IMPROVING THE DIAGNOSIS AND TREATMENT OF CHRONIC NEUROPATHIC PAIN

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Abstract

Chronic neuropathic pain (CNP) occurs as a consequence of injury to the nervous system. Despite recent advances, CNP lacks objective diagnostic criteria, is often unrelenting and refractory to treatment. The primary aims of this thesis are twofold; the identification of CNP biomarkers using both human cohorts and an animal model (spinal nerve ligation; SNL) of neuropathic pain, and to provide clarity on the role of GTP cylcohydrolase I (*GCH1*) in CNP.

Analysis of *GCH1* and related genes and metabolites was conducted. As biomarkers, nitrite/nitrate and neopterin did not differentiate controls from CNP patients. However, significant differences were observed with biopterins, whilst correlations were observed between *GCH1*, nitrite/nitrate and neopterin, which were notably stronger in patients than controls. Analysis in human cohorts and in the SNL model also inferred that downregulation of *GCHFR* may contribute to BH_4 synthesis.

In order to provide clarity on the role of the *GCH1* pain protective haplotype, reporter gene assays were used. This demonstrated a potential regulatory role for the *GCH1* 5' SNP (rs8007267). *In silico* prediction of transcription factor binding sites suggested that this may be mediated by the aryl hydrocarbon nuclear translocator. The use of electrophoretic mobility shift assays showed strong specific binding with probe pertaining to the major allele. Further analysis is required to elucidate transcription factor binding, potentially facilitated by 2D-PAGE and mass spectrometry.

In order to further elucidate potential CNP biomarkers, microarray analysis and qRT-PCR were performed using blood obtained from CNP patients. Data refinement led to the isolation of 27 potential CNP biomarkers, of which several cross-validated between cohorts. Microarray data, literature evidence, and correlations with previous microarrays provided evidence suggestive of a role for *TIMP1*. Multiple other genes, including *CASP5, TLR4, TLR5, MC1R* and *CX3CR1*, were differentially regulated in CNP. Genes surviving microarray data refinement were subsequently analysed in the dorsal horn of Sprague Dawley and Wistar Kyoto rats after SNL. Several genes, including *Dpp3, Mc1r* and *Timp1*, were similarly differentially expressed in the rodent SNL model, which suggests that these genes may be involved in the pathophysiological mechanisms of CNP, and may also function as potential translational biomarkers of CNP.

This work provides multiple avenues for expansion and further investigation. Clearly, the challenges associated with biomarker discovery in CNP states are considerable, though it is hoped that this thesis provides valuable insight and the necessary foundation for future work.

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List of abbreviations

˚C	degrees celsius
B	biopterin
BH_2	dihydrobiopterin
BH_4	tetrahydrobiopterin
bp	base pairs
BSA	bovine serum albumin
CCI	chronic constriction injury
CFA	Complete Freund's Adjuvant
CIBP	chronic inflammatory back pain
CINP	chemotherapy-induced neuropathic pain
CNBP	chronic neuropathic back pain
CNP	chronic neuropathic pain
CNS	central nervous system
Cq	quantification cycle
CSF	cerebrospinal fluid
CRPS	complex regional pain syndrome
CT	computed tomography
DAHP	2,4-diamino-6-hydroxypyrimidine
CV	coefficient of variation
DAMPs	danger associated molecular patterns
DAVID	Database for Annotation, Visualisation and Integrated Discovery
ddPCR	droplet digital polymerase chain reaction
DH	dorsal horn
DN4	*Douleur neuropathique en 4 questions*
DRG	dorsal root ganglion
FCS	fetal calf serum
fMRI	functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
HEK293	human embryonic kidney 293
HIV	human immunodeficiency virus
HUVECs	human umbilical vein endothelial cells
IASP	International Association for the Study of Pain
IPA	Ingenuity Pathway Analysis
kb	kilobases
LANSS	Leeds Assessment of Neuropathic Symptoms and Signs
mb	megabases
min	minute
LPS	lipopolysaccharide
NeuPSIG	Neuropathic Pain Special Interest Group
NMDA	N-methyl-d-aspartate
NNT	number needed to treat
NPQ	Neuropathic Pain Questionnaire
NPSI	Neuropathic Pain Symptom Inventory
PAMPs	pathogen associated molecular patterns
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism

