: Neuropathic pain Special Interest Group; NfL: neurofilament light chain; QST: quantitative sensory testing; CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio.

6.3.3. Continued levels of posttraumatic stress are present in a subgroup of participants.

Measures of depression, anxiety, and stress show similarly low values both acutely and six-months after injury (Table 6.1). Scores for posttraumatic stress using the Impacts of Events-Revised questionnaire show signs of improvement but are not significantly better at six-months (median = 15, IQR = 27.5) compared to the acute phase (median =26.5, IQR = 28.3 [V = 236, p=0.26]). Twenty-three percent of participants also have signs of 'probable PTSD' using the Impact of Events-Revised scale. The PTSD-8 questionnaire does indicate significant improvement at follow-up

(median = 14, IQR = 10) compared to acute WAD2 (median = 17, IQR = 10 [V = 284, p = 0.03]). However, 36% of follow-up WAD2 participants are still classified as 'likely PTSD'.

6.3.4. Majority of WAD2 participants receive treatment related to their whiplash injury.

Overall, 58% (29/50) of my WAD2 follow-up cohort report receiving treatment due their whiplash injury (Table 6.1). Nearly half of these participants (46%) received physiotherapy due to their whiplash injury and only 6% received osteopathic or chiropractic treatment. Approximately one-third of follow-up participants are taking medication due to their whiplash symptoms (28%). Of those, the most commonly used medications six-months after injury include paracetamol (28%) and nonsteroidal anti-inflammatories (16%). Other treatments at follow-up include amitriptyline (2%), citalopram (2%), transcutaneous electrical nerve stimulation (2%), and topical methyl salicylate (Icy Hot, 2%).

6.3.5. Bedside neurological assessment indicates improvement but persisting hypoaesthesia for a subgroup of WAD2.

Neurologic dysfunction is identified in 63% people with WAD2 acutely and 37% at follow-up (Figure 6.4A; Supplemental Table 6.1). Abnormal bedside neurological assessment results at follow-up include 17% with loss of function, 13% with gain of function, and 7% with mixed (loss and gain) presentations (Figure 6.4A). Loss of

function at follow-up is predominantly from small nerve fibres (13.3% pin prick, 13.3% thermal coins) and 6.7% from light touch hypoaesthesia (Figure 6.4B). Abnormalities in gain of function tests at follow-up include 16.7% from pin prick and 3.3% from light touch hypersensitivity (Figure 6.4B). Upper extremity neurological assessment (C5-T1 innervation territories) shows limited abnormal findings (<11% of participants) with a mixed presentation of widespread loss and gain of function (Figure 6.4C). The identified upper extremity nerve dysfunction is predominantly seen on the symptomatic versus contralateral side (Figures 6.4C-6.4D). The greatest amount of nerve dysfunction at follow-up is in participants' primary pain areas (41% loss of function, 16% gain of function; Figure 6.4E). Continued loss of function is identified at the index finger, but to a lesser extent on both symptomatic and contralateral sides (Figures 6.4F-6.4F).

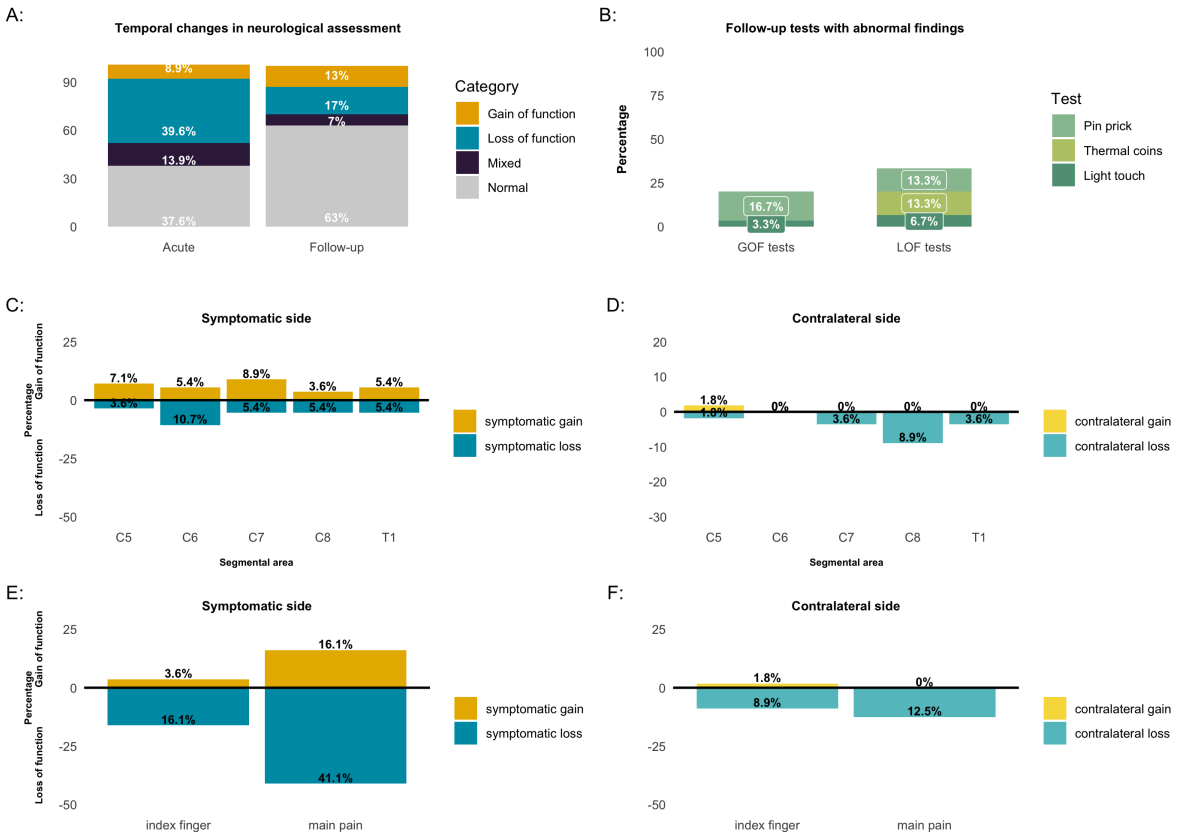


Figure 6.4. Continued upper extremity hypoaesthesia is present for a subgroup of WAD2 participants six-months after injury using bedside neurological assessment. (A) Acute and follow-up categorisation of upper extremity neurological assessment, including strength, reflexes, light touch, pin prick, and thermal coins. (B) Type and percentage (%) of results contributing to abnormal findings of neurological gain of function (GOF) and loss of function (LOF).
(C-D) Percentage of neurological loss and gain of function in the C5-T1 innervation territories, including strength, reflexes, light touch, pinprick, and thermal coins. Panel C shows most symptomatic side and panel D shows the contralateral side. (E-F) Percentage of bedside small fibre testing for neurological loss and gain of function within the symptomatic index finger and main pain area. Tests included pinprick, warm and cool coins. Panel E shows the most symptomatic side and panel F shows contralateral side. N=56 WAD2 follow-up participants were included.

6.3.6. QST identifies continued sensory hypoaesthesia six-months after injury for a subgroup of participants with WAD2.

Figure 6.5 and Supplemental Tables 6.1 and 6.2 show results for all QST testing performed at the index finger and leg comparing controls, acute WAD2, and follow-up WAD2. Means and standard deviations for all QST parameters and participants are listed in Supplemental Table 6.1. At group level, there is a significant decrease in detection thresholds for cold ($p < 0.001$), warm ($p < 0.001$), thermal sensory limen ($p < 0.0001$), mechanical ($p < 0.0001$), and vibration detection ($p < 0.01$) in people with acute WAD2 compared to controls. At follow-up, sensory detection is reduced for warm ($p < 0.05$), thermal sensory limen ($p < 0.001$), mechanical detection ($p < 0.0001$). There is also reduced hyperalgesia to pressure pain thresholds ($p < 0.001$) compared to controls. Reduced pressure pain thresholds are the only significantly different QST parameter between acute and follow-up WAD2 participants ($p < 0.001$). QST at the leg shows no significant differences in thermal detection or pain pressure thresholds for controls, acute, or follow-up participants (Figure 6.5B).

Though most QST parameters improved, a subset of WAD2 present with continued dysfunction at six-month follow-up. Loss of function in at least one QST parameter (defined as z-score < -1.96) is present in 64% (32/50) of WAD2 participants at follow-up (Supplemental Figure 6.2A, Supplemental Table 6.1). Over one-third of follow-up participants (38% [19/50]) present with loss of function in two or more

QST parameters. QST parameters with higher prevalence of hypoaesthesia at follow-up include mechanical detection, pressure pain thresholds, and warm detection at the leg (Supplemental Figure 6.2A).

Gain of function using QST (defined as z-score > 1.96) is present in 43% (22/50) of WAD2 follow-up participants (Supplemental Figure 6.1B). Thirty-nine percent (20/50) have gain of function in only one QST parameter and five percent have gain in two parameters (3/50). Most QST parameters at follow-up show a reduced prevalence of gain of function (Supplemental Figure 6.2B). Compared to acute WAD2, there is a slightly greater prevalence of follow-up WAD2 participants with higher cold and heat pain thresholds ($z > 1.96$). Cold pain increased from 10% to 12% and heat pain thresholds increased from 3.9% to 10.2%. This suggests a larger subgroup with heat hypersensitivity six-months after injury.

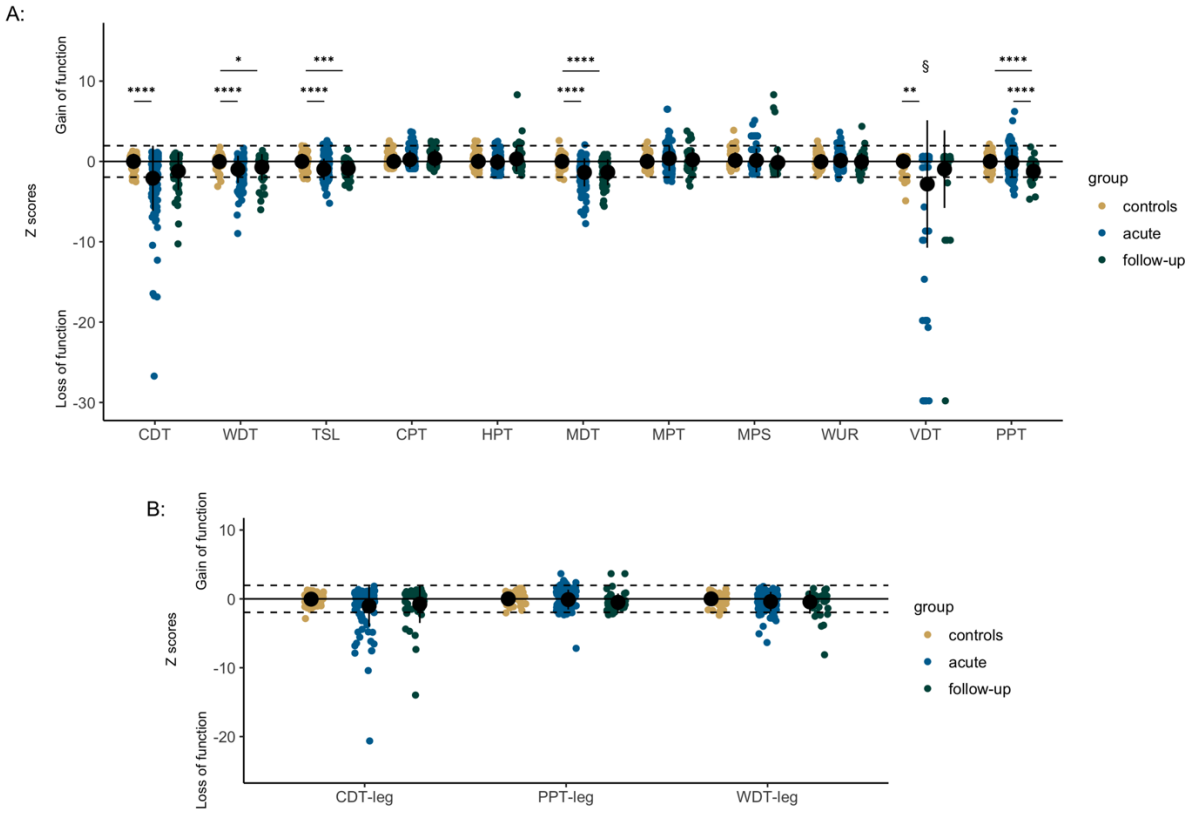


Figure 6.5. Reduced sensory detection thresholds over the index finger persist six-months after injury. (A) QST measured over the index finger in acute WAD2, follow-up WAD2, and controls. Due to ceiling effect of VDT parameter, there are 3 acute WAD2 VDT z-scores less than -30 and out of figure range (indicated as §). The three missing values include z-score = -39.76, -79.7, -59.79. (B) QST measured over the tibialis anterior in acute WAD2, follow-up WAD2, and controls. Index finger data includes n=124 acute WAD2, n= 50 follow-up WAD2, and n=61 controls. Leg data includes n=111 acute WAD2, N=47 follow-up WAD2, and n=33 controls. One-way ANOVA tests with Tukey's Honest Significant Difference post hoc testing were used for comparison between controls, acute, and follow-up participants. *P $<$ 0.05; **P $<$ 0.01; ***P $<$ 0.001; ****P $<$ 0.0001.
Abbreviations: CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio.

6.3.7. Acutely elevated NfL levels resolve by six-month follow-up for WAD2 participants.

As discussed in Chapter 5, there is significantly increased serum NfL concentrations in people with acute WAD2 ($p < 0.05$), but no difference at six-month WAD2 follow-up compared to acute WAD2 or controls (Figure 6.6, Table 6.3). Participants with paired acute and follow-up samples show overall reductions in NfL z-scores over time (Figure 6.6C). Using the previously established serum NfL z-score cut-offs indicating pathologically high NfL (z-score >1.5)[388] indicate two percent (1/41) have serological signs of nerve pathology at follow-up (Supplemental Table 6.3).

Table 6.3. Serum NfL levels at six-months are no longer elevated for participants with WAD2.

	Controls	Acute WAD2	Follow-up WAD2	F	Sig.	Acute v control	Acute v follow-up	Follow-up v control
Serum NfL concentration (pg/mL) median (IQR)	5.24 (3.5)	7.13 (5.3)	5.61 (4.6)	3.62	**P = 0.03**	**p-adj = 0.04**	P-adj = 0.20	P-adj = 0.69
Serum NfL z-scores (mean/SD)	-0.766 (1.2)	0.016 (1.4)	-0.55 (1.1)	5.58	**P < 0.01**	**P-adj = 0.01**	P-adj = 0.05	P-adj = 0.75

Aligned ranks transformation ANOVA with Tukey's post hoc testing was used to compare absolute serum NfL concentrations. One-way ANOVA with Tukey's Honest Significant Difference post hoc testing was used to compare NfL z-scores Bolded text indicated statistical significance (set at $p < 0.05$). N=30 controls, N=91 acute WAD2, N=41 follow-up WAD2 were included for analysis.
Abbreviations: NfL: Neurofilament light chain; SD: standard deviation; IQR: interquartile range. P-adj: adjusted p-value. F: F-statistic of one-way ANOVA; Sig: p-value for overall one-way ANOVA analysis.

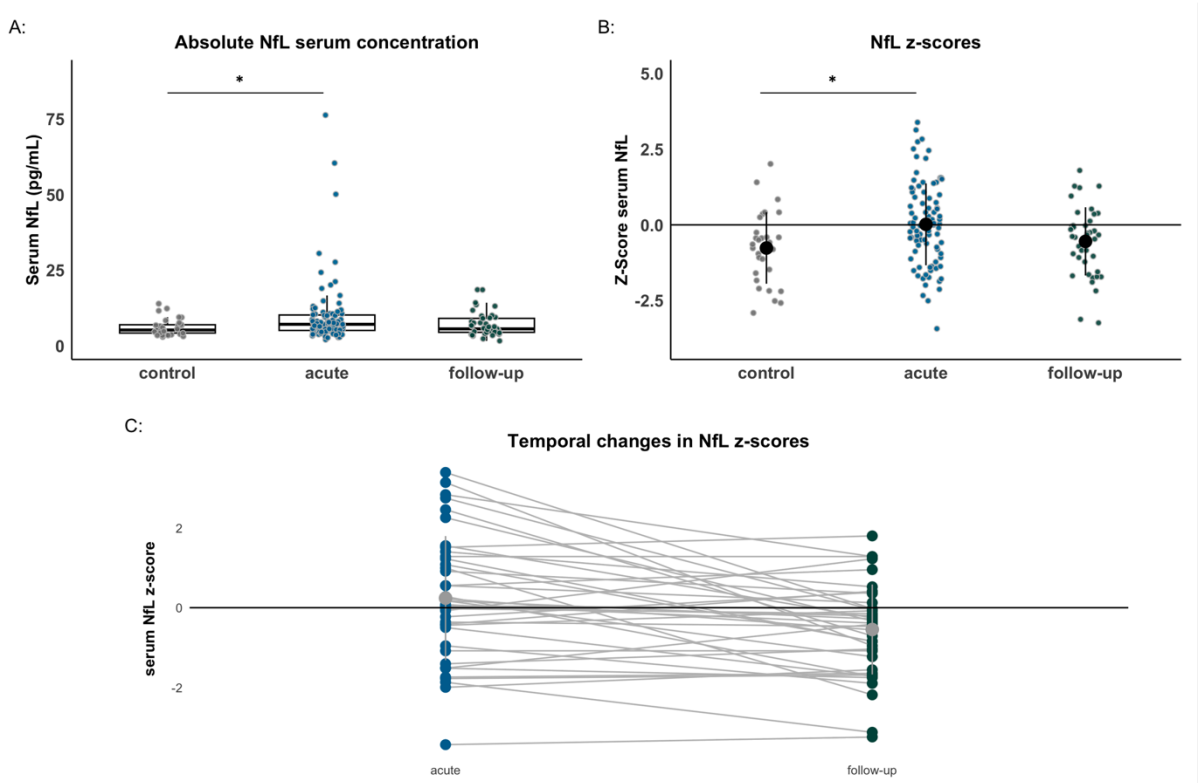


Figure 6.6. NfL levels decrease six-months after injury for WAD2 participants. (A) Absolute serum NfL concentrations (pg/mL) in control, acute WAD2, and follow-up WAD2 participants were compared using aligned ranks transformation ANOVA with Tukey's post hoc testing. (B) Serum NfL z-scores in control, acute, and follow-up WAD2 participants were compared using a one-way ANOVA with Tukey's Honest Significant Difference post hoc testing. (C) Paired serum NfL z-scores for acute and follow-up WAD2 participants with grey lines indicating match acute and follow-up samples.

6.3.8. Intraepidermal nerve fibre densities are preserved six-months after injury.

There are no significant difference in nerve fibre densities at the index finger or leg comparing acute WAD2, follow-up WAD2, and controls (Table 6.4, Figures 6.7A, 6.7C). Similarly, there are no significant differences in the number of Meissner corpuscles at the index finger comparing acute WAD2, follow-up WAD2, and controls (Table 6.4, Figure 6.7B).

Figure 6.7D shows similar trends of intraepidermal nerve fibre densities at the index finger for paired acute and follow-up participant samples. However, there are limited paired acute and follow-up WAD2 skin biopsy samples due to technique errors in obtaining samples or participant's refusal at follow-up (N = 17 total). Figure 6.7E demonstrates a slight increase in intraepidermal nerve fibre densities for paired samples six-months after injury compared to acute WAD2, though is from a limited sample size (N = 19 total paired samples). Using the previously described cut-off scores for nerve pathology from skin biopsies data at the index finger (Chapter 5), 8.7% (2/23) have signs of intraepidermal nerve pathology at follow-up (Supplemental Table 6.3).

Table 6.4. Intraepidermal nerve fibre densities at the index finger and distal leg are preserved six-months after injury.

	Acute WAD2	Follow-up WAD2	Controls	F	Sign.	Acute v control	Acute v follow-up	Follow-up v control
Index finger								
IENFD med/IQR (fibres/mm)	9.9 (6.1)	10.8 (10.1)	8.72 (3.8)	1.25	P = 0.30	P-adj = 0.39	P-adj = 0.91	P-adj = 0.37
Meissner's corpuscles med/IQR, (number/mm)	0.62 (0.7)	0.54 (0.5)	0.64 (0.5)	0.30	P = 0.74	P-adj = 0.86	P-adj = 0.75	P-adj = 0.97
Distal leg								
IENFD med/IQR (fibres/mm)	7.8 (6.3)	10.4 (5.9)	8.7 (6.1)	0.90	P = 0.41	P-adj = 0.97	P-adj = 0.39	P-adj = 0.63

Index finger skin biopsies were taken from the volar aspect of the proximal phalanx of the index finger for all participants. Index finger skin biopsies for acute WAD2 was taken from the most symptomatic side and from the non-dominant side for controls. Aligned ranks transformation ANOVA with Tukey's post hoc testing was used for all comparisons. Index finger data includes N=62 acute WAD2, N=23 follow-up WAD2, and N=38 controls. Distal leg data include N=39 acute WAD2, N=24 follow-up WAD2, and N=21 controls.
Abbreviations: IENFD: intraepidermal nerve fibre density; IQR: interquartile range; Med: median.

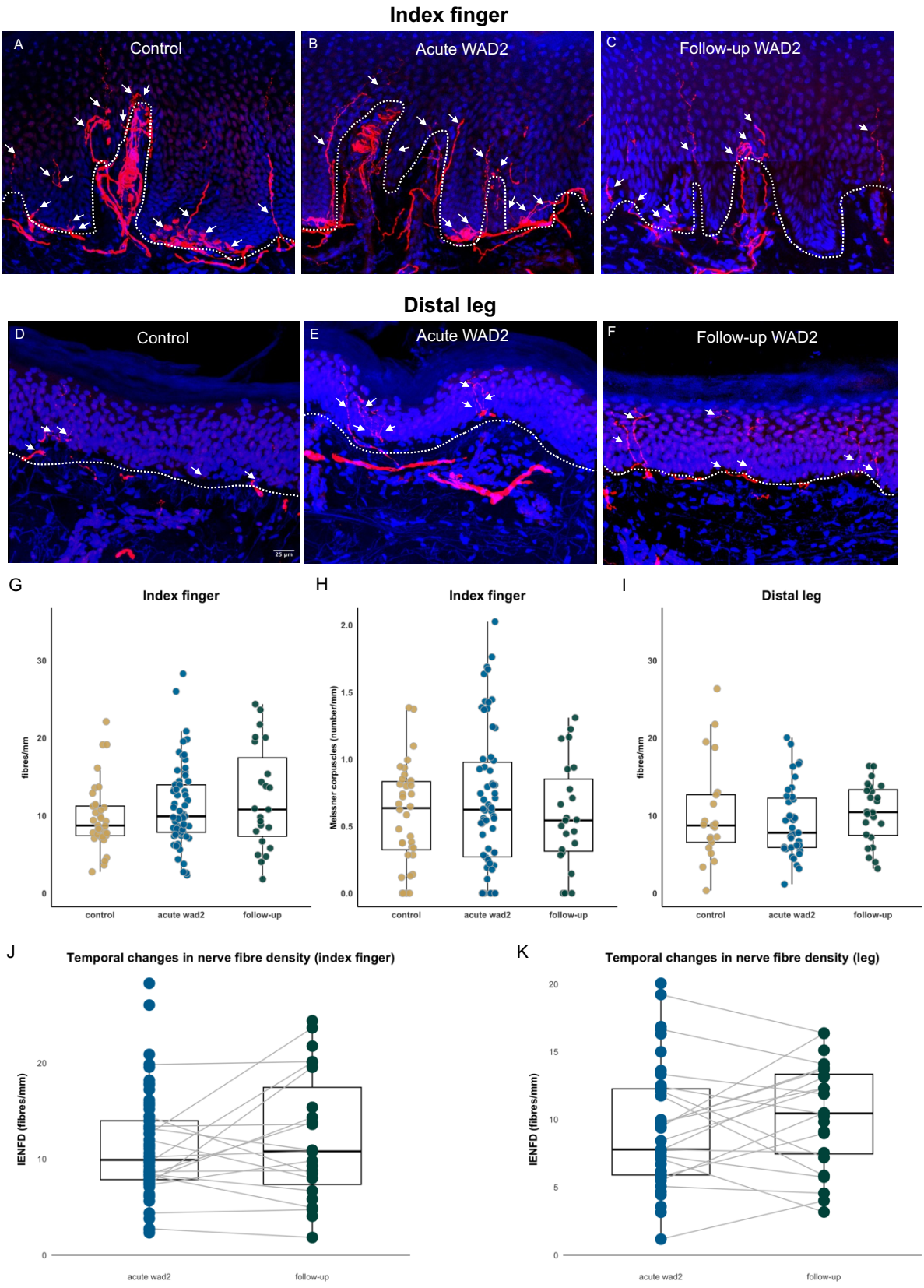


Figure 6.7. Intraepidermal nerve fibre densities at the index finger and leg are preserved at six-month follow-up.

(A-F) representative images of skin immunohistochemistry taken at the index finger and distal leg in a participant with acute WAD2, follow-up WAD2, and age- and sex-matched healthy control. Arrows

indicate epidermal small nerve fibres and the dashed line indicates the dermal-epidermal border. (G) Comparison of intraepidermal nerve fibre densities at the index finger in acute WAD2, follow-up WAD2, and controls (median/IQR). (H) Comparison of Meissner corpuscles at the index finger in acute WAD2, follow-up WAD2, and controls (median/IQR). (I). Comparison of intraepidermal nerve fibre densities at the distal leg in acute WAD2 and controls. (J-K) Paired intraepidermal nerve fibre densities for acute and follow-up WAD2 participants at the index finger and leg. Grey lines indicate paired acute and follow-up samples. Aligned ranks transformation ANOVA with Tukey's post hoc testing was used for all comparisons. Total index finger samples used for analysis includes n = 62Acute WAD2, n= 23 follow-up WAD2, n= 36 controls. Total leg samples used in analysis includes n= 39 acute WAD2, n= 23 follow-up WAD2, n= 21 controls. Paired acute follow-up samples for WAD2 participants includes N=17 at the index finger and N= 19 at the leg.
Abbreviations: IENFD: intraepidermal nerve fibre density; IQR: interquartile range.

6.3.9. Measures of nerve pathology are not prognostic in WAD2.

Overall, acute measures of nerve pathology, including NfL and bedside testing for small fibre loss of function at the index finger, are not prognostic for neck-related disability in acute WAD2 (Table 6.5). This includes acute absolute serum NfL concentrations (coefficient estimate: 0.01, standard error: 0.06, p=0.86, adjusted r^2= 0.25); acute age and BMI adjusted NFL z-scores (coefficient estimate: 0.36, standard error: 0.57, p=0.53, adjusted r^2= 0.25); and bedside tests for small fibre loss of function at the index finger (coefficient estimate: 0.40, standard error: 1.40, p=0.78, adjusted r^2= 0.25).

Similarly, acute measures of nerve pathology using NfL and bedside small fibre loss of function testing in acute WAD2 are not prognostic for whiplash-related symptoms six months after injury. This includes absolute acute serum NfL concentrations (coefficient estimate: 0.01, standard error: 0.25, p=0.97, adjusted r^2= 0.11); acute age and BMI adjusted NFL z-scores (coefficient estimate: -0.52, standard error: 2.31, p=0.82, adjusted r^2= 0.09); and bedside tests for small fibre loss of function at the

index finger (coefficient estimate: 7.67, standard error: 6.28, p=0.23, adjusted r^2= 0.10).

Table 6.5. Acute nerve pathology does not predict neck-related disability or whiplash-related symptoms at six-months, results of multivariable linear regression.

Neck-related disability				
Variable	**Coefficient estimate**	**Std Error**	**P-value**	**Overall model adjusted R^2 value**
Abs NfL concentrations	0.01	0.06	0.86	0.25
NfL z-scores	0.36	0.57	0.53	0.25
Clinical loss of function (index finger)	0.40	1.40	0.78	0.25
Whiplash-related symptoms				
Abs NfL concentrations	0.01	0.25	0.97	0.11
NfL z-scores	-0.52	2.31	0.82	0.09
Clinical loss of function (index finger)	7.67	6.28	0.23	0.10

Each multiple linear regression model included follow-up WAD2 Neck Disability Index scores as the dependent variable with NfL as the independent variable controlling for age, sex, and initial Neck Disability Index scores. N=60 acute WAD2 participants included in regression models.
Abbreviations: NfL: neurofilament light chain.

6.3.10. Trends of acute variables associated with persistent whiplash symptoms and neck-related disability six-months after injury.

I performed exploratory secondary analyses to investigate potential differences in variables related to persisting moderate-severe whiplash symptoms and neck-related disability using kernel density estimation. Acute WAD2 clinical variables with a higher density trend in moderate-severe whiplash symptoms at six-months include higher initial whiplash symptoms and neck-related disability, higher painDETECT and NeuPSIG neuropathic pain grading scores, and higher Pain Catastrophisation and Impact of Events Scale-Revised scores (Supplemental Figure 6.3). Acute QST

parameters with higher densities for moderate-severe whiplash symptoms at follow-up include higher heat pain thresholds and reduced mechanical detection thresholds (Supplemental Figure 6.4). No other QST parameters show clear trends towards persistent moderate-severe pain levels at follow-up. Lastly, there are trends for the use of any treatment for whiplash symptoms and physiotherapy treatment for more moderate-severe neck-related disability at six-month follow-up (Supplemental Figure 6.5). The use of ibuprofen/paracetamol or weak opioids acutely after injury also trends towards higher levels of neck-related disability at follow-up. However, clear trends between groups are difficult to distinguish.

6.3.11. RNA-sequencing of WAD2 blood samples six-months after injury reveals sex-dependent effects in persistent pain.

Six-month follow-up blood samples from a total of 30 WAD2 participants were used for RNA-sequencing analyses. There are 15 participants per group (minimal persistent pain and moderate/severe persistent pain) with equal distribution of sexes between groups (n = 7 females/group). All samples were assessed for RNA degradation and passed initial QC with a RIN > 8. Quality Control of the raw sequencing reads was performed using FastQC/MultiQC and assessed the yield (Mean = 28.2, SD = 5.75 million read pairs), the per sequence / per base Phred quality score show no reduction of the mean Phred quality at the read extremities and all samples have an average Phred > 35, the GC content, the length distribution and over-representing sequences or adapter contaminations. An over-representation

of adapter sequences was found so reads were trimmed for Illumina adapters using trimmomatic. The percentage of uniquely mapped reads is excellent (Median = 91.75, IQR = 1.8) and cook's distance analysis reveals no outliers.

There is only one significantly differentially expressed gene when comparing minimal pain and moderate/severe pain controlling for age group and gender. This is the pseudogene ENSG00000243495 (Table 6.6 and Supplemental Figure 6.6). Stratifying moderate/severe pain versus minimal pain between sexes and analysing males and females separately, while controlling for age, does show significantly differentially expressed genes (Figures 6.8A - 6.8B). In females, there are four significantly down-regulated genes in the moderate/severe pain group (SWT1, HLA-DQA1, HEBP1, NECTIN2) and one pseudogene is upregulated (TEKT4P2). There is only one significantly downregulated gene for moderate/severe pain versus minimal pain in males (MXRA7) and one significantly upregulated gene (HLA-G). Principal component analysis showed that most of the observed variance can be attributed to sex differences, rather than differences between moderate/severe and minimal pain. (Figure 6.8C).

Table 6.6. Differentially expressed genes in participants with moderate/severe pain versus minimal pain six-months after WAD2.

Differential gene expression (combined group)								
	Base Mean	log2Fold Change	Lfc SE	stat	P-value	P-adj	wt	symbol
ENSG00000243495	0.29	14.87	2.98	4.98	6.26^{E-07}	0.03	1	NA
Differential gene expression (females)								
ENSG00000116668	1444.90	-0.68	0.15	-4.48	7.59^{E-06}	0.06	1	SWT1
ENSG00000196735	942.10	-1.20	0.21	-5.78	7.48^{E-09}	0.00	1	HLA-DQA1
ENSG00000013583	278.00	-0.94	0.17	-5.51	3.53^{E-08}	0.00	1	HEBP1
ENSG00000130202	132.22	-2.43	0.52	-4.68	2.89^{E-06}	0.04	1	NECTIN2
ENSG00000188681	20.31	1.66	0.36	4.59	4.34^{E-06}	0.04	1	TEKT4P2
Differential gene expression (males)								
ENSG00000182534	144.38	-1.41	0.31	-4.61	4.07^{E-06}	0.09	1.48	MXRA7
ENSG00000204632	44.28	1.51	0.34	4.50	6.71^{E-06}	0.09	1.51	HLA-G

Abbreviations: lfc: log fold change; p-adj: adjusted p-value; wt: weight.

Correlations of log2 normalised gene expression, using the condition-aware regularised log transformation, and measures of whiplash symptoms, neck-related disability, and nerve pathology are shown in Table 6.7. HLA-DQA1 has a strong negative correlation with both whiplash-related symptoms (r = - 0.61, p = 0.02) and the likelihood of neuropathic pain using painDETECT (r = -0.62, p = 0.01). All other genes (SWT1, HEBP1, NECTIN2, MXRA7, HLA-G) do not demonstrate any significant correlations after strict comparisons for multiple testing.

Table 6.7. Correlation of log2 normalised gene expression and clinical measures of whiplash symptoms, disability, and nerve pathology at six-month follow-up.

	HLA-DQA1	SWT1	HEBP1	NECTIN2	MXRA7	HLA-G
NDI total score	-0.40 (0.79)	-0.51 (0.15)	-0.17 (1.0)	-0.29 (1.0)	0.04 (1.0)	0.39 (0.98)
Average pain (painDETECT)	-0.20 (1.00)	-0.28 (1.0)	-0.08 (1.0)	-0.08 (1.0)	0.03 (1.0)	0.45 (0.45)
PainDETECT total score	**-0.62 (0.01)**	-0.29 (1.0)	-0.35 (1.0)	-0.14 (1.0)	-0.04 (1.0)	0.26 (1.0)
Whiplash symptoms	**-0.61 (0.02)**	-0.54 (0.10)	-0.32 (1.0)	-0.33 (1.0)	-0.06 (1.0)	0.36 (1.0)
Neuropathic pain grading (NeuPSIG)	-0.54 (0.09)	-0.42 (0.61)	-0.23 (1.0)	-0.32 (1.0)	-0.05 (1.0)	0.03 (1.0)
CDT	0.18 (1.0)	0.05 (1.0)	0.02 (1.0)	0.10 (1.0)	-0.14 (1.0)	0.11 (1.0)
WDT	0.11 (1.0)	0.44 (0.54)	0.18 (1.0)	0.14 (1.0)	0.12 (1.0)	0.17 (1.0)
MDT	0.27 (1.0)	0.16 (1.0)	-0.09 (1.0)	-0.18 (1.0)	0.23 (1.0)	-0.17 (1.0)

Data are presented as Pearson correlation coefficients and adjusted p-values using Holm's method in brackets. Bolded text indicated statistical significance ($p < 0.05$). CDT, WDT, and MDT were taken from the index finger at six-month follow-up.

Abbreviations: NDI: Neck Disability Index, NeuPSIG: Neuropathic Pain Special Interest group of the International Association for the Study of Pain; CDT: cold detection thresholds; WDT: warm detection thresholds; MDT: mechanical detection thresholds.

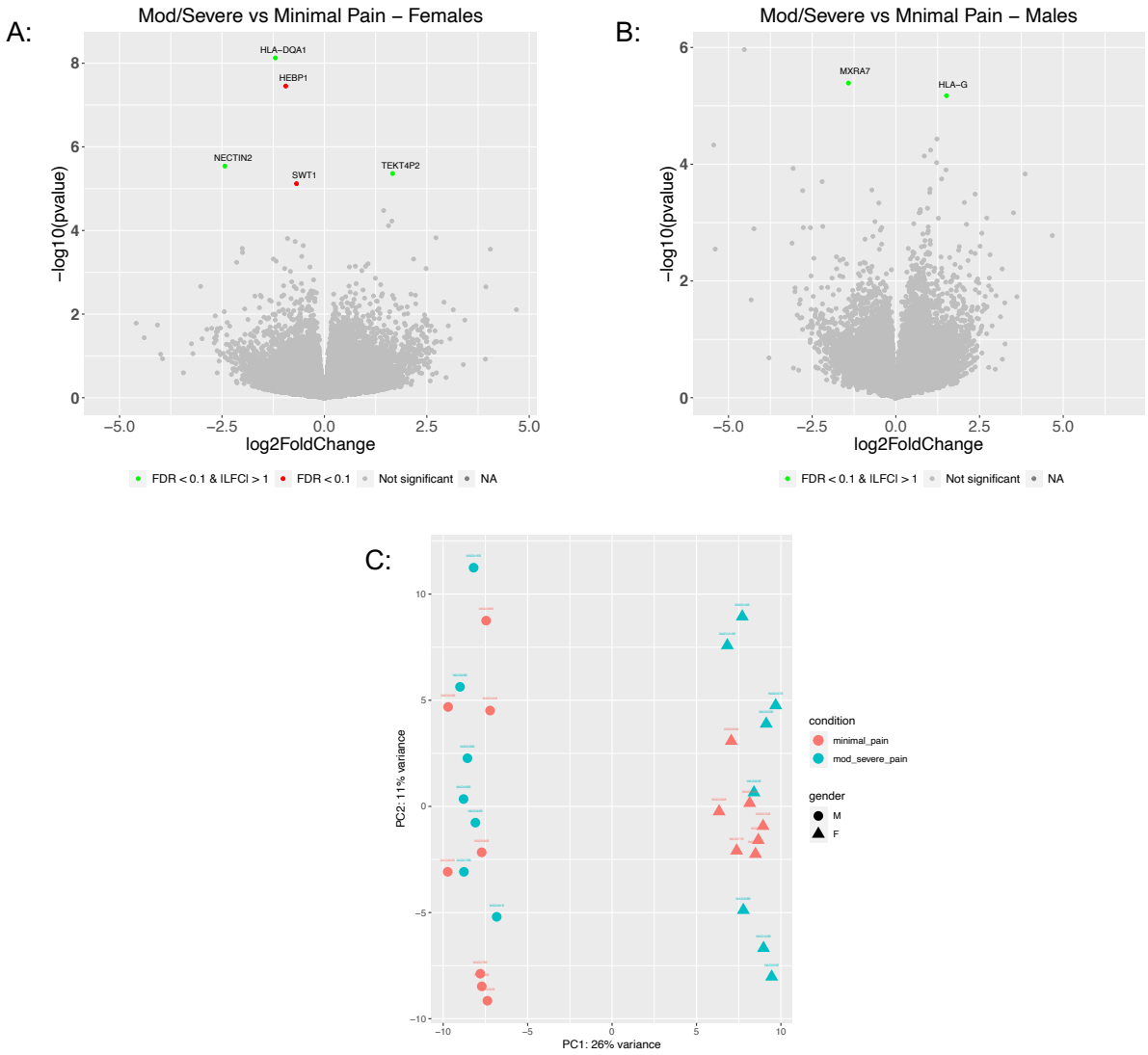


Figure 6.8. Differential gene expression and principal component analysis from RNA-sequencing of blood samples six-months after whiplash injury. (A-B) Differential expression analysis comparing moderate/severe pain to minimal pain in WAD2 participants separated by sex. (C) Principal component analysis plot separated by sex comparing moderate/severe pain versus minimal pain showing variance between principal components 1 and 2.
Abbreviations: PC: Principal component; FDR: false discovery rate; LFC: log fold change

6.4. Discussion

The main aims of this chapter were to assess the temporal development of nerve pathology and assess its prognostic role for neck-related disability and whiplash-related symptoms in WAD2. Acute signs of nerve pathology and neuropathic pain improved for most participants six-months after injury. There was however a subset of participants with WAD2 that presented with continued dysfunction. Neuropathic pain at follow-up ranged from 3% - 23% using the painDETECT and NeuPSIG grading scale, respectively. Similarly, there were indications of both functional and structural nerve pathology six-months after injury. Functional nerve pathology measured with clinical assessment and QST was the most prevalent pathological measure at follow-up (range 24% - 64%). Structural signs of nerve pathology ranged from 2% using serum NfL and 8.7% from skin biopsies.

The selected measures of nerve pathology did not appear prognostic for neck-related disability or whiplash-related symptoms in WAD2. Multivariable linear regression modelling suggests measures of acute nerve pathology were not prognostic in my WAD2 cohort. Exploratory analysis however suggested that higher levels of acute neuropathic pain, posttraumatic stress symptoms, and decreased mechanical detection thresholds may be related to non-recovery, though heterogeneity was present. A detailed molecular understanding of persistent symptoms was further analysed using RNA-sequencing from blood samples.

A secondary aim included exploring molecular mechanisms of moderate/severe persistent pain using RNA-sequencing. Transcriptional profiling in participants with moderate/severe pain versus minimal pain six-months after injury did not show any significant changes. Though, there were several significantly expressed genes that were apparent after stratifying groups by sex. Significantly dysregulated genes showed a sex-dependent pattern of immune system regulation, though multiple biological functions were implicated in the identified genes. The results of my RNA-sequencing analysis point towards sex-dependent biological mechanisms of persistent pain. While these results are intriguing, this preliminary analysis needs further exploration and validation.

The overall results of this chapter show improving but continued signs of neuropathic pain and nerve pathology six-months after whiplash injury. These findings warrant greater clarity. I will discuss each element in greater detail in the below sections.

Most WAD2 participants experience persistent symptoms six-months after injury.

Though whiplash-related symptoms showed signs of improvement for WAD2 participants six-months after injury, persistent symptoms continued for most participants. Thirty-seven percent reported continued moderate-severe whiplash-related symptoms and 68% reported persistent moderate-severe neck-related disability six-months after injury. Comparable to previous longitudinal WAD cohorts, recovery trajectories for neck-related disability revealed over half of my WAD2 cohort continued

to have at least moderate levels of neck-related disability disability[212,352]. Posttraumatic stress symptoms also remained elevated for a subgroup of my WAD2 participants at six-months, ranging from 23% - 36%. This aligns with previous longitudinal cohorts indicating persistent posttraumatic stress symptoms lasting months to years after whiplash injury[174,212,352,453].

Most participants (67%) continued to have whiplash-related symptoms six-months after injury. This included over one-third experiencing moderate-severe symptoms. Persistent neuropathic pain also continued to be present for 21% using the NeuPSIG grading criteria. As seen in Figure 6.2, only those with some level of acute neuropathic pain continued to have neuropathic symptoms at six-months. Equally, participants who did not present with acute neuropathic pain did not develop neuropathic pain at six months. Although no previous WAD cohorts have tracked neuropathic pain longitudinally, one study in participants with broader traumatic injuries showed very similar findings. Rosenbloom et al. show that 21.4% of participants with acute neuropathic pain after traumatic injury continued to have moderate-severe neuropathic pain at 4-month follow-up[454]. Similarly, I identified trends of higher levels of acute neuropathic pain with participants who continue to have moderate-severe pain at six-months. This is important, as neuropathic pain has greater psychological burden and requires different treatment strategies than non-neuropathic conditions[195].

The overall treatment patterns in my WAD2 cohort align with UK National Institutes of Health and Care Excellence guidelines and illustrate typical treatment patterns for WAD2[63]. Sixty-eight percent of my WAD2 cohort reported seeking treatment for their whiplash-related symptoms. This included physiotherapy (46% [23/50]) and osteopathic or chiropractic treatment (6% [3/50]). Additionally, 28% (14/50) reported continued medication use due to their whiplash injury, which predominantly included non-steroidal anti-inflammatories and paracetamol (28%, and 16%, respectively). No participants who attended a follow-up appointment reported continued use of a weak or strong opioid. Interestingly, only two follow-up participants reported using a first-line neuropathic pain medication (amitriptyline and citalopram)[84].

Though neuropathic pain was present in a subgroup of both acute and follow-up participants, current international treatment guidelines for whiplash do not include neuropathic pain medications[75,283]. Interestingly, my longitudinal data show neuropathic pain at follow-up was only present in WAD2 participants who had reports of at least possible neuropathic pain at the acute stage (using the NeuPSIG grading). WAD2 participants initially considered unlikely to have neuropathic pain did not present with neuropathic painlike symptoms six-months later. Thus, the temporal neuropathic pain profiles in my cohort suggest that early identification and treatment of neuropathic pain may be beneficial for some participants with WAD2. In fact, my acute data showed that only one out of 130 participants reported taking a first-line neuropathic pain medication acutely after whiplash injury (Chapter 5). Detailed

discussion of the clinical implications of neuropathic pain in WAD2 is included in Chapter 7. Overall, the assessment and identification of neuropathic pain in WAD2 may change future treatment strategies.

My exploratory analyses revealed higher follow-up neck-related disability for participants receiving treatment for their whiplash injury. This trend appeared similar for both conservative treatment (physiotherapy) as well as medication (nonsteroidal anti-inflammatories, paracetamol, or weak opioids). Though the observational nature of my cohort study makes it difficult to assess the effects of treatment on WAD2 recovery. The analysis of treatment trends in recovery only used data dichotomised by the reported history of treatment (e.g., yes or no statements to receiving physiotherapy). My study would have benefited from greater treatment details, including the amount, duration, and timelines of participants' whiplash treatments. Another caveat to my treatment data is that it is all based on participant self-report; detailed analysis of participants' treatment records would have given greater clarity to understanding the effects of their treatment plan on recovery.

Aetiological considerations for the identified nerve pathology in WAD2.