PET	positron emission tomography
PHN	postherpetic neuralgia
PNI	peripheral nerve injury
PNS	peripheral nervous system
qRT-PCR	quantitative real time polymerase chain reaction
QST	quantitative sensory testing
RIN	RNA integrity number
sec	second
SERT	serotonin transporter
S-LANSS	Leeds Assessment of Neuropathic Symptoms and Signs (self-reporting)
SNI	spared nerve injury
SNL	spinal nerve ligation
SNP	single nucleotide polymorphism
SNRI	serotonin noradrenaline reuptake inhibitor
SNT	sciatic nerve transection
SSRI	selective serotonin reuptake inhibitor
StEP	Standardised Evaluation of Pain
TCA	tricyclic antidepressant
TENS	transcutaneous electrical nerve stimulation
TGN	trigeminal neuralgia
TRPV1	transient receptor potential vanilloid 1
VAS	visual analogue scale
VRS	verbal rating scale

Chapter 1 Introduction

1.1 Introduction

According to the International Association for the Study of Pain (IASP), pain is described as an 'unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (IASP 1979). The definition presented by IASP represents a culmination of theories presented over several centuries (Moayedi *et al.* 2013). Prior to the gate control theory, pain was considered to be a 'sensory phenomenon with emotions described as reactions' (Melzack *et al.* 1965), though the wider multi-dimensional aspects of pain were subsequently described, encompassing sensory-discriminative, motivational-affective, and cognitive-evaluative aspects (Casey 1968, Turk *et al.* 2010). Pain is therefore best summarised as a complex amalgamation of somatic mechanisms and psychological influences (Bennett 2011).

The differentiation between pain and nociception has long-since been established (Descartes 1662), with pain recently regarded as a 'homeostatic emotion' (Craig 2003). Acute pain is a necessary and relatively short-lived indicator of a specific disease or injury, thereby representing a vital biological function. However, chronic pain, which persists for at least 3 months and fails to relent beyond recovery from the causative injury or organic disease, is generally thought to possess no clearly defined biological purpose (Grichnik *et al.* 1991, Turk *et al.* 2011). It has, however, been argued that the vital need to avoid potentially life-threatening injury places the axis of sensitivity and specificity within the nervous system towards sensitivity, which may predispose one to the development of chronic pain (Brodal 2010, Bäckryd 2015). Indeed, the vital importance of sensitive detection of noxious stimuli is demonstrated in people with a congenital insensitivity to pain. They are unable to detect painful stimuli, fail to undertake appropriate avoidance measures, and subsequently are at great risk of serious and potentially life-threatening injury (Verheyen *et al.* 2007, Basbaum *et al.* 2009, Cox *et al.* 2010).

1.2 Chronic Pain

Chronic pain is a tremendous global health problem. A staggering one in five adults suffer from moderate to severe chronic pain, and in Europe alone, one in five of those afflicted with chronic pain have endured it for over 20 years (Breivik *et al.* 2006). Chronic pain implicates people of all populations, regardless of ethnicity, age, gender and geographical location, although frequencies

and distributions are not universal. Amongst the leading predicted causes of death worldwide by 2030, diseases and incidences associated with chronic pain, including chronic neuropathic pain (CNP), are highly represented (WHO 2008). These include stroke (central post stroke pain), diabetes mellitus (painful diabetic peripheral neuropathy), HIV (HIV-associated neuropathy), lung cancer (related skeletal metastases and chemotherapy-induced peripheral neuropathy) and road-traffic accidents.

Traditionally perceived as merely a symptom of disease, chronic and recurrent pain is gradually becoming considered a disease its own right (Niv *et al.* 2001). Osteoarthritis, herniated discs, traumatic injury and nerve damage are amongst the most frequent causes of chronic pain (Breivik *et al.* 2006), thus highlighting a diverse range of medical specialties wherein patients with chronic pain seek treatment. Indeed, many clinicians managing patients exhibiting symptoms of CNP are often restricted by both time and skill to complete extensive neurological examination (Bennett *et al.* 2007b). This illustrates the potential for mismanagement, suboptimal treatment and failure to implement optimal and individualised treatment by specialist pain clinicians. Such circumstances undoubtedly contributes to the impetus for novel diagnostic methodology.