Signs of nerve pathology improved, but did not resolve, six-months after whiplash injury. Though I cannot definitively conclude the origin of the persistent nerve pathology, the results of my longitudinal deep phenotyping shed light on this question. Group level data of preserved intraepidermal nerve fibre densities in the index finger

and resolving NfL levels suggests an acute injury to the cervical dorsal roots or central nervous system is possible for a subgroup of WAD2 participants (as discussed in Chapter 5). These data point towards a preganglionic versus postganglionic injury for a subset of participants. My findings of widespread upper and lower extremity hypoaesthesia suggests potential injury to multiple nerve roots, spinal cord tracts, or the brain may occur for some WAD2 participants. Further contextualising my results with the temporal resolution of NfL suggests this is more likely to occur from cervical dorsal roots or spinal cord than a mild brain injury, as discussed in detail below. Taken together, my results suggest a subgroup of WAD2 participants experience a spectrum of nerve pathology resulting from their whiplash injury. The role of such nerve pathology in recovery currently remains less well understood.

NfL levels in acute WAD2 reduce by six-month follow-up timepoint.

The temporal pattern of NfL indicates an acute axonal injury is present for a subgroup of WAD2 and resolved by the six-month follow-up appointment. Although NfL levels cannot localise the site of nerve pathology, I will integrate my current findings with the literature to better understand the anatomical origins. As previously discussed in Chapter 5, a postganglionic peripheral nerve injury is unlikely to demonstrate upper and lower extremity sensory hypoaesthesia with preserved nerve fibre densities from skin biopsies. Thus, the two most likely causes of acutely elevated NfL levels in the presence of preserved nerve fibre densities in the index finger are from the cervical

dorsal roots or central nervous system. I will discuss both possibilities in the sections below.

Acutely elevated NfL in WAD2 is unlikely to result from TBI.

Levels of NfL in my WAD2 cohort were significantly reduced six months after injury. This reduction limits the likelihood it initially resulted from a TBI. Participants with TBI consistently show prolonged elevation of NfL levels from one to five years after injury[430-432]. These higher levels are seen regardless of TBI severity. A longitudinal study by Shahim et al. showed that serum NfL levels were increased in participants with mild, moderate, and severe TBI five years after injury compared to controls[430]. The prolonged elevation of NfL in TBI is thought to reflect ongoing neurodegenerative pathology[291]. Compared to my WAD2 cohort, serum NfL concentrations were higher even in mild TBI compared to whiplash participants (30 pg/mL in mild TBI vs 7 pg/mL in WAD2)[430]. I also did not find any significant correlations with acute levels of NfL in WAD2 and headache severity (Chapter 5), which I used as the most similar proxy parameter for TBI symptoms in my cohort. Data from my deeply phenotyped cohort, together with current evidence in TBI, does not strongly support the hypothesis for my acutely elevated levels of NfL to be from mild TBI.

Another recent deeply phenotyped cohort of participants with non-TBI traumatic injuries had increased plasma NfL levels compared to controls[291]. Plasma NfL measures were taken up to 10 days after their non-TBI injury and elevated compared to healthy

controls (median plasma NfL concentration for non-TBI injury ranged from 12 to 15 pg/mL)[291]. This study strictly controlled for any indications of even mild TBI, as well as 'any clinically apparent peripheral sensory or motor abnormalities'[291]. Vertebral fractures were present for some non-TBI trauma participants with the authors suggesting these findings may be originating from subtle spinal cord injury[291]. When considering my clinical phenotype, subtle nerve root or spinal cord pathology may be one explanation for the signs of nerve pathology in my cohort.

Spinal cord pathology may account for acutely elevated NfL in WAD2.

Widespread upper and lower extremity sensory hypoaesthesia, paired with preserved nerve fibre densities at the index finger, may indicate subtle spinal cord pathology in my WAD2 cohort. Indeed, the temporal profile of NfL levels in my cohort share similar patterns to longitudinal cohorts with spinal cord injury.

Though there is limited longitudinal data, two studies have assessed the temporal patterns of NfL in spinal cord injury more than one week after injury. Both studies show a progressive decrease in NfL over time with NfL peaking approximately 30 days after injury[455,456]. Both studies also reported NfL levels progressively increased with the severity of spinal cord injury[455,456]. It is difficult to directly compare NfL values between cohorts due to differences in blood sampling techniques (plasma vs serum) and analytical platforms. However, numerous studies indicate much greater absolute concentrations of acute NfL in frank spinal cord injury compared to my WAD2 cohort

(spinal cord injury NfL values ranging from 90 pg/mL to 8,000 pg/mL)[434,455,456]. Less severe injury to the spinal cord or cervical nerve roots may share greater similarities to the NfL levels in my WAD2 cohort.

Preservation of intraepidermal nerve fibre densities at the index finger remain six-months after injury.

As discussed in Chapter 5, the overall findings of acute nerve pathology are unlikely to arise from a postganglionic peripheral nerve injury. The preservation of both intraepidermal nerve fibres and Meissner corpuscles at the index finger six months after injury further supports this hypothesis. One previous study, by Farrell et al., showed a small decrease in intraepidermal nerve fibre densities at the index finger and less pronounced levels of hypoaesthesia at the index finger compared to my cohort[40] . As I discussed in Chapter 5, this contrast likely stems from the significant difference in our cohorts. The previous study only included participants with persistent pain and prolonged chronicity of symptoms (median symptom duration of five years). The preservation of nerve fibres within my WAD2 cohort may result from several factors, including the fact that my group-level comparisons included participants who had recovered, in addition to a subgroup with lower levels of persistent pain and neck-related disability compared to the cohort with only participants with persistent pain[40].

Acute injury proximal to cervical spinal ganglia is one explanation for the widespread upper extremity hypoaesthesia at both acute and follow-up time points. As I have

discussed in Chapters 1 and 5, preclinical and human cadaver studies suggest that spinal nerve roots are more susceptible to injury than peripheral spinal nerves[411,415,418,457]. However, another explanation for the preserved intraepidermal nerve fibre densities and somatosensory hypoaesthesia may be due to injury to the dorsal nerve root or spinal cord. Previous small cohort studies in WAD2 have identified potential spinal cord changes at the chronic stage of injury (1 – 4 years post-injury)[115,423,424]. A more subtle peripheral nerve injury primarily demonstrating functional neurological changes may also be possible. Future research assessing the role of central nervous system pathology in WAD could help further elucidate these findings.

Sensory hypoaesthesia continues for a subgroup of WAD2 six-months after injury.

My clinical neurological assessment and QST showed improvements but continued widespread upper extremity hypoaesthesia. Over one-third of participants presented with abnormal neurological assessments six-months after injury. Additionally, QST over the index finger demonstrated continued signs of hypoaesthesia. Aligned with my systematic review in WAD (Chapter 2), sensory loss of function affected both large and small nerve fibres and was present throughout the upper extremity[401]. Only one previous study assessed sensory hypoaesthesia longitudinally. This study, by Chien et al., reported acute hypoaesthesia to cold detection but normalisation for WAD2 participants three months after injury[17]. This study however did find reduced heat, electrical, and vibration detection thresholds at 6-months in WAD2 participants who did not recover[17].

The absence of sensory changes over the leg at group-level is also supported by a recent systematic review highlighting strong evidence that widespread hyperalgesia in the lower extremity does not occur at any timepoint in WAD[158]. Previous WAD studies, included in this review, highlight increased pressure pain thresholds at the leg are not apparent at group level, but occurred in a subset with worse recovery[158]. I found a similar pattern with the absence of group level differences for QST parameters at the leg, but a sign of both loss and gain of function in a minority of WAD2 follow-up participants (range 4% - 14%). Taken together, these findings suggest the presence of nerve pathology at the leg in a subgroup of WAD2 participants that are typically considered without such dysfunction.

QST indicated greater levels of upper extremity hypoaesthesia at follow-up compared to bedside neurological assessment, which may indicate the higher sensitivity of QST. Sensory profiling of clinical assessment and QST showed greater similarities in the acute phase (53.8% clinical assessment vs 66% QST). This may be due to the more pronounced deficits in the acute stage, along with greater acute large nerve fibre deficits, as apparent on QST profiles. The higher prevalence of hypoaesthesia from QST at six-month follow-up may result from the higher sensitivity of testing, as my clinical bedside tests had only moderate agreement and were not correlated with their corresponding QST parameters. Overall, this suggests that nearly two-thirds of participants with WAD2 (64%) show signs of continued somatosensory hypoaesthesia six-months after injury. A recent systematic review of QST in WAD found only one

study that tracked loss of function parameters in longitudinal cohorts[158]. Most studies have only focussed on signs of somatosensory hyperalgesia. Interestingly, my QST findings indicate sensory hypoaesthesia is the predominant neurological dysfunction (64% follow-ups) while hyperalgesia was present in only 43% of follow-ups (QST z-scores ±1.96). The extent and detail of such somatosensory hypoaesthesia has not before been reported and may help us to better understand underlying mechanisms contributing to persistent whiplash symptoms in a subgroup of participants. However, the implications of this dysfunction on recovery are incompletely understood.

Measures of acute nerve pathology do not appear prognostic for WAD2.

Acute measures of nerve pathology, including NfL and bedside small nerve fibre tests, were not prognostic for whiplash-related symptoms or disability at six-month follow up in my WAD2 cohort. As discussed in Chapter 1, the most consistent factors related to WAD prognosis found in the literature include high initial neck-related disability, negative expectations of recovery, signs of posttraumatic stress, cold hyperalgesia, and high frequency of acute healthcare utilisation[36,50]. However, little is currently known about the role of nerve pathology and its prognostic ability in WAD[36]. Previous systematic reviews of prognostic factors for WAD suggest a preliminary negative association with recovery and the presence of frank nerve lesion (WAD grade 3)[36,50].

My findings may indicate that more subtle signs of nerve pathology may not be prognostic. I selected NfL and bedside small fibre testing at the index finger as clinically

relevant and representative measures of nerve pathology. Their prognostic ability may be limited because of the heterogenous nature of my WAD2 cohort. This includes the fact that only a subgroup of my cohort presented with acute signs of nerve pathology (24.6% with hypoaesthesia at the index finger). Similarly, only 13% were classified as having significantly elevated acute NfL levels (z-score $>$ 1.5). This may limit the prognostic ability of these measures of nerve pathology my WAD2 cohort.

Numerous studies have shown NfL to be highly prognostic in many neurodegenerative conditions of the central nervous system (e.g., multiple sclerosis[290], Alzheimer's disease[390], amyotrophic lateral sclerosis[334]). NfL has also been shown to be prognostic in cohorts with traumatic injury, such as TBI[291,389]. Both neurodegenerative and TBI cohorts typically include much more pronounced nerve pathology than identified in my cohort. Although NfL was not prognostic using strict multivariable linear regression, my exploratory analyses did indicate variables of nerve pathology to be related to moderate-severe pain at six-month follow-up (e.g., reduced QST detection, higher likelihood of neuropathic pain). These exploratory findings highlight the need for continued evaluation of acute prognostic factors in WAD2.

The varied clinical phenotypes of whiplash injuries contribute to the challenge in identifying robust prognostic factors for the past decades[36,50,458]. The emphasis on understanding acute prognostic factors stems from the significant percent of participants who experience persistent symptoms (approximately 50%). Trajectory

analyses from previous longitudinal studies in WAD suggests recovery typically occurs within the first 2-3 months following injury[22,459]. Signs of at least partial recovery were seen for all participants, regardless of their injury severity (mild, moderate, or chronic severe)[22]. Thus, the initial acute phase of injury is likely an important time to aid in participants' recovery.

Although many studies have assessed prognostic factors related to WAD, few have been externally validated[36,48]. One of the most extensively studied tools, known as WhipPredict, is one of few prognostic tools to be externally validated in WAD[34]. This clinical prediction tool has shown high accuracy, reproducibility, and simple clinical use[34]. WhipPredict uses known factors of poor prognosis in WAD (initial neck-related disability, age, sex, and posttraumatic stress hyperarousal) and was created for clinical use. The WhipPredict was recently used in a high-quality stratified randomised controlled trial, which did not appear more effective than usual care[68]. These findings, paired with my prognostic data, highlight our currently limited understanding of prognosis, recovery and, best ways of treatment stratification in whiplash.

RNA-sequencing reveals sex-dependent transcriptional responses associated with the severity of persistent pain in WAD2.

Although prognostic factors can help us determine a participant's clinical endpoint, they do not indicate mechanisms of recovery. To address this gap in our understanding, I performed RNA-sequencing to analyse differences in gene expression between minimal

and severe whiplash-related symptoms. The results from my RNA-sequencing represent a preliminary analysis of mechanisms related to persistent pain in WAD2. While these findings provide an essential initial step, it is important to emphasise further validation is required. Despite the exploratory nature, my current results offer intriguing initial insights.

While only one differentially expressed pseudo-gene was identified at initial group-level comparison, there were significant differences in moderate/severe pain versus minimal pain when stratified by sex. This is not necessarily an unexpected finding, as females have consistently shown to have worse prognosis and disproportionality worse outcomes in WAD[36,50]. Yet, the cause for the identified sex differences in recovery remains unknown. One debated hypothesis for this finding is that females with poor recovery after whiplash trauma have greater psychosocial contributions to persistent pain. This however has not shown to be true. A large cohort study (N = 740) showed no difference in coping strategies and neck pain in different sexes with WAD[460]. Another study assessed a range of psychosocial variables in people with chronic WAD, including depression, fear, and personality traits, and did not find a gender difference[461]. These findings suggest psychosocial factors after whiplash are not sex specific. Nevertheless, the question remains as to why females are less likely to recover.

Though studies assessing molecular mechanisms in WAD are limited, previous work by Linnstaedt et al. have shown sex-dependent differences acutely after injury. In a large,

prospective observational cohort (N = 948) assessed within 24 hours after injury, a moderate effect between the μ-opioid receptor gene (OPRM1, A118G allele) and pain levels were identified from DNA in blood samples in females at six-week follow-up[462]. This study also demonstrated that the presence of the G allele had contrasting effects for sex. Women with a G allele and distress had less pain while men with a G allele had increased pain six weeks after injury. Another study by the same research group showed sex-dependent changes in microRNA-19b on persistent pain related to circadian function[55]. This study suggests that acute expression of microRNA-19b had an inverse relationship between sexes in the development of persistent pain six-months after injury[55]. These studies shed light on initial understanding of sex-based differences in recovery, but further validation is needed to confirm these findings for persistent pain in WAD.

There is increasing awareness of sex-based differences in the pathophysiology of persistent pain. Persistent pain is more prevalent in females when taken from multiple persistent pain conditions[463,464]. Genetic studies of participants with chronic pain have also shown sex-dependent differences in molecular pathways related to recovery[463,465]. One such sex-dependent genetic association with persistent pain is related to the neuroimmune system[464,466,467]. The results of my current RNA-sequencing analysis also demonstrate significant variation in sex-dependent immune dysfunction in WAD2-related persistent pain. The small number of dysregulated genes in my analysis limited the use of gene ontology enrichment to assess biological function. Consequently, I will

discuss each significantly dysregulated gene, with the exclusion of the pseudogene TEKT4P2.

Human leukocyte antigen (HLA)

The two most dysregulated genes in both females and males were in the HLA system. Interestingly, females with moderate/severe persistent pain showed a downregulation in HLA-DQA1 while males who recovered showed an upregulation in HLA-G. The HLA complex, located on chromosome six, includes a diverse range of functions and includes over 200 genes[468]. Both identified HLA genes in WAD2 are related to the immune system; HLA-G is considered a major histocompatibility complex class I and HLA-DQA1 is considered a class II molecule. HLA-G has been shown to be pro-inflammatory and associated with tissue remodelling[469].

The significant increase in HLA-G in males may suggest a more robust inflammatory process possibly facilitating recovery for males with minimal pain in my cohort. Though not directly related to HLA-G, a recent study using similar inclusion and RNA-sequencing methodologies of participants with persistent pain after WAD suggests a potentially similar pattern of protective inflammatory response six-months after whiplash injury. This study, led by Parisien et al., found only one dysregulated gene (an increase in SAMD15 mRNA expression) in participants with minimal pain six-months after whiplash injury[470]. They then validated these findings using the UK Biobank linking higher SAMD15 levels to decreased risk for chronic pain. Another

comparable study of transcriptional profiling from blood in participants with low back pain suggests that an increased acute inflammatory response may be protective against developing chronic pain three months after injury[168]. Although neither study performed sex-stratified analyses, these findings may highlight the protective role of a pro-inflammatory response after whiplash injury that may differ depending on sex.

The downregulation of HLA-DQA1 in females with moderate/severe persistent pain presents the opposite biological perspective. HLA-DQA1 is a class II molecule expressed by a number of immune cells, including activated T cells, B cells, dendritic cells, and macrophages[471]. One consequence of low HLA-DQA1 expression levels is lower levels of protective regulatory T cells[472] and a possible blunted immune response after injury. Although HLA-DQA1 expression is not sufficiently understood, a systematic review and meta-analysis of genetic association studies suggests that HLA-DQB1 (which forms a function unit with HLA-DQA1[468]) was associated with a significantly increased risk for developing neuropathic pain[473]. Interestingly, I also identified significant moderate correlations with HLA-DQA1 and whiplash symptoms and the likelihood of neuropathic pain at six-month follow-up. Together, this data highlight interesting preliminary support for the role of HLA-DQA1 and persistent neuropathic pain.

Mutations in the HLA-DQ system have also been associated with neurologic consequence. This has been related to peripheral neuropathy with gluten sensitivity[474],

sporadic cerebellar ataxia in gluten sensitivity[475], and diabetic peripheral neuropathy[476]. These preliminary associations do not provide causal evidence, however, are interesting in combination with the findings of neuropathic pain and nerve pathology in my WAD2 cohort. Additional transcriptional changes may help shed light on the involvement of molecular mechanisms affecting the nervous system.

Nectin Cell Adhesion Molecule 2 (NECTIN-2)

NECTIN-2, which is widely expressed on neuronal and non-neuronal cells, was downregulated in females with moderate/severe persistent pain. NECTIN-2 encodes proteins associated with T cell signalling[477] and cellular adhesion[478]. Recent reports have indicated the neuronal functions of Nectin-2 to include astrocyte homeostasis and synapse formation in neurons in the central nervous sytem[479]. A study using an epigenome wide analysis of the prefrontal cortex in mice after spared nerve injury showed decreased Nectin-2 expression six and 12 months after injury[480]. Nectin-2 deficit mice showed significant neurodegenerative effects in the brain, suggesting acute pain may result in epigenetic changes in the prefrontal cortex of mice. These preclinical findings, in combination with my clinical data, highlight a possible role of NECTIN-2 in the maintenance of persistence pain after whiplash injury.

Heme Binding Protein 1 (HEBP1)

Another gene shown to be downregulated in females with moderate/severe persistent pain is HEBP1. It is hypothesised that the HEBP1 protein functions primarily to bind

heme but may also involve synthesis of porphyrin[481,482]. Neurovascular implications of hebp1 have been recently assessed with the identification of hebp1 expression in neurons. However conflicting results between its role in the central versus peripheral nervous system have been reported. Increased levels of Hebp1 have been shown as an early marker in a mouse model of Alzheimer's Disease[483]. This study further elucidated the role of hebp1 in causing cellular toxicity and heme-induced cell death[483]. These findings however are opposite to a study assessing the role of hebp1 in mouse models of peripheral nerve injury. Using a peripheral cavernous injury model reflective of prostate cancer, this study suggested increased expression of Hebp1 was associated with increased peripheral neurovascular regeneration in mice after injury[484].

Although the role of HEBP1 in human injury models is not well understood, the downregulation in females with moderate/persistent pain may be relevant to inflammation or neurovascular components. Additional understanding is needed to better interpret the role of HEBP1 in persistent whiplash symptoms.

SWT1 RNA Endoribonuclease Homolog (SWT1)

There is currently limited biological understanding of the final two dysregulated genes. SWT1, which was downregulated in females with moderate/severe persistent pain, is a protein encoding gene within the nucleus. The expression of SWT1 in the immune cell types is thought to have higher immune cell type basophil expression. The non-

specific function within the nucleus may be one explanation for the range of findings identified for this gene.

Matrix Remodelling Associated 7 (MXRA7)

Lastly, MXRA7 was significantly downregulated in males who did not recover. MXRA7 is thought to be involved in cellular adhesion and matrix remodelling[485]. Though incompletely understood, reduced levels of Mxra7 have recently been associated with impaired wound healing in mice[486]. Reduced expression in males with moderate/severe persistent pain after whiplash injury continue to underscore towards sex-dependent factors related to persistent whiplash symptoms.

Protein-level changes in inflammatory profiles of persistent pain in WAD.

Limited research has been done assessing the role of the immune system in persistent pain after WAD, but preliminary findings from a small longitudinal cohort study provide insight into altered profiles of inflammatory proteins. This study measured protein levels of TNF-α, IL-1B, and c-reactive protein levels within three weeks of whiplash injury and again three months later[51]. Notably, there were significant differences in protein expression levels and temporal patterns among participants with varying levels of neck-related disability three-months after injury. Participants with lower disability levels demonstrated higher levels of TNF-α acutely and three-months after injury compared to the WAD group with higher disability and healthy controls[51]. Higher levels of c-reactive protein three-months after injury were only identified in the

higher disability group compared to low disability and controls. Integrating these protein-level findings with my mRNA expression data suggests a potential role of immune dysfunction in the recovery from WAD, which may occur in a sex-dependent manner.

Limitations

There are limitations to consider in this chapter. First, the statistical comparisons for intraepidermal nerve fibre densities (finger and leg) and QST at the leg were not fully powered, as highlighted in Chapter 5. The number of follow-up skin biopsies were limited by participants declining a follow-up biopsy, as well as technical error while obtaining the biopsy (i.e., insufficient depth of skin biopsies preventing visualisation of dermal/epidermal border).

Eighteen healthy control index finger skin biopsies were collected and counted by another researcher (AS). As described in my methods in Chapter 4, I identified excellent inter-rater reliability in counting of intraepidermal nerve fibres between myself and AS. However, this combined cohort may have contributed to larger variance in nerve fibre densities within my cohort. Additional leg biopsies would have also aided in the understanding of the cause of the subgroup with somatosensory hypoaesthesia at the leg.

Another limitation is the incomplete understanding of participants' treatments throughout the study. Obtaining greater detailed information about participants treatments would have provided additional understanding about their recovery. In retrospect, I would have collected details regarding the duration, amount, and reported response to treatments throughout their injury. These treatment details could have allowed for greater understanding of recovery pathways.

I would also include greater details related to whiplash-related symptoms using follow-up questionnaires. Though painDETECT questionnaires were included for all follow-ups, we only received detailed pain phenotypic information for those that attended an in-person session. To address this limitation, I would have included a full body diagram of symptoms as well as detailed questions about whiplash-related symptoms instead of only pain rating scale, which may not be specifically related to whiplash symptoms. These additional insights could have improved our understanding of recovery from whiplash-related symptoms.

6.5. Conclusion

While many participants showed improvements over time, persistent symptoms were present in most WAD2 participants six-months after injury. This included 37.5% with moderate-severe whiplash-related symptoms and 68% reporting moderate-severe neck-related disability. Neuropathic pain continued to be experienced by 3% - 23% of participants. Nerve pathology was also present for a subgroup of follow-up WAD2

participants, including functional hypoaesthesia (range 24% – 64%) and structural changes (range 2% - 8.7%). Acute measures of NfL and bedsides tests of small nerve fibre pathology were not prognostic of neck-related disability or whiplash-related symptoms in my WAD2 cohort. Intriguingly, blood samples from participants with persistent moderate-severe whiplash symptoms revealed sex-dependent downregulation in immune-related genes compared to participants with minimal persistent pain. Overall, my findings help advance our understanding of the pathomechanisms and recovery trajectories for participants with WAD2.

Supplemental Table 6.1. One-way ANOVA results assessing QST parameters between controls, acute WAD2, and follow-up WAD2.

	Acute vs follow-up	Acute vs HC	Follow-up vs HC
CDT	0.23	**P < 0.001**	0.12
WDT	0.54	**P < 0.001**	**0.048**
TSL	0.88	**P < 0.0001**	**P < 0.001**
CPT	0.70	0.39	0.18
HPT	0.12	0.90	0.33
MDT	0.99	**P < 0.0001**	**P < 0.0001**
MPT	0.79	0.22	0.70
MPS	0.55	0.99	0.63
WUR	0.65	0.62	0.99
VDT	0.09	**P < 0.01**	0.84
PPT	**P < 0.001**	0.80	**P < 0.001**
CDT – leg	0.65	0.14	0.60
WDT – leg	0.97	0.33	0.35
PPT – leg	0.21	0.91	0.23

Data are presented as adjusted p-values from a one-way ANOVA with Tukey's Honest Significant Difference post hoc testing. Bolded text indicates statistically significant (set at $p < 0.05$).
Abbreviations: CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio.

Supplemental Table 6.2. Means and standard deviations for each QST parameter by group.

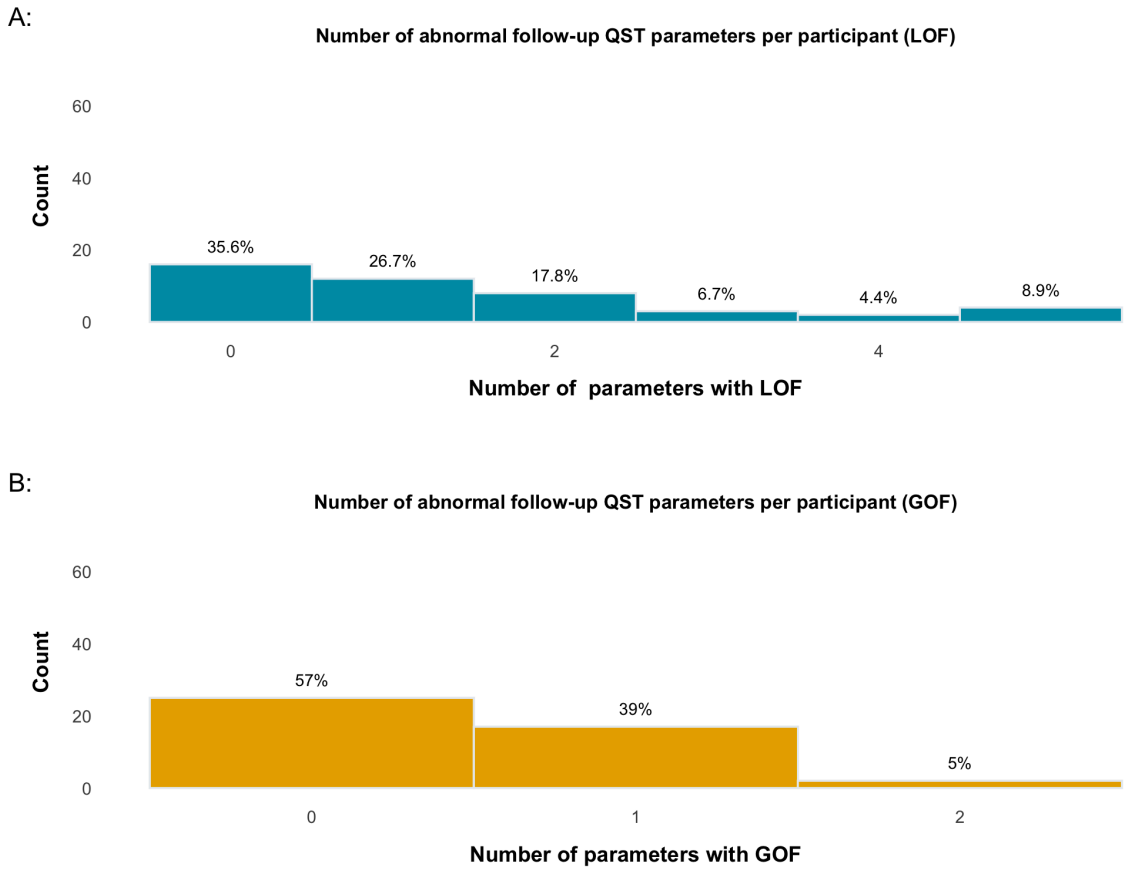
	Acute	Controls	Follow-up
Mean CDT	-2.090	0.000	-1.213
Sd CDT	4.047	0.957	2.339
Mean WDT	-0.982	-0.024	-0.712
Sd WDT	1.694	0.957	1.621
Mean TSL	-0.976	0.000	-0.881
Sd TSL	1.322	0.957	1.025
Mean CPT	0.222	0.000	0.368
Sd CPT	1.140	0.957	1.115
Mean HPT	-0.076	0.000	0.311
Sd HPT	1.007	0.957	1.559
Mean MDT	-1.366	0.000	-1.339
Sd MDT	1.742	0.957	1.602
Mean MPT	0.357	0.000	0.210
Sd MPT	1.535	0.957	1.334
Mean MPS	0.133	0.136	-0.125
Sd MPS	1.486	1.000	1.914
Mean WUR	0.110	-0.036	-0.043
Sd WUR	0.977	0.960	1.080
Mean VDT	-4.154	0.000	-0.961
Sd VDT	11.969	0.966	4.830
Mean PPT	-0.146	0.000	-1.217
Sd PPT	1.723	0.957	1.212
Mean CDT (leg)	-1.254	-0.036	-0.750
Sd CDT (leg)	3.808	0.953	2.749
Mean WDT (leg)	-0.391	0.000	-0.446
Sd WDT (leg)	1.405	0.953	1.691
Mean PPT (leg)	-0.105	0.000	-0.491
Sd PPT (leg)	1.439	0.953	1.277

Abbreviations: CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio; Sd: standard deviation.

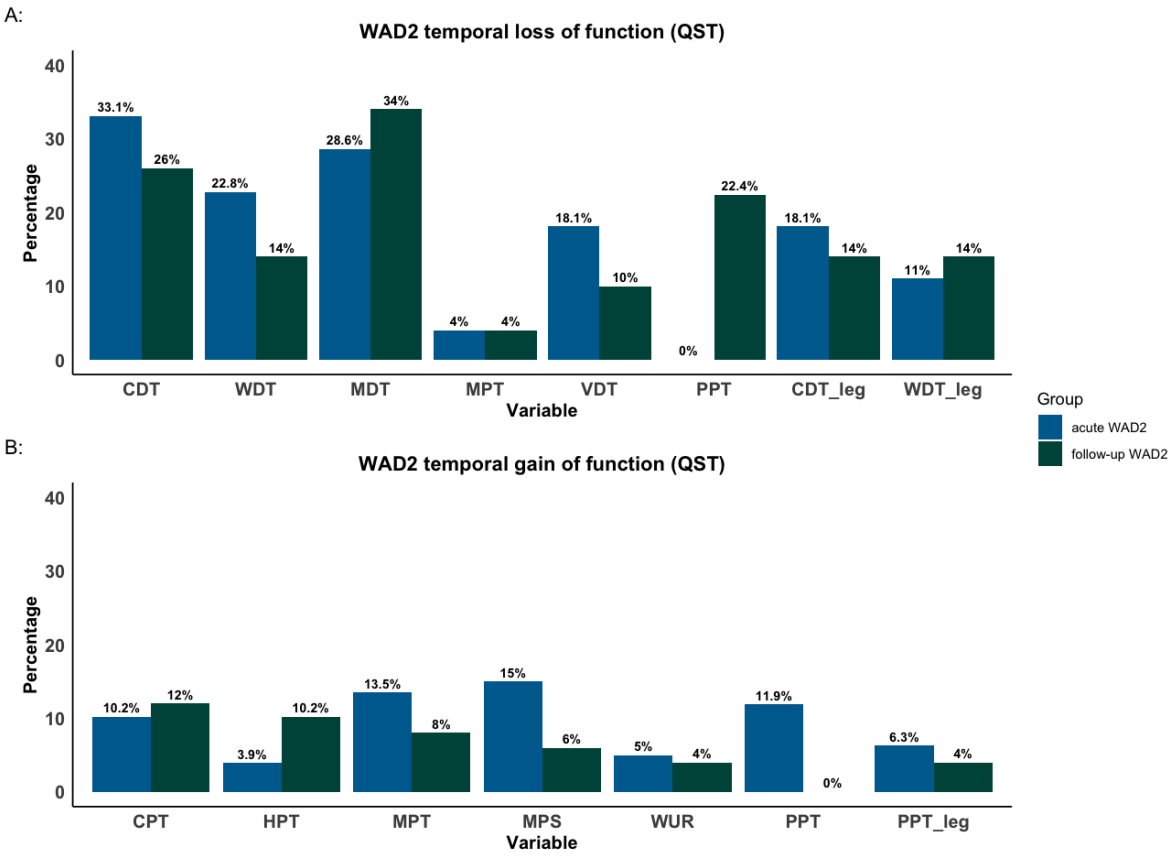
Supplemental Table 6.3. Number of acute WAD2 participants with abnormal intraepidermal nerve fibre densities at the index finger based on healthy control estimates per age decade.

Prevalence of small fibre pathology from skin biopsies at index finger		
	Acute	Follow-up
TOTAL	1.6% (1/62)	6.1% (2/23)

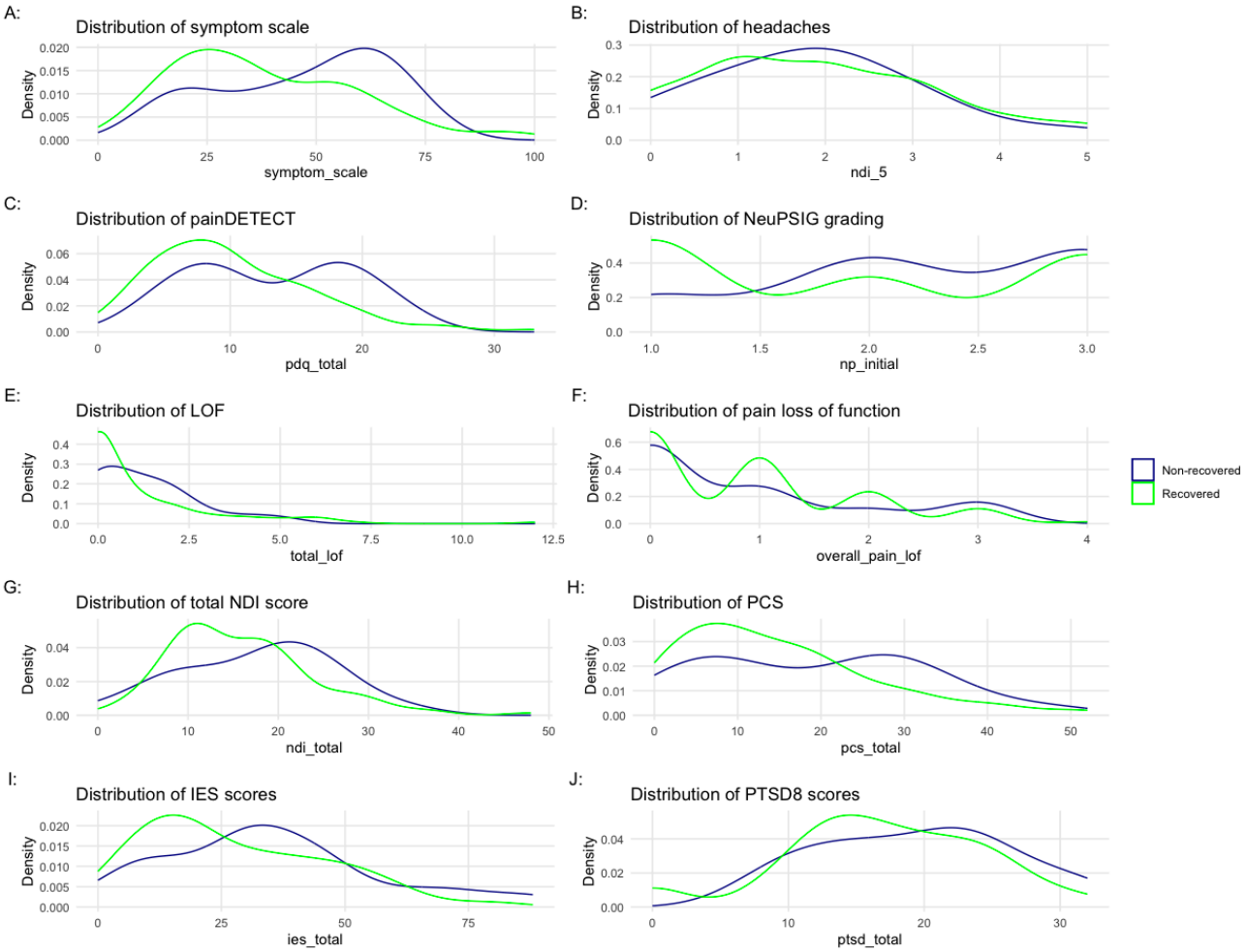
Linear regression including healthy control participants' age and index finger intraepidermal nerve fibre densities was calculated. Corresponding linear regression in Supplemental Figure 6.5 (regression equation: Y = -0.1191*X + 14.72). Derived from N= 36 healthy control participants.



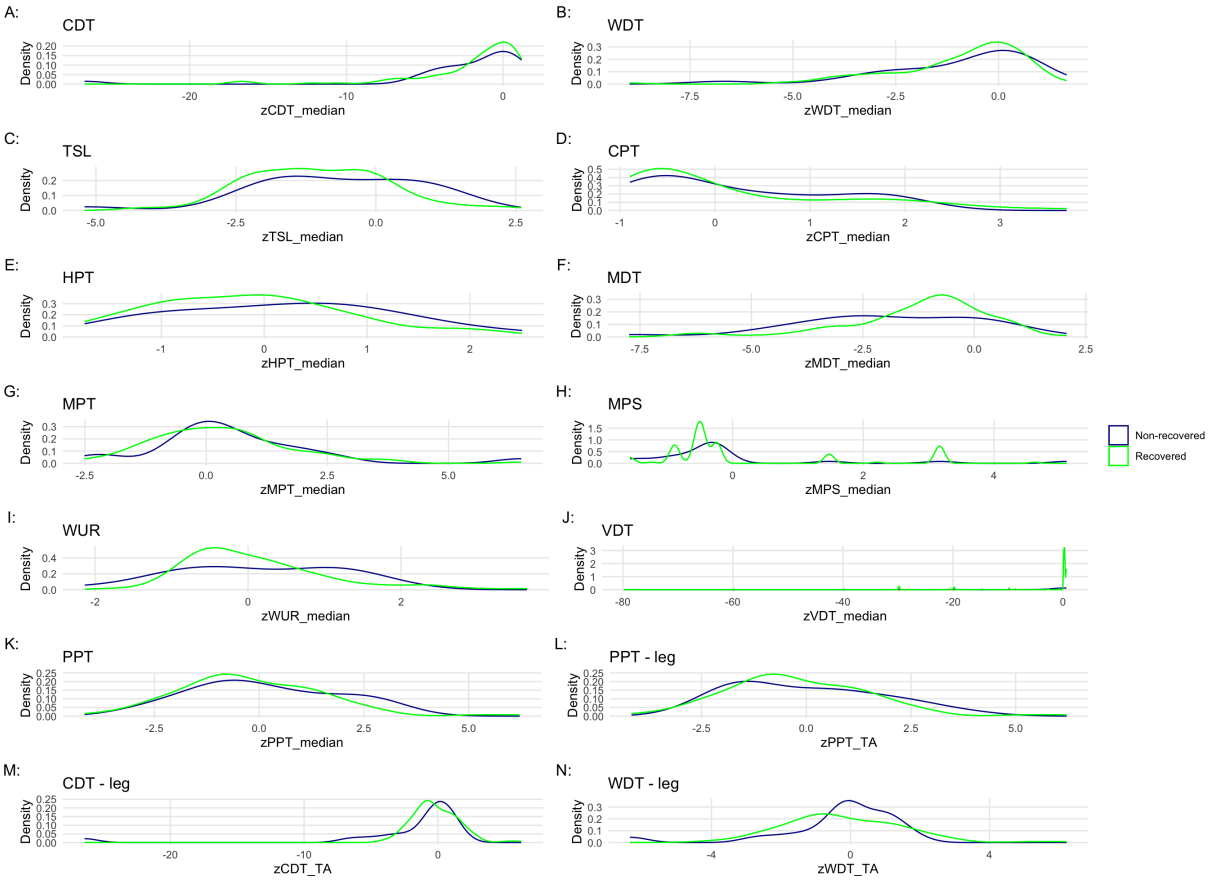
Supplemental Figure 6.1. Both loss and gain of function continue for WAD2 participants six-months after injury. Number of QST parameters outside of z-scores ± 1.96 for (A) loss of function and (B) gain of function. QST loss of function parameters (z-scores < -1.96) included: CDT, WDT, TSL, MDT, MPT, VDT, CDT-leg, WDT-leg). QST gain of function parameters (z-scores > 1.96) included: CPT, HPT, MPT, MPS, WUR, PPT, PPT-leg.
Abbreviations: LOF: loss of function; GOF: gain of function; CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio.



Supplemental Figure 6.2. Percentage of WAD2 participants with QST z-scores outside of standard range (z-score = ± 1.96). (A) indicates temporal changes in loss of function measures. PPT is included in both loss and gain of function due to the presence of hypoaesthesia to PPT over the hand at six-month follow-up in a subgroup of participants. (B) indicates temporal changes in gain of function measures at both acute and follow-up timepoints.
Abbreviations: CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio.

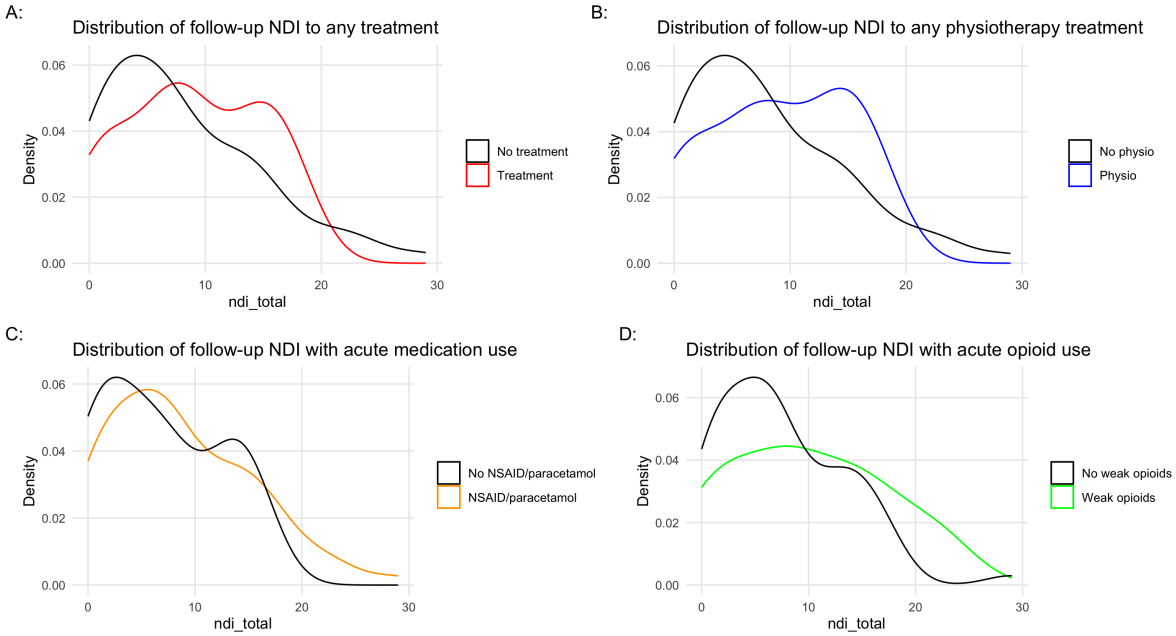


Supplemental Figure 6.3. Kernel density estimation plots showing the distribution of acute clinical variables for participants with WAD2 dichotomised into minimal vs moderate-severe whiplash symptoms (minimal symptoms = <30/100 symptom VAS; moderate-severe ≥30/100).
Abbreviations: PCS: Pain Catastrophisation Scale, PTSD: posttraumatic stress disorder, IES: Impact of Events Scale-Revised. NDI: Neck Disability Index; LOF: loss of function; NP: neuropathic pain; PDQ: painDETECT.



Supplemental Figure 6.4. Kernel density estimation plots showing the distribution of acute QST parameters for participants with WAD2 dichotomised into minimal vs moderate-severe whiplash symptoms (minimal symptoms = <30/100 symptom VAS; moderate-severe ≥30/100). NDI was selected as it was also the primary endpoint used in the multivariable linear regression analysis assessing prognosis.

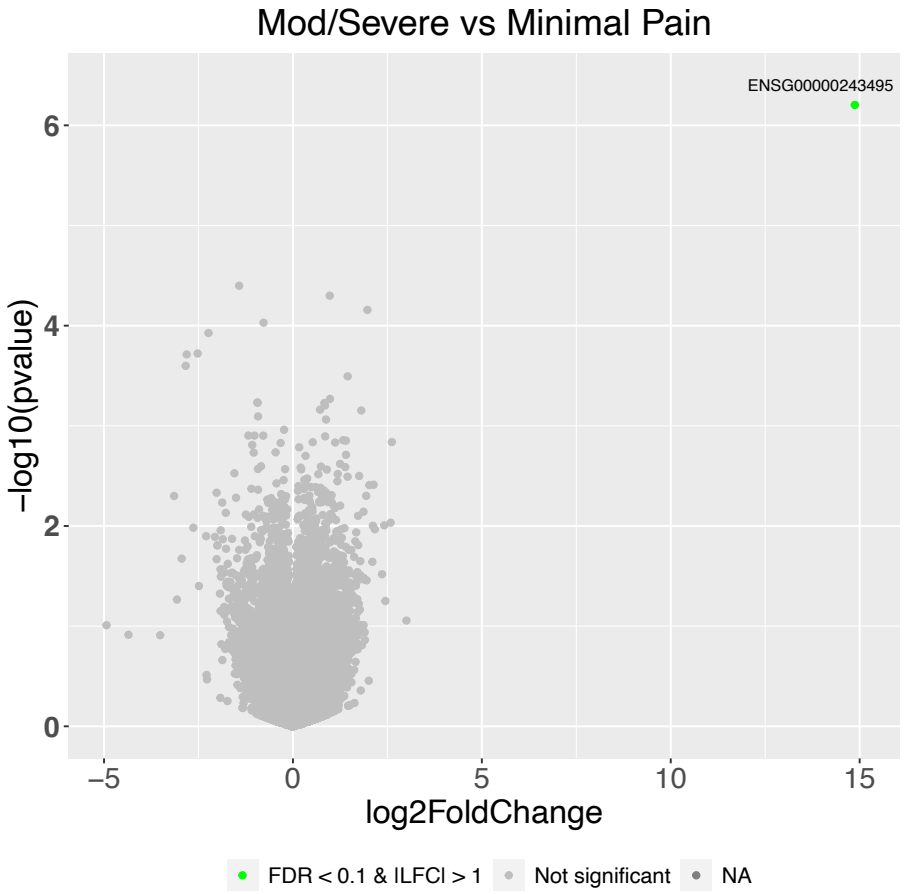
Abbreviations: CDT: cold detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warm detection threshold; WUR: wind-up ratio.



Supplemental Figure 6.5. Kernel density estimation plots showing the distribution of different treatment responses to follow-up NDI scores in WAD2 participants Group based on participants who did or did not receive (A) any treatment, (B) physiotherapy, (C) NSAID/paracetamol, or (D) weak opioids throughout the course of the study.

The NDI was selected for treatment response as it had the greatest amount of follow-up data (N=81 follow-up WAD2 participants). Categories of any treatment and physiotherapy was taken from participants who attended follow-up appointments due to small percentage having received physiotherapy by the time of initial appointment (n=56 total Follow-up WAD2). Acute medication use (panels C and D) was calculated from acute use of medication at baseline appointment. All treatment data are from WAD2 participants' subjective reports. Weak opioids included: tramadol (n=2), codeine, co-codamol, co-dydramol, or co-proxamol.

Abbreviations: NDI: Neck Disability Index; NSAID: non-steroidal anti-inflammatory; Physio: physiotherapy.



Supplemental Figure 6.6. Differential gene expression from RNA-sequencing of blood samples six-months after whiplash injury. Differential expression analysis comparing moderate/severe whiplash-related symptoms to minimal whiplash-related symptoms in all WAD2 participants (N=30 total follow-up WAD2 blood samples).

7
Discussion

7.1. Summary of main findings

I sought to answer two primary questions in my thesis:

1. *What is the temporal pattern of nerve pathology in participants with WAD2?*

2. *Are acute measures of nerve pathology prognostic?*

As background for my primary cohort study, I have completed two systematic reviews with meta-analyses. My first systematic review summarised our current understanding of the literature regarding measures of nerve pathology and neuropathic pain in people with WAD. My second systematic review aggregated the findings of blood-based markers of nerve pathology in patients with peripheral neuropathy. The aim of my second review was to identify the most promising blood-based marker to measure nerve pathology in my longitudinal cohort of patients following whiplash injury.

Systematic review and meta-analysis of nerve pathology and neuropathic pain in WAD.

I identified clinical questionnaires for symptoms suggestive of neuropathic pain in 34% of people with WAD (range 25% - 75%). The mean prevalence of nerve

pathology was 13% (range 0% - 100%) using neurological examination and 32% (range 10% - 100%) from electrodiagnostic testing. Participants independent of WAD severity (Quebec Task Force grades I - IV) demonstrated significantly impaired sensory detection thresholds over the index finger compared with controls, including mechanical, current, and thermal detection. Participants with WAD had significantly heightened nerve mechanosensitivity compared with controls on median nerve pressure pain thresholds and neurodynamic tests.

Systematic review of blood-based biomarkers in peripheral neuropathies.

I identified sixteen different blood-based biomarkers associated with nerve involvement. The most studied biomarker was NfL, which demonstrated significantly higher levels compared with controls. Neurofilament heavy chain, transmembrane protease serine 5, and nerve growth factor were also significantly dysregulated. There were no significant differences in levels of S100B, brain-derived neurotrophic factor, or neuron-specific enolase in participants with peripheral neuropathy compared with control participants.

Longitudinal cohort study

My primary DPhil project included a longitudinal cohort of participants with WAD2. This study built upon recent findings of chronic nerve pathology and answered novel objectives by assessing the temporal development of nerve pathology in WAD2 and its ability to predict neck-related disability. I measured nerve

pathology in 4 primary ways: 1) clinical bedside neurological assessments; 2) quantitative sensory testing; 3) serum concentrations of NfL; and 4) intraepidermal nerve fibre densities using histological staining from skin biopsies.

To better understand participants' recovery, I assessed the prognostic role of the acute measures of nerve pathology. I also used participants' blood samples to identify a molecular signature associated with recovery from injury. An overview of my main cohort findings is summarised below.

Temporal profiles of nerve pathology and neuropathic pain in WAD2

A substantial portion of participants with WAD2 had signs of neuropathic pain and nerve pathology after injury (Figure 7.1). Neuropathic pain was present in over one-third of acute participants and improved for many participants six-months after injury (35.4% acutely to 24% at follow-up using NeuPSIG grading system). An even larger subset of WAD2 participants presented with signs of nerve pathology. Approximately two-thirds of WAD2 participants presented with signs of nerve pathology using QST both acutely and six-months after injury. The clinical neurological assessment showed frequent levels of nerve pathology (53.8%) that improved six-months later (24%).

Signs of structural nerve pathology were measured using serum concentrations of NfL and intraepidermal nerve fibre densities at the index finger. Acute measures of

NfL were significantly higher in WAD2 participants compared to age- and sex-matched controls. NfL levels in WAD2 were significantly reduced by six-month follow-up assessment, reaching control levels. Serum NfL cut-offs suggestive of nerve pathology indicated 13% of participants surpassed this cut-off acutely and only 2% at six-months after injury. Intraepidermal nerve fibre densities at the index finger remained preserved for both acute and follow-up stages of WAD2. Thresholds for identifying nerve pathology using skin biopsy at the index finger are less well known and thus more challenging to quantify. Using healthy control participant data per age decade, I estimated small fibre nerve pathology from skin biopsies present in 1.6% acutely and 8.7% at follow-up.

Measures of nerve pathology do not appear prognostic in WAD2.

Neither NfL nor bedside clinical tests of small fibre loss of function at the index finger were prognostic for neck-related disability or whiplash-related symptoms six-months after injury. This finding may result from the heterogeneity in nerve pathology present within the cohort. Future studies assessing the prognostic role of nerve pathology in participants with more frank nerve lesions (WAD grade 3) could provide more definitive evidence on the importance of such measures in varying severities of whiplash injuries.

RNA-sequencing of blood samples reveals sex-dependent effects in persistent pain.

Transcriptional blood profiling revealed significant gene dysregulation for moderate/severe persist pain versus minimal persistent pain when stratified by sex six months after injury. I also identified significant negative correlations with whiplash-related symptoms and neuropathic pain for three genes (HLA-DQA1, SWT1, AND HEBP1). Further analyses of these preliminary results are needed to validate and substantiate my findings, which is discussed in detail below. My preliminary RNA-sequencing results do shed light on potential sex-dependent molecular pathways of recovery in WAD2.

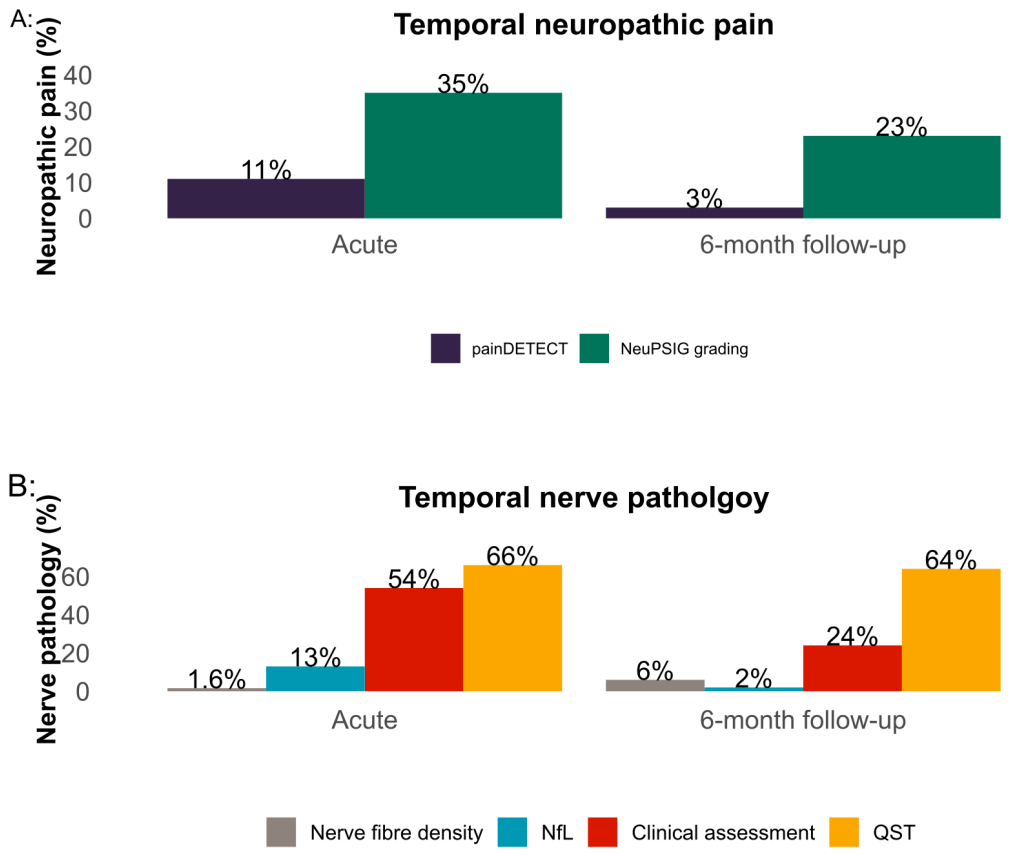


Figure 7.1. Summary of temporal signs of neuropathic pain and nerve pathology in WAD2. (A) Summary of the temporal profile of neuropathic pain using both painDETECT questionnaire and Neuropathic Pain Grading scales (NeuPSIG). (B) Summary of the temporal profiles of nerve pathology in WAD2.

Neuropathic pain was defined using the NeuPSIG grading scale of 'probable' and the painDETECT cut-off scores >18/38. Nerve pathology was defined in the following categories: the presence of loss of function upon clinical neurological assessment (strength, reflexes, light touch, pin prick, or temperature); QST z-scores < -1.96 (including CDT, WDT, TSL, MDT, MPT, VDT, CDT-leg, WDT-leg), serum NfL z-score > 1.5; intraepidermal nerve fibre density cut-offs were calculated per age decade based on included healthy control cohort. A full description of the nerve fibre density cut-off is included in Chapter 5, Supplemental Table 4.
Abbreviations: NeuPSIG: Neuropathic Pain Special Interest Group, NfL: Neurofilament light chain, QST: Quantitative sensory testing.

7.2. Clinical implications

The identification of nerve pathology and neuropathic pain in WAD2 create important clinical considerations. This includes routine assessment of neuropathic pain in clinical settings, the limited validity and accuracy of clinical neurological assessments, and the classification of whiplash injuries.

Neuropathic pain is present for approximately one-third of WAD2 participants.

Neuropathic pain conditions are associated with greater psychosocial burden and require different treatment strategies compared to non-neuropathic conditions[195]. Unsurprisingly, recent qualitative evidence corroborates the personal challenges for participants with WAD. These challenges include participants distancing from normalcy, difficulty finding the 'right' healthcare provider, and a mismatch in beliefs and expectations about their recommended treatment strategies[281]. An improved understanding of participants' symptom profiles, including neuropathic-related symptoms, may help reduce the personal challenges for participants following whiplash injuries. The limited number of studies assessing neuropathic pain in WAD may contribute to clinical difficulties for participants experiencing such symptoms.

Addressing the current limitations in effective treatments for WAD requires exploring new approaches. The currently used homogenous treatment strategies may be one reason for the overall ineffectiveness of WAD treatment[64,65]. One area for future research involves treatment stratification based on pain mechanisms. Recognising different types of pain may require distinct treatment strategies[195], the clinical classification and treatment for neuropathic pain may be one approach to improving outcomes for a subgroup of people with whiplash injury. Treatment based on pain phenotyping however has shown ineffective for some conditions. Though our knowledge of pain pathomechanisms is improving, they remain incompletely understood. This is evidence by previous trials stratifying pain mechanisms failing to show efficacy[487-489] and emphasises the heterogeneity within the pain experience.

There is limited evidence for stratified treatment based on pain classifications in WAD and this may be one way forward. The results of the acute WAD2 cohort highlight the need to screen for neuropathic pain in the acute phase after whiplash injury. Current international treatment guidelines for whiplash injuries do not include recommendations for the assessment or treatment of neuropathic pain (e.g., specific physiotherapeutic approaches or analgesic medications). Although no previous clinical trials have assessed its efficacy in WAD, two recent feasibility studies have been conducted assessing two first-line neuropathic pain medications for the treatment of acute whiplash injuries (using pregabalin[73] and duloxetine[74]). This gives justification to assess if a targeted treatment could improve outcomes for

participants with WAD2. High quality randomised controlled trials are needed to assess if neuropathic focussed treatment is beneficial for a subgroup who present with such symptoms following whiplash injuries.

Sensory hypoaesthesia was present for nearly two-thirds of WAD2 participants, but clinical bedside tests showed limited validity.

The clinical neurological assessment identified signs of widespread upper extremity hypoaesthesia. This was predominantly from small nerve fibres (approximately 64% of the overall identified loss of function). This highlights the significance of performing a detailed neurological assessment (including small fibre testing) throughout the cervical spine and upper extremities. It is also critical to evaluate neurological function over time, as small nerve fibre pathology has been shown to precede findings of inherent large fibre pathology in patients with focal nerve injury[278,279]. The progressive worsening of nerve pathology may require diagnostic imaging to rule out sinister pathology or indicate the need for surgery or other invasive treatments. However, one important caveat to consider is the limited validity of bedside neurological assessments to detect warm, cold, and mechanical stimuli compared to their corresponding QST measure. This highlights the potential limitations in clinically detecting more subtle signs of nerve pathology.

More valid and reliable clinical tests are needed, as nearly two-thirds of WAD2 participants had signs of nerve pathology using QST (66% acutely and 64% at follow-

up). Future studies comparing different clinical tools to QST are required to reliably identify clinical neurological dysfunction. A recent study using a different set of simple bedside sensory tools has been shown to be valid and reliable in participants with chronic neuropathic-like pain[490]. Implementing this battery of bedside testing may demonstrate a more valid and reliable approach to detecting nerve pathology in participants with WAD2.

Classification of WAD2 does not accurately represent the clinical phenotype.

I identified both functional and structural nerve pathology in participants with WAD2. This goes against the commonly used Quebec Task Force criteria for such injuries[10]. Although my cohort did not include any participants with frank sensorimotor cervical radiculopathy, over 60% of WAD2 participants had abnormal neurological findings. My results further highlight the clinical heterogeneity present within the WAD classification system, which has been widely criticised since its inception[276]. Newer classification schemes have been proposed that better integrate the complex array of biopsychosocial sequelae of whiplash injuries[14,18]. The overall clinical considerations should therefore focus on conducting an in-depth clinical examination and place less emphasis on the simple WAD classification schema originally proposed.

7.3. Potential aetiological causes of nerve pathology in WAD2.

My findings reveal a substantial subgroup of WAD2 participants present with dysfunction to the nervous system. Nearly two-thirds of acute WAD2 participants presented with signs of functional nerve pathology identified using clinical neurological assessment and QST. But where is the origin of this pathology? The most parsimonious explanation is an injury to the cervical nerve roots or spinal cord within a subset of WAD2 participants. Supportive evidence for this hypothesis includes signs of widespread hypoaesthesia, acutely elevated NfL levels, and preserved intraepidermal nerve fibre densities. This theory however cannot be definitively confirmed with my current data. Future research is needed to assess and quantify the anatomical origins of this pathology, as described below.

7.4. Thesis limitations

The primary limitations included in this thesis have been discussed at the end of each chapter and are summarised here in three main categories: 1) sample size of skin biopsy analysis; 2) cohort generalisability; and 3) potential differences between assessors. Though I achieved full statistical power for almost all analyses included in this work, skin biopsy analyses and comparison of QST parameters over the leg were not fully powered. Second, the generalisability of my participants was limited by the fact that most participants within the cohort were from the Oxfordshire area. We also did not collect detailed information regarding race or sociodemographic status of participants. These factors may limit the ability to compare to other WAD2

cohorts. Lastly, the multicentre nature of this study creates that potential for variance in outcomes between assessors. Healthy control skin biopsy data collected from a previous cohort was also used in my current study. Strategies to mitigate these inter-rater effects included the selection of valid and reliable clinical measures, piloting sessions between assessors of the study, and investigating inter-rater reliability of intraepidermal nerve fibre counts. Differences however may still have occurred.

7.5. Future directions

The findings in my thesis present exciting opportunities for several directions of future research.

1. Stratify WAD2 participants.

One tool to better understand the clinical heterogeneity within the WAD2 cohort is through participant stratification. My deeply phenotyped cohort allows for potential stratification to better understand the underlying pathological mechanisms. In the future, I plan to use two primary approaches to perform such stratification. The first includes a clinical stratification to distinguish different pain classification. The second involves the use of unbiased dimensionality reduction and machine learning techniques.

1.1 Clinical stratification

Treatments based on specific pain mechanisms may be one potential way to improve outcomes for participants with WAD[491,492]. As described above, approximately one-third of acute participants with the most common type of whiplash injury (WAD2) present with neuropathic pain. However, only one participant (0.8%) received a first-line neuropathic pain medication acutely after injury. This finding suggests that nearly one in three participants did not receive treatments targeting their pain mechanisms. Based on the significant prevalence of neuropathic pain in my cohort, it would be sensible to assess the effectiveness of first-line neuropathic pain medications in participants stratified by the presence of clinically determined neuropathic pain.

1.2. Unbiased stratification

Data reduction and machine learning techniques are an increasingly common approach to identify patterns and subgroups within deeply phenotyped cohorts. Such statistical approaches can provide an unbiased strategy to distinguish participants and identify potentially novel clusters that are not clearly identified in clinically derived methods. The advancement of artificial intelligence creates novel ways to assess this complex pain phenotyping and make it more explainable. One example of such an approach has been done using machine learning for feature selection and cluster allocation to QST variables identifying pain conditions[493]. I could incorporate a similar interpretable machine learning approach to identify novel clusters and

features within my deeply phenotyped dataset. One advantage of this approach is the potential to understand pain mechanisms more deeply. This unbiased approach might however be challenging to clinically implement and limit its usefulness.

2. Integrate datasets to better understand the interplay between nerve pathology and neuroinflammation.

The data from my project will provide important insights that will be compared and analysed separately from the Pain Challenge data (e.g., serum inflammatory markers, T2-weighted weighted imaging of the brachial plexus). However, combined they are a rich dataset that allows us to explore additional questions. Evaluating the association between the temporal patterns in the concentrations of immune-related proteins will provide important details to better understand my identified gene dysfunction at mRNA level. Additional questions we will assess include the association of NfL concentrations with changes of neuroinflammation as apparent on magnetic resonance imaging; Correlations with measures of nerve pathology and nerve mechanosensitivity; and associations between nerve pathology and serological measures of inflammation. Combining these datasets will provide valuable insights into the association of nerve pathology and inflammation in the onset and recovery of WAD2 injuries.

3. Gain a deeper understanding of the molecular mechanisms of persistent symptoms.

A logical next step to better understand the results of my RNA-sequencing is to perform deconvolution analysis to estimate the proportions of cell types in my samples using methods such as CIBERSORT[494]. This could help to characterise the composition of blood cell types between sexes in moderate/severe and minimal persistent pain groups. I could also perform genome-wide association studies of chronic pain within the UK Biobank to further validate potential single nucleotide polymorphisms associated with my identified dysregulated genes. These would be my next steps related to my findings in blood-based gene dysregulation.

4. Evaluate signs of spinal cord pathology using the now collected magnetic resonance images.

Even though the combination of my phenotypic findings point towards central rather than peripheral nervous system pathology, the presence or extent of spinal cord pathology within my cohort remains unknown. Our project has collected MRI of the cervical spine as part of the broader aims for this cohort study[349]. This provides an invaluable opportunity to assess for the presence and extent of spinal cord pathology identified at both acute and follow-up timepoints. One useful tool to assess potential changes to the spinal cord from MRI is the Spinal Cord Toolbox[495]. Examples of data derived from the Spinal Cord Toolbox include spinal cord cross-sectional area, diffusion metrics, intensity profiles, lesion segmentation, and quantification of changes to spinal cord tracts and pathways. A previously published paper used these methods to demonstrate changes to specific white matter tracts of the spinal cord

in participants with chronic WAD2[115]. Although the MRI parameters in our study were optimised for the brachial plexus and not for the spinal cord, I will assess the viability of using the Spinal Cord Toolbox for our images. This could aid in understanding the presence and extent of spinal cord pathology present in my cohort.

7.6. Concluding remarks

Neurological sequelae have been a consequence of transportation-related trauma since its inception, as evidenced by Professor Erichsen's seminal lecture in 1866. Yet, one could argue if there has been improvements for these patients within the last two centuries. My motivation to undertake a DPhil stemmed from a deep-seated desire to improve outcomes for people suffering from whiplash injuries. The personal and societal burden of whiplash injuries continues to affect millions worldwide. This is emphasised by the still relatively unknown pathomechanisms and unidentifiable pathology for most people. Moreover, nearly 50% of people continue to experience persistent symptoms after a whiplash injury today. Fortunately, the field has progressed from initially considering whiplash as a purely psychosis-induced malady to more deeply understanding its spectrum of biopsychosocial symptoms. But does the work presented in my thesis contribute to improving outcomes for these patients?

While my current work may not prompt immediate change in clinical care for WAD, it lays important groundwork for improvement in three primary ways. First, the presence of neuropathic pain in approximately one-third of my cohort offers insights

to distinct and already available treatment options. Second, my deeply phenotyped dataset demonstrates clear signs of nerve pathology in roughly two-thirds of participants who are typically classified without such dysfunction. These novel findings help us better understand the heterogenous nature of WAD2 injuries. Lastly, I identified differences in transcriptional profiles between males and females with persistent whiplash-related symptoms that correlated with whiplash-related symptoms and neuropathic pain. Each of these elements provide important foundational work that could lead to potential step-changes in how we evaluate and treat people with WAD.

Significant work however lies ahead. The complex and subjective nature of pain makes it elusive to understand. Nevertheless, I am optimistic it can improve. The revolutionary technological advances and increasingly collaborative spirit of science are all reasons for hope. As Dr J. J. Bonica stated, "The proper management of pain remains, after all, the most important obligation, the main objective, and the crowning achievement of every physician." As a clinician, I find these words inspiring and a call to continue with this meaningful vocation. I hope the work presented in this thesis acts as a foundation to ultimately help improve outcomes for people suffering from whiplash injuries.

Scientific outputs

1. Matesanz-García L, Billerot C, **Fundaun J,** Schmid AB. Effect of Type and Dose of Exercise on Neuropathic Pain after Experimental Sciatic Nerve Injury: a Preclinical Systematic Review and Meta-analysis. J Pain. 2023 Jan.

2. **Fundaun J***, Ridehalgh C*, Bremner S, Cercignani M, Young R, Trivedy C, Novak A, Greening J, Schmid A*, Dilley A*. Does peripheral neuroinflammation predict chronicity following whiplash injury? Protocol for a prospective cohort study. BMJ Open. 2022 Dec 15;12(12):e066021. *Contributed equally.

3. **Fundaun J,** Kolski M, Molina-Álvarez M, Baskozos G, Schmid AB. Types and Concentrations of Blood-Based Biomarkers in Adults With Peripheral Neuropathies: A Systematic Review and Meta-analysis. *JAMA Netw Open.* 2022;5(12):e2248593.

4. **Fundaun J,** Thomas ET, Schmid AB, Baskozos G. The power of integrating data: advancing pain research using meta-analysis. Pain Rep. 2022 Oct 4;7(6):e1038.

5. **Fundaun J**, Kolski M, Baskozos G, Dilley A, Sterling M, Schmid AB. Nerve pathology and neuropathic pain after whiplash injury: a systematic review and meta-analysis. Pain. 2022 Jul 1;163(7):e789-e811.

Invited presentations

1. Ridehalgh C, **Fundaun J**, Farrell S. Neuroimmune Contributions to Persistent Musculoskeletal Pain: Evidence and Clinical implications. World Conference of Musculoskeletal and Manual Physical Therapy - IFOMPT 2024, Switzerland. **Conference Symposium.**

2. Schuster V, Bradley L, **Fundaun J**, Kolski M, Courtney C. The Relationship between Sleep and Neuropathic Pain in Individuals with Diagnosed Peripheral Nerve Pathology. American Physical Therapy Association Combined Section Meeting 2023, Boston, MA. Poster.

3. Tejos-Bravo M, Muhammad F, **Fundaun J**, Panduro T, Espiosa A, Stewart M, Beer S, Novak A, Schmid AB. Role of serum biomarkers in suspected cauda equina syndrome (Preliminary results of a potential screening method using Single-Molecule Array). Royal College of Emergency Medicine Annual Scientific Conference 2023, Poster.

4. **Fundaun J,** facilitator. Co-created the most attended conference workshop entitled 'Transition from acute to chronic pain'. APDP 2023 Conference.

5. **Fundaun J**, Ridehalgh C, Baskozos G, Tejos-Bravo M, Greening J, Bremner S, Dilley A, Schmid AB. Acute nerve pathology and neuropathic pain after whiplash injury: preliminary cohort findings. NeuPSIG Congress 2023, poster.

6. Ridehalgh C, **Fundaun J**, Greening J, Bremner S, Schmid AB, Dilley A. The relationship between neuropathic pain measures and heightened nerve mechanosensitivity in people after acute whiplash. NeuPSIG Congress 2023. Poster.

7. Tejos-Bravo M, Muhammad F, **Fundaun J**, Panduro T, Espiosa A, Stewart M, Beer S, Novak A, Schmid AB. Serum Neurofilament Light Chain Levels In Suspected Cauda Equina Syndrome: Preliminary Results Of A Potential Screening Method Using Single-Molecule Array. NeupSIG Congress 2023, poster.

8. **Fundaun J**, Ridehalgh C, Baskozos G, Tejos-Bravo M, Greening J, Bremner S, Dilley A, Schmid AB. Is acute nerve pathology present after whiplash injury: preliminary cohort findings. Advanced Pain Discovery Platform Conference 2023, poster.

9. **Fundaun, J.** Podcast discussing whiplash injuries for physiotherapists, Guida Galattica per Fisioterapisti. https://youtu.be/p5fKvVzxPss

10. **Fundaun J,** Kolski M, Alvarez MA, Baskozos G, Schmid AB. Blood-based biomarkers of peripheral neuropathy: a systematic review and meta-analysis. IASP World Congress 2022. Toronto, CA. Poster.

11. **Fundaun J,** Kolski M, Dilley A, Baskozs G, Sterling M, Schmid Nerve Pathology And Neuropathic Pain After Whiplash: A Systematic Review And Meta-Analysis. EFIC Congress 2022, Dublin, Ireland. Poster.

12. **Fundaun J,** Kolski M, Dilley A, Baskozs G, Sterling M, Schmid A. Peripheral nerve pathology and neuropathic pain in whiplash-associated disorders. International Association for the Study of Pain Neuropathic Special Interest Group Data Blitz, 4 November 2021.

13. **Fundaun J,** Kolski M, Dilley A, Baskozos G, Sterling M, Schmid A. Peripheral nerve pathology and neuropathic pain in whiplash-associated disorders: a systematic review. Physiotherapy UK Conference 2021. Platform.

14. **Fundaun J,** Kolski M, Dilley A, Baskozos G, Sterling M, Schmid A. Peripheral nerve pathology and neuropathic pain in whiplash-associated disorders: a systematic review. International Association for the Study of Pain Virtual Congress, June 2021. Poster.

15. **Fundaun J,** Kolski M, Dilley A, Baskozos G, Sterling M, Schmid A. Peripheral nerve pathology and neuropathic pain in whiplash-associated disorders: a systematic review. Council for Allied Health Professions Conference 2021. Rapid 5 Platform Presentation.

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Appendices

Appendix A. The Power of Integrating Data Manuscript

Big Data and Pain
Review

OPEN

PAIN REPORTS

The power of integrating data: advancing pain research using meta-analysis

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Abstract

Publications related to pain research have increased significantly in recent years. The abundance of new evidence creates challenges staying up to date with the latest information. A comprehensive understanding of the literature is important for both clinicians and investigators involved in pain research. One commonly used method to combine and analyse data in health care research is meta-analysis. The primary aim of a meta-analysis is to quantitatively synthesise the results of multiple studies focused on the same research question. Meta-analysis is a powerful tool that can be used to advance pain research. However, there are inherent challenges when combining data from multiple sources. There are also numerous models and statistical considerations when undertaking a meta-analysis. This review aims to discuss the planning and preparation for completing a meta-analysis, review commonly used meta-analysis models, and evaluate the clinical implications of meta-analysis in pain research.

Keywords: Meta-analysis, Random-effects, Common-effect, Fixed-effect, Meta-regression, Network, Individual participant data, Prevalence

1. Introduction

The field of pain research has grown substantially in recent years.[53,93] The rapid increase in research output creates an important need to synthesise these findings. One commonly used tool to combine and analyse data in health care research is using meta-analysis. Often combined with a systematic review of the literature, a meta-analysis aims to quantitatively synthesise the results of multiple studies that answer the same research question.[37,41,76] Meta-analyses help to understand what is currently known, identify gaps in the literature, and formulate new research questions.

Systematic reviews and meta-analyses are considered the pinnacle of evidence-based medicine.[8,12] High-quality meta-analyses can guide clinical decision making, inform national and international clinical guidelines, update health care policies, and influence research priorities and funding. This is relevant for both clinicians and scientists and can include a variety of pain-related research topics. Examples range from understanding treatments effects in preclinical models of painful conditions,[54,55,95] measuring the efficacy of physiotherapy[17,26,30] or pain medications,[21,28,64] to assessing associations of functional magnetic resonance imaging to placebo treatment.[96,97]

Rigorous meta-analyses have the potential to provide important insights for pain research.[40] However, aggregating data to answer impactful clinical questions can be challenging.[8] There are inherent difficulties when combining data sets and selecting the most appropriate statistical method for a meta-analysis.[27,52] The overall aims of this review are to discuss the preparatory considerations for completing a meta-analysis, review commonly used meta-analysis models, and evaluate the clinical implications of meta-analysis in pain research.

2. Planning and design

Detailed planning and preparation are critical to avoid common pitfalls when conducting a meta-analysis. Methodological errors include poorly designed search strategies, analysing overly dissimilar data, synthesising poor-quality studies, and changing outcomes without properly reporting.[19,27] These pitfalls can lead to misinterpretation and inaccurate conclusions of the literature. A

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detailed prospectively registered protocol provides transparency and can mitigate these errors thus strengthening the results and allowing for further scrutiny from the scientific community. Preregistration of a meta-analysis protocol (before completion of data extraction) in an academic journal or an online repository, such as PROSPERO[67] or the Open Science Framework,[22] is now a requirement for publication in most high-quality journals. **Figure 1** highlights a brief summary of the steps and considerations for undertaking a meta-analysis.

For both systematic reviews and meta-analyses, there are several guidelines to help standardise the study design and reporting of results (eg, Cochrane Handbook,[37] PRISMA guidelines,[65] prospective meta-analysis,[77] preclinical systematic reviews[79]). The EQUATOR Network (https://www.equator-network.org) and CAMARADES (https://www.ed.ac.uk/clinical-brain-sciences/research/camarades) are excellent resources for clinical and preclinical reporting guidelines, respectively. There are also many tools to assess the quality of studies included in a meta-analysis (eg, GRADE,[4] Cochrane Risk of Bias tool,[36] Newcastle–Ottawa Scale,[80] etc). The overall quality and subsequent impact of a meta-analysis can significantly improve through proper study design and appropriate planning.

3. Meta-analysis models

There are numerous statistical models to consider when completing a meta-analysis. This review does not provide a comprehensive overview of all available options but will discuss commonly used models in pain research. In each section, we will introduce the model, discuss relevant considerations, and present an illustrative example related to pain research. **Table 1** describes a summary of the models discussed in this review. For comprehensive details regarding meta-analysis model application, selection, and statistical methods, refer previous studies.[3,33,34,76]

Combining data for a meta-analysis focuses on creating an overall effect size estimate of improved precision. An effect size is a quantification of the relationship between 2 entities that incorporates both its direction and magnitude (eg, standardised mean difference, odds, and risk ratios).[37] It is important to consider the weighting of individual study estimates to improve the precision of the overall estimate for the true effect size of pooled data. The most common method for calculating study weight for continuous data is the inverse-variance method.[9,37] This method uses the inverse of the variance of the effect size estimate (ie, one over the square of its standard error) to determine the weight given to each study.[9,29] Similarly, there are other approaches available to calculate the study weight of binary data (eg, Mantel-Haenszel,[74] Peto[94]). However, determining the most appropriate model to calculate study weight for a meta-analysis remains controversial.[9,33,76]

Effect sizes are graphically depicted using forest plots. Forest plots include critical components of a meta-analysis, including the type of model used, results and weighting of individual studies, the overall effect sizes, confidence intervals, and between-study heterogeneity. **Figure 2** includes a detailed description of an example forest plot from our previous systematic review and meta-analysis.[23]

3.1. Common-effect model

The common-effect model, also known as the fixed-effect model, is a meta-analysis method that assumes that all included studies share a common effect.[9] This implies that there is only one true underlying effect (in both magnitude and direction), and the between-study differences are only the result of sampling error, the within-study variance.[9] For example, this may be applicable when analysing multiple groups (data sets) from a large study performed by a single research group on the same population of individuals and similar experimental paradigms.[9]

3.1.1. Considerations

A common-effect model is applicable if heterogeneity, between-study variance, is not present or when the distribution of the intervention effects is nearly symmetrical.[37] However, another consideration when choosing between common and random-effect models is accounting for the number of included studies. A small number of studies could overinflate the effect size estimation for random-effects models.[9] Thus, a common-effect

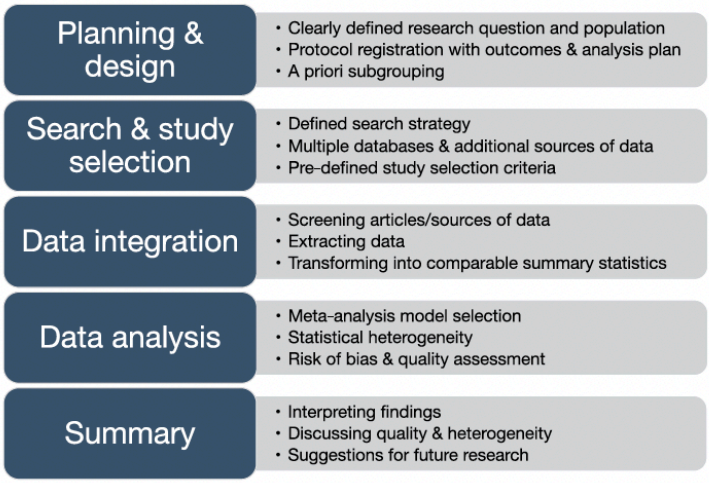

Figure 1. Considerations for completing a systematic literature review with meta-analysis.

Table 1

Summary of meta-analysis models and corresponding statistical considerations.

Meta-analysis models	Main aim	Considerations
Common-effect	Synthesises the common effect measure between studies	Strengths: Estimates the assumed common underlying treatment effect between studies May be more appropriate for meta-analysis with few included studies Limitations: Problematic when combining the effects of multiple studies because maintaining the assumption that there is no other variance is unlikely Interpretation of results is focused only on the included population
Random-effects	Synthesises the average effect measure between studies	Strengths: More generalisable and less restrictive than the common-effect model Considers heterogeneity of included studies More likely to fit the sampling distribution Limitations: A small number of studies may overinflate effect size estimation High levels of heterogeneity can limit the representation of the identified effect
Meta-regression	Explores potential associations and relationships between studies	Strengths: Assesses strength and direction of relationships Ability to assess multiple covariates simultaneously Limitations: Requires adequate number of studies Must limit covariates based on background subject knowledge
Multivariate	Simultaneously analyses multiple outcomes from the included studies	Strengths: Useful when analysing multiple main outcomes Produces a summary statistic for each outcome Reduces the impact of reporting bias by allowing inclusion of more data Limitations: Correlations measured across studies may not reflect the underlying association between treatment effects Correlation estimates can be less precise and prone to large bias
Network	Assesses available interventions for a clinical condition and makes direct and indirect comparisons across studies to determine the most effective interventions	Strengths: Beneficial for clinicians to decide on the best treatment for patients who fit the review question Limitations: Assumes that heterogeneity variance across different comparisons within the network meta-analysis model is the same Transitivity and inconsistency must be assessed and addressed
Individual participant data	Summarises original data taken from individual participants from multiple studies	Strengths: Increased power to detect differential treatment effects across individuals in randomised controlled trials Ability to identify confounding factors in observational studies Limitations: Difficult to coordinate and obtain individual data Increased time and resource requirements in order to complete
Prevalence	Used to estimate the frequency of a disease occurring within a predefined population	Strengths: A useful tool for clinicians, researchers, and policymakers to better understand the burden of disease Limitations: Variation in the underlying population, case definition, and disease severity is likely to contribute to heterogeneity in the results Transformation of the prevalence proportions may be necessary to obtain confidence intervals that do not lie in extreme ranges and variances that do not result in the undue weighting of studies

model may produce more robust estimators when comparing only a small number of studies. Conversely, a common-effect model in the presence of heterogeneity can lead to an underestimation of the confidence interval's width because the between-study variance is not taken into account.

3.1.2. Example

Veluchamy et al. performed meta-analyses investigating the association of genetic variants on the susceptibility to neuropathic pain.[91] The authors performed meta-analyses of genome-wide association studies from 3 large comparable cohorts of patients with neuropathic pain in the United Kingdom. Using common-effect meta-analyses of each single-nucleotide polymorphism,[60] they identified a novel genome-wide significant locus at chromosome 12q23.1 mapping to SLC25A3 (odds ratio = 1.68, 95% confidence interval [CI]: 1.40–2.02). Experimental models have suggested that SLC25A3 is believed to have a role in developing neuropathic pain; however, further research is required to better understand the underlying mechanisms implicated with these findings.[91] This study illustrates the use of common-effect models to provide important insights into the potential genetic associations to neuropathic pain.

3.2. Random-effects model

In contrast to the common-effect model, the random-effects model allows for the distribution of the true effect size, ie, different

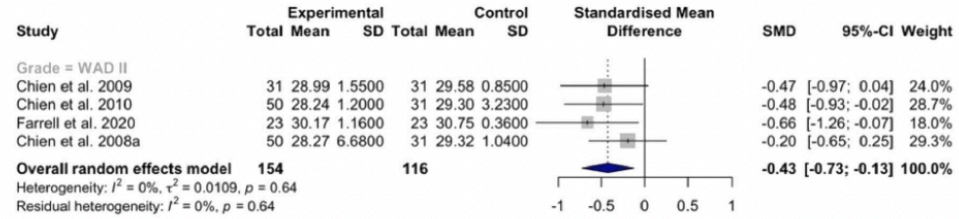

Figure 2. Example forest plot of cold detection thresholds taken at the index finger in patients with whiplash associated disorder (WAD) compared with control subjects. A random-effects model was used to account for potential between-study variance. The left side of the figure displays the total number of participants and corresponding means/standard deviations (SD) for cold detection thresholds of the WAD and control groups. Individual study standardised mean differences (SMD) are depicted by the grey squares (varying in size depending on study weight). The black lines extending from the squares represent the 95% confidence intervals (CI). The overall random-effect meta-analysis summary is shown in bolded text and blue diamond. The overall effect size estimate (blue diamond) does not cross the zero line, indicating that cold detection thresholds were significantly decreased in the WAD groups compared with the control group ($P < 0.05$). The individual and overall SMD, CI, and corresponding study weight values are shown on the right side of the forest plot. Between-study heterogeneity values (shown as Higgins I^2 and τ^2) were low and not considered important. This figure was originally published by Fundaun et al.[23]

effect estimates for each study, and considers additional levels of variance.[9] In many instances, it is difficult to assume that all studies included in a meta-analysis share one underlying effect size. For instance, studies may measure the same biomarker for a painful condition, but they could have variation in the duration of diagnosis, the timepoint of biomarker analysis, or differences in the type of analytic platform used. Therefore, a random-effects model may be more appropriate because it considers both the within-study and between-study variance (heterogeneity).

3.2.1. Considerations

Random-effects meta-analysis models estimate the variance of the true effect size distribution, which is known as tau^2.[3,9] There are multiple methods described to estimate tau^2. Examples of tau estimators include the restricted maximum likelihood,[92] DerSimonian–Laird,[18] Paule–Mandel,[66] or Sidik–Jonkman.[78] There is still dispute regarding which estimator is most appropriate.[42,44,51] In the bias-variance trade-off context, a random-effect meta-analysis is less biased than a common-effect analysis, but it can produce estimators with more variance.

Tau^2 is necessary to calculate the pooled effect size and indicates the between-study variance. However, tau^2 does not describe the source of the heterogeneity present between the studies. The quantification of heterogeneity is commonly expressed through measures, including Cochrane Q, showing the variation excess to sampling error, and Higgins I^2 statistic, showing the excess percentage of the observed Q vs the expected Q.[34,35] These measures help to understand the extent of between-study heterogeneity present within a meta-analysis but do not identify its source. One method to explore the source of heterogeneity is through subgroup analysis and meta-regression, as discussed below.[90]

3.2.2. Example

Georgopoulos et al.[25] performed a systematic review and meta-analysis to determine whether quantitative sensory testing (QST) parameters were prognostic of pain and disability in various musculoskeletal conditions. Taken from 37 studies, random-effect meta-analyses identified initial QST measures as prognostic for pain (mean r = 0.31, 95% CI: 0.23–0.38, n = 1,057 participants) and disability (mean r = 0.30, 95% CI: 0.19–0.40, n = 290 participants). This study highlights the potential impact of using QST as a prognostic tool to stratify patients with musculoskeletal pain.

3.3. Meta-regression

The interpretation of a meta-analysis is often limited due to potential confounding variables from combining studies. One way to "explore" the potential associations and relationships between the studies, while controlling for covariates, is using meta-regression.[2] Like linear regression, meta-regression evaluates whether there is a linear relationship between the variables using weighted summary statistics from the included studies. Meta-regression evaluates both the strength and direction of association between the covariates within an analysis.[2]

3.3.1. Considerations

Common-effect meta-regression models do not consider between-study variance, making random-effects models more appropriate for meta-regression.[7,84] Compared with subgroup analysis, meta-regression provides more detailed consideration for the strength and direction of relationships between the covariates. The selection of covariates (eg, age, sex, comorbidities, etc) should be limited in number, based on background subject knowledge, and should be determined a priori.

3.3.2. Example

Niesters et al. used meta-regression to understand sex differences in opioid analgesia.[62] These results indicated that there was no effect of age or study size on analgesia. However, they identified significantly greater effects of patient-controlled analgesia in women compared with men (effect size = 0.22, 95% CI: 0.02–0.42). Further analysis, which only included studies using morphine-based analgesia, showed even greater effect in women (effect size = 0.36, 95% CI: 0.17–0.56). With the increasing evidence of sex differences in pain mechanisms and processing,[58] meta-regression could be an important tool to highlight sex differences in pain research.

3.4. Multivariate methods

Meta-analyses are often focused on a clinical topic with multiple correlated measures. The most appropriate way to analyse this type of data is through multivariate meta-analysis approaches. Multivariate meta-analysis simultaneously estimates the effect of multiple correlated outcomes.[15,45,71] Due to the inherent variance included in multivariate methods, random-effects models are commonly used.[45,46,76] Classic examples of multivariate analysis

include assessing both the systolic and diastolic blood pressure or the sensitivity and specificity of a diagnostic test.[45]

3.4.1. Considerations

Multivariate approaches are critical to consider when there are missing data or when the summarised effects depend on other correlated outcomes.[71,76]

Unfortunately, correlated outcomes are often assessed using multiple univariate analyses. This univariate approach can produce bias and overestimate the overall effect.[69] This approach is also a common criticism of meta-analysis[19] because it does not adequately assess the influence of multiple correlated outcomes on each other.[45,76] Multivariate approaches help overcome this problem by accounting for the inherent dependence of certain outcomes in an analysis.[56]

3.4.2. Example

Tagliaferri et al. analysed the contributions of multiple factors (pathological and psychological biomarkers) related to persistent nonspecific low back pain.[82] They concluded that there were significant contributions of all studied biomarker categories to persistent low back pain (nervous system, spinal imaging, and psychosocial). However, psychosocial factors showed the greatest effect (Hedges g = 0.90, 95% CI: 0.69–1.10) compared with the nervous system (Hedges g = 0.31, 95% CI: 0.13–0.49) or spinal imaging measures (Hedges g = 0.55, 95% CI: 0.37–0.73). Due to the often complex and multifactorial nature of painful conditions, multivariate meta-analysis methods may elucidate important underlying factors that can facilitate patient stratification in various painful conditions.

3.5. Network meta-analysis

Network meta-analysis allows researchers to compare 3 or more interventions simultaneously by combining all of the available evidence both directly and indirectly across studies.[37] By combining 2 different sets of interventions (ie, interventions A and B in study 1, and interventions B and C in study 2), it is possible to estimate the effects between 2 indirect interventions (interventions A and C). Network meta-analyses are beneficial for clinicians because they allow comparisons across the available evidence to rank the efficacy of different interventions for a clinical condition. This renders the findings more clinically relevant to the appropriate patient. It is particularly an advantageous method of meta-analysis because it allows for the comparison of interventions that have previously never been compared in primary studies.

3.5.1. Considerations

Pairwise meta-analyses of the directly compared interventions should be performed before performing the network meta-analysis so that the statistical heterogeneity for each comparison can be directly evaluated. After this, the network meta-analysis model can be developed. Several models can be utilised for this: If there are no trials with multiple arms, meta-regression (described above) can be used; If multiarm trials are included, hierarchical models could be used within a Bayesian framework, or alternatively, a multivariate meta-analysis approach can be taken. Researchers should also prespecify how heterogeneity will be assessed within the model.

Network meta-analyses assume that there is consistency or agreement between the direct and indirect comparisons. However, this is not always the case and researchers must check for both global inconsistency across all comparisons and local inconsistency or "hotspots" within comparisons. If either of these are identified, it is important to closely examine the potential effect modifiers of studies within inconsistent loops. Network meta-regression models can also explore how the effect modifiers can affect the results. In addition, sensitivity analyses excluding studies that may be contributing to inconsistency can improve the robustness of the results.

3.5.2. Example

Ho et al. performed a network meta-analysis comparing the effectiveness of various psychological interventions for chronic low back pain.[39] This included 97 randomised controlled trials with 17 treatment nodes. They performed traditional pairwise meta-analyses for all direct comparisons and used random-effects network meta-analysis to combine the direct and indirect evidence. The mean rank and relative treatment rankings for each node were estimated, and the authors determined that the most highly ranked intervention for the primary outcome (physical functioning) at postintervention was cognitive behavioural therapy delivered with physiotherapy care (mean rank = 2.2, standardised mean differences = 1.01, 95% CI: 0.58–1.44).

3.6. Individual participant data methods

Individual participant data (IPD) meta-analysis is a method of obtaining and analysing raw individual level data from single studies instead of traditional group-level summary statistics.[70,81] Individual participant data enables the identification of covariates or subgroups that traditional meta-analyses of aggregate data are not able to detect.[27,70,72,85] As such, IPD meta-analysis is considered the benchmark for integrating data from clinical studies.[73,81] With the increased need for personalised and stratified pain management, IPD meta-analysis has the potential to uncover the important and targeted treatment options that single randomised controlled trials are not powered to detect.[11,14,16,83] This is particularly relevant for pain research because most clinical trials are not adequately powered to detect subgroup differences or identify relevant covariates. Recent advances in statistical modelling of IPD meta-analysis have shown promise and can be reviewed in detail here.[33,70,72,76]

3.6.1. Considerations

Because IPD meta-analyses are more time and resource intensive, they should only be undertaken when traditional meta-analyses cannot adequately answer a clinical question.[72] One such area for using IPD is to detect differences in treatment effects between individuals and account for covariates. With the often-disappointing results of potentially promising pain medications of the past few decades,[13,20,21,47,50] IPD meta-analysis may provide important insights on how to identify significant subgroup differences in treatment effects. However, IPD meta-analyses are not always possible, and there are significant challenges with data sharing policies,[75] data set harmonization,[1] and obtaining full data sets.

3.6.2. Example

Hayden et al. initially performed a systematic review and traditional meta-analysis that suggested that exercise therapy to be more

effective in decreasing pain and improving function in patients with persistent low back pain[31]. However, this study used aggregate level data and was unable to identify which individuals may be more likely to benefit from exercise therapy. Then, the authors performed an IPD meta-analysis to identify different treatment effects of exercise among individual patients with persistent low back pain[32]. The overall IPD meta-analysis for persistent low back pain suggested that exercise was more beneficial than usual care or no treatment on pain at short-term follow-up (mean effect = −10.7, 95% CI: −14.1 to −7.4). This review also identified potential novel covariates of participants who may respond more favourably to an exercise intervention for persistent low back pain, including not having heavy physical work demands, normal body mass index, and any medication use for low back pain. These covariates could be used in future research to assess a stratified treatment approach for subgroups of patients with low back pain.

3.7. Prevalence

Prevalence meta-analysis is used to estimate the frequency of a disease occurring within a predefined population.[5] Prevalence meta-analyses, such as the Global Burden of Disease Study,[1] are valuable tools for researchers, clinicians, and policymakers to better understand disease burden and therefore direct resources and research appropriately. There are a variety of considerations to make when conducting a prevalence meta-analysis: the choice of method, model, variance estimation technique, whether the prevalence proportions need to be transformed, and method of heterogeneity assessment.

3.7.1. Considerations

Currently, there are no reporting guidelines for prevalence meta-analyses. This results in reviews of varying quality.[10] The main challenge with undertaking a prevalence meta-analysis is assessing heterogeneity.[57] Within prevalence studies, there is likely to be variation in the underlying population, case definition, disease severity, and other biases, and therefore, a random-effects model should be utilised. To address heterogeneity, reviewers should assess for covariates that may explain heterogeneity and stratify the results into appropriate subgroups or perform meta-regression.

Transformation of the prevalence proportions may be necessary to obtain confidence intervals that do not lie in extreme ranges and variances that do not result in the undue weighting of studies. The most commonly recommended transformation is the Freeman–Tukey double–arcsine, followed by the logit, log, and arcsine transformations.[10]

3.7.2. Example

Murray et al.[59] conducted a meta-analysis examining the prevalence of chronic pain in young adults. They examined possible sources of heterogeneity by classifying studies by location of chronic pain, demographic, geographic, and psychosocial factors related to chronic pain as well as study-level characteristics such as population type, sampling area, years of data collection, and assessment method. The authors calculated heterogeneity using the I^2 statistic and the Q test, and they found a very high degree of heterogeneity, with prevalence rates of chronic pain in young adults ranging from 1% to 41%, I^2 = 99%, Q(42) = 5473.3. There was high heterogeneity even when the results were stratified by pain subtype.

4. Implications

The abundance and diversity of pain research creates unique opportunities to use meta-analysis in many areas (see examples in **Fig. 3**). These techniques are highly relevant for pain researchers and are currently being used to understand many aspects of pain. For example, there are several, large, multidisciplinary consortia actively collecting data to be meta-analysed.[1,1,68] This enables large sample sizes and adequate power to detect significant effects for a range of biological and clinical variables, which cannot be identified in smaller studies.[43]

The complex pathological mechanisms of pain[6,48,86] contribute to diverse and challenging clinical presentations. One approach to better understand and improve treatment for these pain phenotypes is through patient subgrouping, ie, stratification.[87] Examples of data for patient stratification include clinical

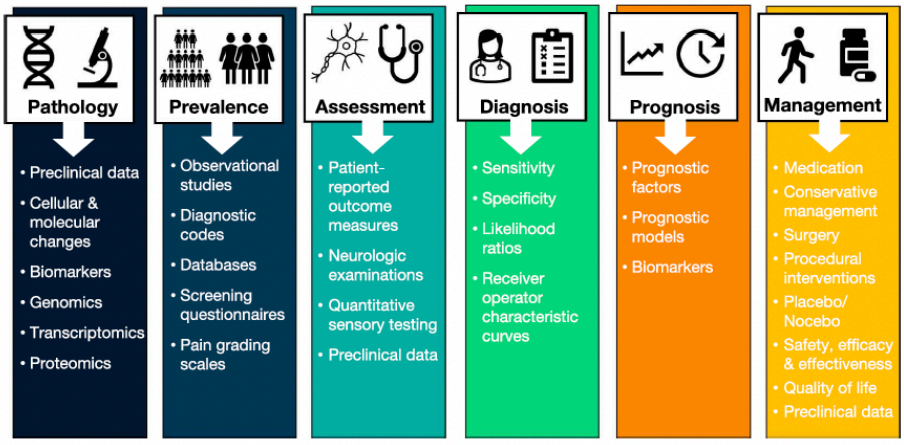

Figure 3. Examples of potential categories and data types that could be meta-analysed in the field of pain research.

examination measures, QST, physiological and psychological factors, and molecular profiling.[83] Meta-analysis can be a powerful tool to identify, organise, and analyse data to improve patient stratification.[38]

To continue advancing pain research, it is imperative to recognise what is currently known. Meta-analyses provide critical summaries of all available evidence to inform clinical practice and impact national and international guidelines[41,49,89] and resource allocation. Although there are many different models and statistical considerations, meta-analysis is an important technique to understand and integrate these data. Meta-analyses can provide robust syntheses of published and unpublished data and can be planned prospectively through consortia and collaboration.

5. Conclusions

Meta-analysis can be used as a powerful tool to quantitatively synthesise important questions in pain research. In this review, we have highlighted several models and statistical methods to consider for the selection and interpretation of a meta-analysis. Although careful methodological consideration must be taken, meta-analyses can provide important summaries to facilitate scientific discovery and clinical advancement in pain research.

Disclosures

The authors have no conflicts of interest to declare.

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Appendix B. Clinical proforma

Code:_____ Date:_____/_____/_____

CLINICAL SCREENING PROFORMA

Interviewer:_____ Consent: ☐

Age: _____ Sex: M ☐ F ☐ Dominant Hand: Right ☐ Left ☐ Either ☐

Height:_____ Weight:_____

Occupation:_____ At Work: Y ☐ N ☐

Hospital of recruitment? _____

HISTORY

Collision: rear ☐ forward ☐ side ☐ Your speed: slow ☐ fast ☐ stopped ☐

Their speed: slow ☐ fast ☐ stopped ☐
(<10 mph)

Date Injury:_____ Anticipation Y ☐ N ☐

Currently involved in litigation due to collision? Y ☐ N ☐

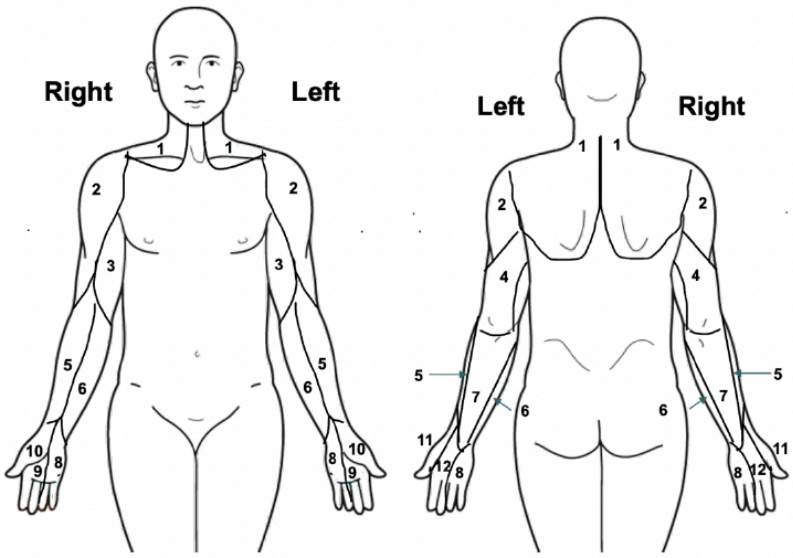
Symptom history:_____

Red flags:_____
(Diplopia, dizziness, drop attacks, dysarthria, dysphagia, ataxia of gait, nausea, numbness and nystagmus)

Current Symptoms: Y. ☐ N. ☐ Comments:_____

Radiating symptoms? Y. ☐ N. ☐ Comments:_____

Subjective sensory changes: Y. ☐ N. ☐ Comments:_____



No symptoms Worst imaginable

1

Code:_____ Date:_____/_____/_____

MANAGEMENT OF SYMPTOMS

	Yes	No	
Physiotherapy	☐	☐	_____
Osteopathy / Chiropractic	☐	☐	_____
Drugs	☐	☐	_____
Injections	☐	☐	_____

Other:_____

WAD medication (dose):_____

Other medication (dose):_____

INVESTIGATIONS

	Yes	No	
MRI	☐	☐	_____
X-rays	☐	☐	_____
Nerve Conduction study	☐	☐	_____

Other investigations: _____

Other medical diagnoses (including nerve involvement):_____

NECK ACTIVE MOVEMENT

	0: Full movement	1: Full movement and symptoms	2: Reduced movement; no symptoms	3: Reduced movement and symptoms
Rotation (R)				
Rotation (L)				
Flexion				
Extension				
Lateral flexion (R)				
Lateral flexion (L)				

Description: _____

Code:_____ Date:_____/_____/_____

UPPER EXTREMITY ACTIVE MOVEMENT

	0: Full movement	1: Full movement and symptoms	2: Reduced movement; no symptoms	3: Reduced movement and symptoms
Shoulder flexion				
Shoulder abduction				
Elbow flexion				
Elbow extension				
Elbow supination				
Elbow pronation				
Wrist flexion				
Wrist extension				

Description: _____

NEUROLOGICAL EXAMINATION

TENDON REFLEXES

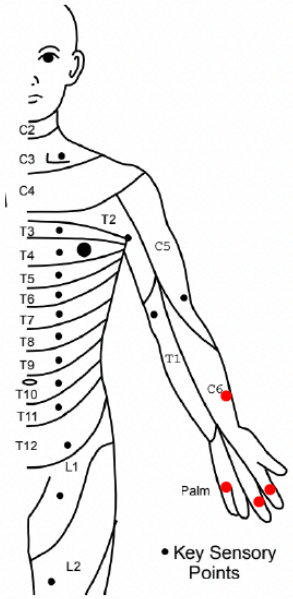
	RIGHT	LEFT
Biceps		
Triceps		

0 Absent
1 Reduced
2 Normal
3 Enhanced

DERMATOME TESTING

RIGHT

	Light touch	Pin prick
C5		
C6 LCN		
C6 MN		
C7		
C8		
T1		



LEFT

	Light touch	Pin prick
C5		
C6 LCN		
C6 MN		
C7		
C8		
T1		

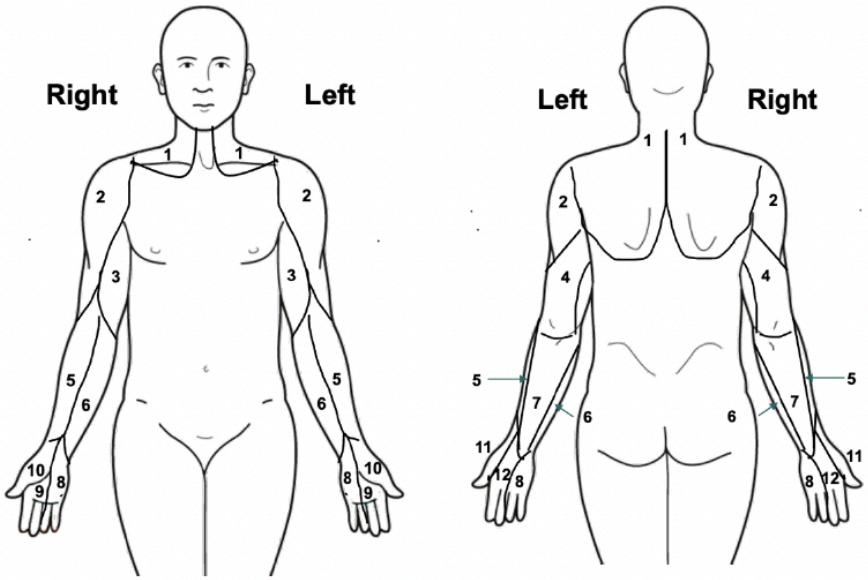
Code:_____ Date:_____/_____/_____

BEDSIDE SENSORY TESTING

	Warm coin		Cool coin		Pin prick	
	R	L	R	L	R	L
Maximal pain area *Skin temp:* ℃						
Index Finger *Skin temp:* ℃						

0 Absent
1 Reduced
2 Normal
3 Enhanced

AREA OF MAXIMAL PAIN



MYOTOME TESTING

	RIGHT	LEFT
Trapezius (C3 **C4**) - Shd shrug		
Supraspinatus (**C5** C6) - Shd abd		
Biceps (C5 **C6**) - Forearm sup + elb flex		
Triceps (radial nerve C6 **C7** C8) - Forearm ext		
Ext pollicis longus (C7 **C8**) – Thumb ext		
Abd pollicis (C8 T1) – Thumb abd		
Interossei (C8 **T1**) – Finger abd/add		

0: No contraction
1: Flicker/trace contraction
2: Active movement, gravity eliminated
3: Active movement, against gravity
4: Active movement, against gravity and resistance
5: Normal power

Code:_____ Date:_____/_____/_____

QUANTITATIVE SENSORY TESTING

MEDIAN (Digit 2) Left Right			
Pain Intensity prior to QST (0-100)		Room T°	°C

		Skin T°	°C			
CDT	WDT	TSL	CPT	VAS	HPT	VAS
°C	°C	°C	°C		°C	
°C	°C	°C	°C		°C	
°C	°C	°C	°C		°C	
		°C				
		°C		PHS		
		°C				

Mechanical Detection Threshold (16 mN)		Mechanical Pain Threshold (8mN)	
felt	not felt	blunt	pricking
	mN		mN
mN		mN	
	mN		mN
mN		mN	
	mN		mN
mN		mN	
	mN		mN
mN		mN	
	mN		mN
mN		mN	

S-R Function (MPS, DMA) – 0-100					
	8			16	
	QT			BR	
	256			512	
	64			32	
	CW			64	
	512			128	
	128			QT	
	16			CW	
	32			8	
	BR			256	

WUR, Wind up-ratio (0-100)	
Single	Series

Vibration detection threshold
2nd MCP
/8
/8
/8

Pressure pain threshold
Thenar:
Kg
Kg
Kg

Stimulus intensity	256 mN

The patient has understood the instructions and complied with them:
Yes ☐ No ☐ Unsure ☐

TIBIALIS ANTERIOR (contralateral)	CDT	WDT	PPT
	°C	°C	Kg
	°C	°C	Kg
	°C	°C	kg

Code:_____ Date:_____/_____/_____

NERVE MECHANOSENSITIVTY TESTS

MOVEMENT

0 = Normal response
1 = Reduced movement; no symptoms
2 = Reduced movement and symptoms

Median nerve biased (ULNT1) WITH INCLINOMETER

	0	1	2	At least partial symptom reproduction	Elbow flexion	Structural differentiation positive
RIGHT						
LEFT						

Median nerve biased (ULNT1) NO INCLINOMETER

	0	1	2	At least partial symptom reproduction	Structural differentiation positive	>90 degrees elbow flexion (Y/N)
RIGHT						
LEFT						

Ulnar nerve biased (ULNT3) NO INCLINOMETER

	0	1	2	At least partial symptom reproduction	Structural differentiation positive	<90 degrees elbow flexion (Y/N)
RIGHT						
LEFT						

DIGITAL PRESSURE

Palpation only

0 = No pain or discomfort
1= local discomfort
2a= local painful response
2b=referred pain/symptoms

Brachial Plexus

Supraclavicular fossa (trunks)

	0	1	2a	2b	Symptoms
RIGHT					
LEFT					

Median nerve

Proximal carpal tunnel

	0	1	2a	2b	Symptoms
RIGHT					
LEFT					

Code:_____ Date:_____/_____/_____

0 = No pain or discomfort
1= local discomfort
2a= local painful response
2b=referred pain/symptoms

Ulnar nerve
Guyon's canal

	0	1	2a	2b	Symptoms
RIGHT					
LEFT					

With algometer
Ulnar nerve
Proximal to cubital tunnel

	0	1	2a	2b	Force at pain onset (kg; avg 3)			Symptoms
RIGHT								
LEFT								

Median nerve
Carpal tunnel

	0	1	2a	2b	Force at pain onset (kg; avg 3)			Symptoms
RIGHT								
LEFT								

CTS TESTS

	RIGHT	LEFT
Positive Tinel's		
Positive Phalen's		

0 = Normal response
1 = WAD symptom reproduction

DIAGNOSIS

WAD1	WAD2	OTHER

Yes No

Blood taken: ☐ ☐ _____

Time of last meal:_____

Questionnaires complete: ☐ ☐ _____

Code:_____ Date:_____/_____/_____

NEUROPATHIC PAIN GRADING SCALE

	Yes	No	
Possible:			
1. Neuroanatomically plausible pain	☐	☐	_____
Probable:			
1. Abnormal light touch	☐	☐	_____
2. Abnormal pin prick	☐	☐	_____
3. Abnormal thermal (coins)	☐	☐	_____
4. Abnormal Reflexes	☐	☐	_____
5. Abnormal Myotomes	☐	☐	_____
6. Post-hoc QST abnormalities	☐	☐	_____
Definite:			
7. Post-hoc MRI abnormalities	☐	☐	_____
8. Post-hoc reduced IENFD	☐	☐	_____

INITIAL GRADE: : ☐ Unlikely ☐ Possible ☐ Probable

FINAL (post-hoc) GRADE: ☐ Unlikely ☐ Possible ☐ Probable ☐ Definite

Code:_____ Date:_____/_____/_____

MRI code:_____

Comments:	**Brachial plexus** T2 stir T1 tse Resolve **Wrist** T1 tirm Trufi Mbep2d

Post Skin Biopsy Note:
Bleeding: Yes / No Lidocaine injected locally ml Digit II left right After care sheet given : yes no Any problems: Sign & date

Post Skin Biopsy Note:
Bleeding: Yes / No Lidocaine injected locally ml Ankle left right After care sheet given : yes no Any problems: Sign & date

Appendix C. Skin biopsy protocol

Free floating skin biopsy protocol

- Cut skin at 50 um and put into well plates with PBS (pH 7.4)
- Pipette off PBS with Pasteur pipette
- Add 500ul 5% fish skin gelatine per well
- Incubate for 1 hour at room temperature on shaker
- Dilute primary antibody in 0.1% Triton in PBS
    - PGP (1:200 dilution, rabbit polyclonal, cat # 516–3344, Zytomed Systems)
    - MBP (1:500 dilution, rat monoclonal, cat #ab7349, Abcam)
- Pipette out fish skin gelatine
- Add 250 ul primary antibody dilution per well
- Incubate over night at 4 degrees on shaker

- Next day: wash 3x1 hour with 0.1% Triton/PBS on shaker at room temperature
- Dilute 2nd antibody in 0.1% Triton in PBS
    - Cy 3 (1:1,000, anti-rabbit, cat # JI711165152, Jackson)
    - Alexa 488 (1:500, anti-rat, cat # A11006, Life Technologies)
- Add 250-300ul AB solution to each well
- Incubate at 4 degrees overnight on shaker in the dark!

- Next day: wash 3x1 hour at room temperature with 0.1% Triton/PBS on shaker (dark)
- Wash 1x1 hour at room temperature with PBS on shaker (dark)
- Transfer the section on glass slides with Pasteur pipette
- Add vectashield (with DAPI) and mount
- Seal with nail polish
- keep in the dark at 4 degrees or freeze for long-term storage

<u>5% fish gelatine solution</u>:

- Use 45% dilution from Sigma (G7765 – 250ml).
- Heat it up at 37% in an incubator or water bath until it becomes more liquid.
- Then add 11ml gelatine to 88.5ml PBS and 500ul TritonX100.

Quanterix™
The Science of Precision Health

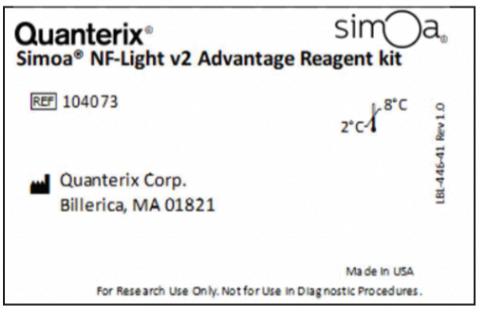
Simoa® NF-LIGHT V2 Advantage Kit

Kit Instructions for SR-X® item 104073

SIMOA NF-LIGHT V2 ADVANTAGE KIT

The Simoa® NF-Light v2 Advantage assay is a digital immunoassay for the quantitative determination of Neurofilament-Light in serum, plasma and cerebrospinal fluid (CSF). This assay is for research use only and not for use in diagnostic procedures.

Note: An Assay Evaluation Kit containing lot-matched Calibrator Concentrate, Calibrator Diluent, and Sample Diluent is available for NF-Light v2 Advantage. Please use item # 104074 to order and refer to the Technical Note on ***Evaluation Kits for Simoa Assays*** on the Quanterix® Customer Portal for details



NF-LIGHT ASSAY DESCRIPTION

Assay format	2-step digital immunoassay: • Sample-bead-detector incubation 30 min at 30°C, 800 rpm • SBG incubation 10 min at 30°C, 800 rpm
Storage conditions	Calibrators and controls: –80°C Reagents: 2–8°C (keep upright)
Total tests/kit	96
Calibrator range: Sample range:	0-450 pg/mL 0-1,800 pg/mL (with 4x dilution Serum/Plasma) 0-45 ng/mL (with 100x dilution CSF)
Typical run setup*	8 calibrators in triplicate (24 tests) 2 controls in duplicate (4 tests) 34 samples in duplicate (68 tests)
Calibrator volume	100 µL per replicate
Sample and control volumes	100 µL per well post-dilution. Minimum required dilution for control, plasma and serum is 4x. Minimum required dilution for CSF is 100x.

***Note:** Each replicate sample is run in a separate well.

NF-LIGHT V2 ADVANTAGE PROTOCOL

Step 1: Reagent and sample warming. Equilibrate reagents, calibrators, controls, and samples to room temperature for 1 hour. Secure RGP bottle in the Simoa heated shaker and let it remain there during sample preparation. At minimum, RGP should shake at 30ºC for ≥30 minutes at 800 RPM (see ***RGP Substrate Preparation for Simoa Assays*** for details). One RGP bottle is sufficient for up to 96 tests and should be used within 8 hours of shaking. Do not store and re-use RGP.

Step 2: Prepare washer and shaker.

- If buffers/DI water have been in bottles on the washer for longer than one month, discard, rinse containers with DI water, and add fresh buffers/DI water.
- Run the **System Flush** washer protocol to rinse the manifold with DI water.
- After the System Flush is finished, connect the Buffer A and Buffer B tubing to the correct buffer reservoirs and ensure that sufficient volume of each buffer is available.
- Ensure sufficient volume is available in the waste bottle.
- Set the temperature and speed on the shaker to the settings indicated in the 'Assay format' section of the table above.

Step 3: Downloads. Download the NF-Light v2 Advantage analysis protocol (http://portal.quanterix.com) for use in Step 14. Download the lot-specific Certificate of Analysis (CoA) from https://www.quanterix.com/coa by entering in the kit lot number (not calibrator lot number) to find the concentrations of the calibrators in this lot. Calibrators are single use.

Step 4: Assay plate preparation. Prepare your assay plate using conical bottom plates provided by Quanterix. This is the only plate type compatible with this protocol! Pipette the appropriate volume of prepared calibrators, controls, and samples into the wells. Place replicates in separate wells. **Calibrators** are run neat with **100 µL** added per well. **Spin samples at 10,000 x g for 5 minutes to precipitate debris.** Spin at least 10 µL more than the volume needed for the run to avoid transferring debris. Plasma or serum samples and controls should be diluted **4x** with Sample Diluent before distributing to individual wells or diluted in the plate by mixing **25 µL of sample** with **75 µL Sample Diluent** in each well.

Step 5: Capture bead addition. Vortex the beads for 30 seconds. Pour beads into a clean reagent reservoir. Using a multi-channel pipettor, dispense **25 µL of beads** into each well, touching off to the samples and changing tips between each column. Pre-wet tips for accurate volume transfer.

Note: Ensure that beads are distributed across the plate within 2 minutes to avoid settling in the reagent reservoir.

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Step 6: Detector addition. Pour the Detector into a clean reagent reservoir. Use a multi-channel pipettor to dispense **20 µL of Detector** into each well, touching off to the samples and changing tips between each column. Pre-wet tips for accurate volume transfer.

Step 7: Sample-bead-detector incubation. Carefully place plate on the shaker, cover the plate with a black lid, secure, and incubate at **30°C** at **800 rpm for 30 minutes**.

Step 8: Washer priming and first wash. Once the first incubation is started, start the **2-step protocol** on the washer to prime the manifold. When the sample-bead-detector incubation is complete, carefully remove the plate from the shaker, place it on the washer magnet (remove lid!), and execute the **first wash of the 2-step protocol**.

Step 9: SBG addition. Keep the plate on the magnet after the last wash aspiration is complete. Pour SBG into a clean reagent reservoir. Use a multi-channel, multi-dispense pipette to dispense **100 µL of SBG** into each well.

Step 10: Bead-SBG incubation: Carefully place plate on the shaker, cover the plate with a black lid, secure, and incubate at **30°C** at **800 rpm for 10 minutes**.

Step 11: Second wash. Place plate on the washer magnet (remove lid!) and continue the **2-step protocol** through the first addition of Buffer B.

Step 12: Buffer B wash/resuspension. Remove the plate from the magnet, place plate on the shaker, cover the plate with a black lid, secure, and shake at 800 rpm for 1 minute. Return the plate to the washer magnet (remove lid!) and press Continue. Repeat the Buffer B wash/resuspension one more time, following washer prompts.

Step 13: Plate drying. After completion of the two Buffer B wash/resuspension cycles, press Continue on the washer for final aspiration. After aspiration, **check all wells to ensure no residual volume or bubbles remain in the wells**. Remove any residual volume with a pipette. If bubbles are observed near the pellet, manually add 100 µL of Buffer B, let the bead pellet on magnet, and then aspirate to remove all liquid. **Leave plate on the magnet and allow to dry for 10 minutes.**

Step 14: Import analysis protocol and update calibrator concentrations. If necessary, import the **NF-light v2 Advantage** analysis protocol into the Quanterix SR-X software. Set up the Plate Layout for the run, ensuring the appropriate calibrator concentrations are entered for each plex (use values on the CoA for Reference Calibrator sets or use the values in the Kit Instructions if calibrators were diluted manually).

Step 15: Start run. After the 10-minute drying step is complete, transfer the plate with the pelleted beads onto the SR-X instrument, load RGP, and start the run. Runs should be started within one hour after drying beads. If the dry plate needs to be stored for a longer period of time, then leave it covered with the black lid at room temperature. The Buffer B wash/resuspension/drying steps should be repeated prior to starting the run if the plate is stored for longer than one hour.

Step 16: Washer maintenance. After the final run of the day is complete, perform another System Flush to ensure the manifold is clean. **Surface cleaning** is necessary daily. Note that buffer buildup on or around the tubing, manifold, or moving parts of the washer may affect future assay performance. If expecting to leave the washer idle for two weeks or longer, it is recommended to discard excess buffer and perform the Idle Maintenance procedure.

Consult the *Simoa Microplate Washer Maintenance Guide* for recommended care of the washer. For additional information on setting up assay runs, data analysis, and general operating procedures, refer to documents on the Quanterix Customer Portal at http://portal.quanterix.com, **which includes the *Quanterix SR-X User Guide* and the *Quanterix SR-X Quick Reference Guide*.**

WARNINGS AND PRECAUTIONS

For research use only. Not for use in diagnostic procedures.

Safety Precautions

CAUTION: Use of this product may require the handling of human specimens. It is recommended that all human-sourced materials be considered potentially infectious and be handled in accordance with the OSHA Standard on Bloodborne Pathogens.[1] Biosafety Level 2[2] or other appropriate biosafety practices[3] should be used for materials that contain or are suspected of containing infectious agents.

Simoa reagents contain methylisothiazolones, which are components of ProClin and are classified per applicable European Community (EC) Directives as: Irritant (Xi). The following are the appropriate Risk (R) and Safety (S) phrases.

R43 May cause sensitization by skin contact.
S24 Avoid contact with skin.
S35 This material and its container must be disposed of in a safe way.
S37 Wear suitable gloves.
S46 If swallowed, seek medical advice immediately and show this container or label.

REAGENTS PROVIDED

Simoa Advantage Reagent Kit for 96 Tests

Bead Reagent	1 bottle (4.4 mL/bottle)	Capture antibody coated beads in buffer with protein stabilizers.
Detector Reagent	1 bottle (3.8 mL/bottle)	Biotinylated detector antibody in buffer with protein stabilizers.
SBG Reagent	1 bottle (12.3 mL/bottle)	Streptavidin-ß-galactosidase (SBG) in buffer with protein stabilizers.
RGP Reagent	3 bottles (3.9 mL/ bottle)	Resorufin ß-D-galactopyranoside (RGP) in buffer with surfactant.
Reference Calibrators	2 sets (1 mL/vial)	Antigen in buffer with protein stabilizers.
Controls	2 sets (0.5 mL/vial	Antigen in buffer with protein stabilizers.
Sample Diluent	2 bottles (14.5 mL/bottle)	Buffer with protein stabilizers, a heterophilic blocker.

Simoa Evaluation Kit (item 104074)

Calibrator Concentrate	1 vial (200µL/vial)	Antigen in buffer with protein stabilizers.
Calibrator Diluent	1 bottle (28.8 mL/bottle)	Buffer with protein stabilizers
Sample Diluent	2 bottles (14.5 mL/bottle)	Buffer with protein stabilizers, a heterophilic blocker.

Additional Equipment and Materials Required

- Quanterix SR-X Instrument
- Simoa Microplate Washer
- Simoa Plate Shaker
- Simoa SR-X System Wash Buffer A and Wash Buffer B
- DI Water
- Simoa SR-X Sealing Oil
- Simoa Discs, Pipette Tips, 96-well Microplates, Plate Lids (included in Simoa disc kit)
- Multi-channel pipettor and tips (for 20-150 µL volumes)
- Multi-channel repeat pipettor and tips (50-1200 µL)

PRINCIPLES OF THE SIMOA BEAD ASSAY

Simoa® Assay Kits contain reagents for running tests to quantify target analyte(s) on the Simoa SR-X Instrument.[4]

Simoa Immunoassays have either 2 or 3 incubation steps. In a 2-step immunoassay, target antibody coated paramagnetic beads are combined with sample and biotinylated detector antibody in the same incubation. Target molecules present in the sample are captured by the antibody coated beads and bind with the biotinylated antibody detector simultaneously.

In a 3-step assay, target antibody coated paramagnetic beads are combined and incubated with sample alone. Target molecules present in the sample are captured by the antibody coated beads. After washing, biotinylated detector antibodies are mixed and incubated with the beads. The detector antibodies bind to the captured target during this additional incubation.

Following a wash, a conjugate of streptavidin-ß-galactosidase (SBG) is mixed with the beads. SBG binds to the biotinylated detector antibodies, resulting in enzyme labeling of captured target. Following a final wash, the beads are resuspended in a resorufin ß-D-galactopyranoside (RGP) substrate solution and transferred to the Simoa Disc. Individual beads are then sealed within microwells in the array. If the target has been captured and labeled on the bead, ß-galactosidase hydrolyzes the RGP substrate in the microwell into a fluorescent product that provides the signal for measurement.

A single labeled target molecule results in sufficient fluorescent signal in 30 seconds to be detected and counted by the Simoa optical system. At low target concentration, the percentage of bead-containing wells in the array with a positive signal is proportional to the amount of target present in the sample. At higher target concentration, when most of the bead-containing wells have one or more labeled target molecules, the total fluorescence signal is proportional to the amount of target present in the sample. The concentration of target in unknown samples is interpolated from the calibration curve.

BIBLIOGRAPHY

1 US Department of Health and Human Services. Biosafety in micro-biological and biomedical laboratories, 4th ed. Washington, DC: US Government Printing Office, May 1999.

2 World Health Organization. Laboratory biosafety manual. Geneva: World Health Organization, 2004.

3 Clinical and Laboratory Standards Institute. Protection of laboratory workers from occupationally acquired infections: Approved guideline, 3rd ed. CLSI Document M29-A3. Wayne, PA: Clinical and Laboratory Standards Institute, 2005.

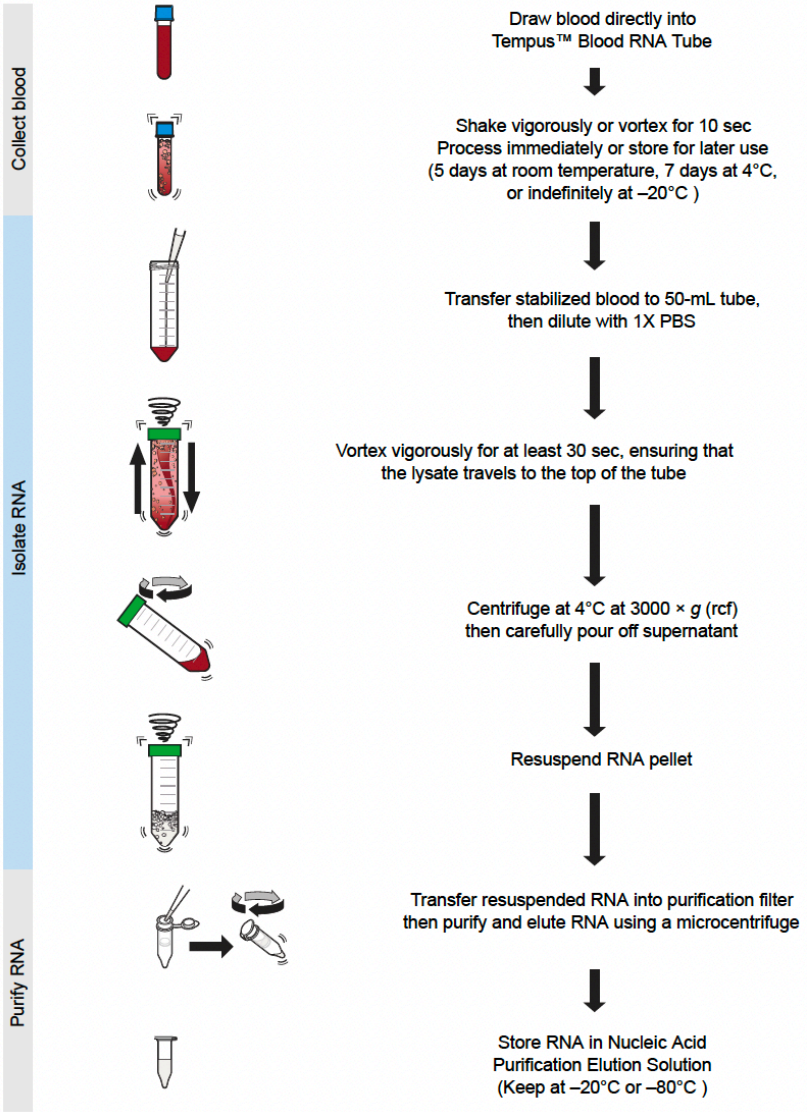
4 Rissin DM, Kan CW, Campbell TG, et al. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. Nat Biotech 2010; 28:595–99.

Appendix E. Blood RNA extraction protocol (Tempus)

Centrifuge protocol workflow

This protocol describes the steps required to use the Spin Kit to purify RNA from a 3-mL sample of whole blood collected in a Tempus tube. This protocol can also be used with the Tempus Sample Kit.

The following diagram provides an overview of the procedure described in this protocol.



Guidelines for extracting RNA

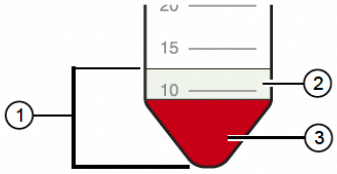
Tempus™ Spin RNA Isolation Kit is used to isolate and purify RNA from whole blood collected with Tempus™ Blood RNA Tubes. The RNA isolated in this procedure contains very low levels of genomic DNA (<0.05% by weight).

- Discard the blood-containing wastes following recognized disinfection procedures and in accordance with all local, state, and national bloodborne/infection regulations.
- Adjust the final concentration of the stabilizing reagent to a final concentration of 1X by diluting the stabilized blood with calcium- and magnesium-free phosphate-buffered saline (PBS) before extracting RNA for purification. Failure to do so results in significantly lower RNA yields.
- Keep the samples on ice as much as possible to prevent possible decrease in RNA yields.

Isolate RNA

1. If the sample is frozen, thaw the sample in the Tempus™ Blood RNA Tube at room temperature (18–25°C).
2. Remove the cap from the Tempus™ Blood RNA Tube, then pour the contents into a clean 50-mL conical tube.

3. Add 3 mL of 1✕ PBS (Ca^{2+}/Mg^{2+}-free) into the conical tube to bring the total volume to 12 mL.



① 12 mL total volume
② 3 mL 1X PBS
③ 3 mL blood mixed with 6 mL 2X stabilizing reagent

Note: If the initial blood sample is less than 3 mL, make up the difference by adding enough 1X PBS to bring the total volume to 12 mL.

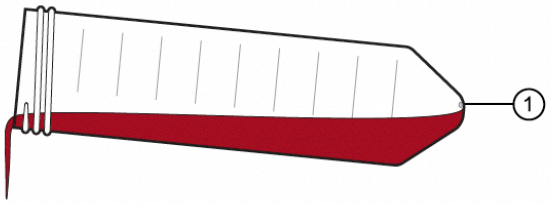
4. Replace the cap on the conical tube, then vortex the tube vigorously (at maximum vortex speed) for 30 seconds to ensure proper mixing of the contents.

   Note: Make sure the conical tube is capped properly to prevent the contents from leaking or spraying out during vortexing.

   Note: Vortexing for less than 30 seconds may cause clogging of the purification column. A layer of froth over the sample after vortexing is normal.

5. Centrifuge the tube at 4°C at 3,000 × *g* (rcf) for 30 minutes.

6. Carefully pour off the supernatant. Handle the conical tube carefully so that you do not dislodge the RNA pellet off the bottom of the tube.



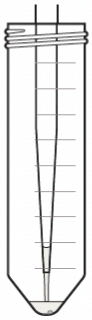
① RNA pellet (transparent and invisible)

7. Leave the conical tube inverted on absorbent paper for 1 to 2 minutes.

8. Blot the remaining drops of liquid off the rim of the conical tube with clean absorbent paper.

9. Add 400 µL of RNA Purification Resuspension Solution into the conical tube, then vortex briefly to resuspend the RNA pellet.

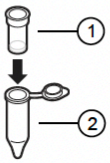
   Note: To prevent washing any blood residue down the inside of the tube, insert the pipette tip into the tube and add the resuspension solution to the bottom of the tube.



10. Proceed to "Purify RNA". The resuspended RNA can be kept on ice while preparing for the next steps.

Purify RNA

1. Label the RNA purification filter, then insert the filter into a waste collection tube.

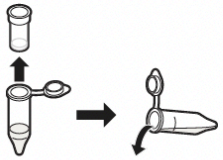


① RNA purification filter
② Waste collection tube

2. Pre-wet the filtration membrane by adding 100 µL of RNA Purification Wash Solution 1 into the purification filter.

3. Add ~400 µL of the resuspended RNA into the purification filter, then centrifuge for 30 seconds at 16,000 × *g*.

4. Remove the purification filter, discard the liquid waste collected in the waste tube, then re-insert the purification filter into the waste tube.



Note: Each time you discard the liquid waste, instead of reusing the waste tube, you can transfer the purification filter into a new collection tube (not provided in the kit). See **15**for ordering information.

5. Add 500 µL of RNA Purification Wash Solution 1 into the purification filter, then centrifuge for 30 seconds at 16,000 × *g*.

6. Remove the purification filter, discard the liquid waste collected in the waste tube, then re-insert the purification filter into the waste tube.

7. Add 500 µL of RNA Purification Wash Solution 2 into the purification filter, then centrifuge for 30 seconds at 16,000 × *g*.

8. Remove the purification filter, discard the liquid waste collected in the waste tube, then re-insert the purification filter into the waste tube.

9. Add 500 µL of RNA Purification Wash Solution 2 into the purification filter, then centrifuge for 30 seconds at 16,000 × *g*.

10. Remove the purification filter, discard the liquid waste collected in the waste tube, then re-insert the purification filter into the waste tube.

11. Centrifuge for 30 seconds at 16,000 × *g* to dry the membrane.

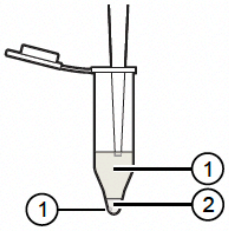
12. Transfer the purification filter to a new, labeled collection tube to collect the eluate.

13. Add 100 µL Nucleic Acid Purification Elution Solution into the purification filter, close the cap, incubate the entire tube for 2 minutes at 70°C, then centrifuge for 30 seconds at 16,000 × *g*.

14. Add ~100 µL of the collected RNA eluate back into the purification filter, then centrifuge for 2 minutes at 16,000–18,000 × *g*. No incubation is necessary.

15. Discard the purification filter, then transfer approximately 90 µL of the RNA eluate to a new, labeled collection tube.

    Note: When transferring the RNA eluate, carefully remove the liquid from the collection tube starting from the top of the liquid to ensure that the pellet is not disturbed.



① ~90 µL of eluate (transfer to new tube)
② ~10 µL (do not disturb)
③ Pellet

16. Replace the cap on the new collection tube, then store the RNA at –20°C, or –80°C for long-term storage.