Whilst acute pain is generally self-limiting with relatively modest or restricted long-term health implications, chronic pain often severely impacts upon long-term quality of life. People with chronic pain often develop additional physiological manifestations, including the loss of weight due to changes in appetite, poor nutrition and restricted mobility. Chronic pain is also synonymous with negative psychological changes, including major depressive disorder and anxiety (Surah *et al.* 2013). These are triggered and exacerbated by multiple factors often associated with inaccurate diagnosis and suboptimal treatment, including loss of employment, social isolation and deprivation of sleep.

The socioeconomic implications of chronic pain are pronounced. Chronic pain is more common in manual laborers (Saastamoinen *et al.* 2005) and deprivation was identified as a factor predisposing patients with type 1 and 2 diabetes mellitus to severe CNP (Anderson *et al.* 2014, Anderson *et al.* 2015). Moreover, persons with chronic non-malignant pain were seven times more likely to cease employment on the basis of poor health than those without chronic pain (Eriksen *et al.* 2003). In the United States, the economic costs of persistent pain are greater than the cost associated with other diseases synonymous with major economic implications, including cardiovascular disease, cancer and diabetes (Gaskin *et al.* 2011).

1.3 Basic anatomy of pain perception

Nociceptive pain is typically divided into somatic and visceral pain. Visceral pain is dull, diffuse and largely associated with pathology of the internal organs. In contrast, somatic pain can either originate in deep tissue, which tends to be relatively dull in nature, or from superficial tissues, which is comparatively sharp and distinct. Primary nociceptive afferent fibres innervating the head and body originate from cell bodies located in the trigeminal and dorsal root ganglion, respectively. These nociceptive fibres are excitatory and release glutamate, in addition to neuropeptides, contributing to afferent central signalling (Dubin *et al.* 2010). They fall into two main classifications, Aδ and C fibres. Myelinated large diameter Aβ fibres exhibit relatively slow conduction velocity and generally respond to innocuous stimuli such as touch sensations (Julius *et al.* 2001). Medium diameter thinly myelinated Aδ fibres and small diameter unmyelinated C fibres are responsive to mechanical, thermal and chemical stimuli. Whilst C-fibre branches are relatively diffuse and spread widely, A-fibres tend to cluster, giving distinct localisation of the stimulus (Dubin *et al.* 2010). The Aδ nociceptors therefore mediate localised, acute and sharp pain sensations, whereas C fibres mediate a delayed, comparatively diffuse and dull response to noxious stimuli (Julius *et al.* 2001).

Variable transduction mechanisms exist pertaining to heat, cold and mechanical stimulation, which if of sufficient strength, trigger depolarisation and subsequent transduction, thereby conferring information pertaining to the duration and intensity of the stimulus (Dubin *et al.* 2010). The intricate transduction mechanisms pertaining to these stimuli have been described (Dubin *et al.* 2010). Both Aδ and C fibres express specific receptors responding to noxious stimuli such prostaglandin and acid-sensing receptors, promoting impulse transmission to the DRG and subsequently, to the dorsal horn (DH). The DH allows for modulation of signalling, permitting excitation and 'wind up' or inhibition (Figure 1.1). The primary afferent nociceptors (Aδ and C fibres) synapse with second afferent neurons in the DH, which is histologically divided into distinct laminae. Specifically, these afferent terminals release excitatory neurotransmitters and interact with inhibitory and excitatory interneurons and descending pathways, to modulate the activity of secondary afferent neurons. Second order neurons decussate and travel via the spinothalamic tract to the ventral posterolateral nucleus of the thalamus. Third order neurons travel from thalamus to the somatosensory cortex and anterior cingulate cortices, enabling the sensory discriminative and affective-cognitive responses to pain (Millan 1999).

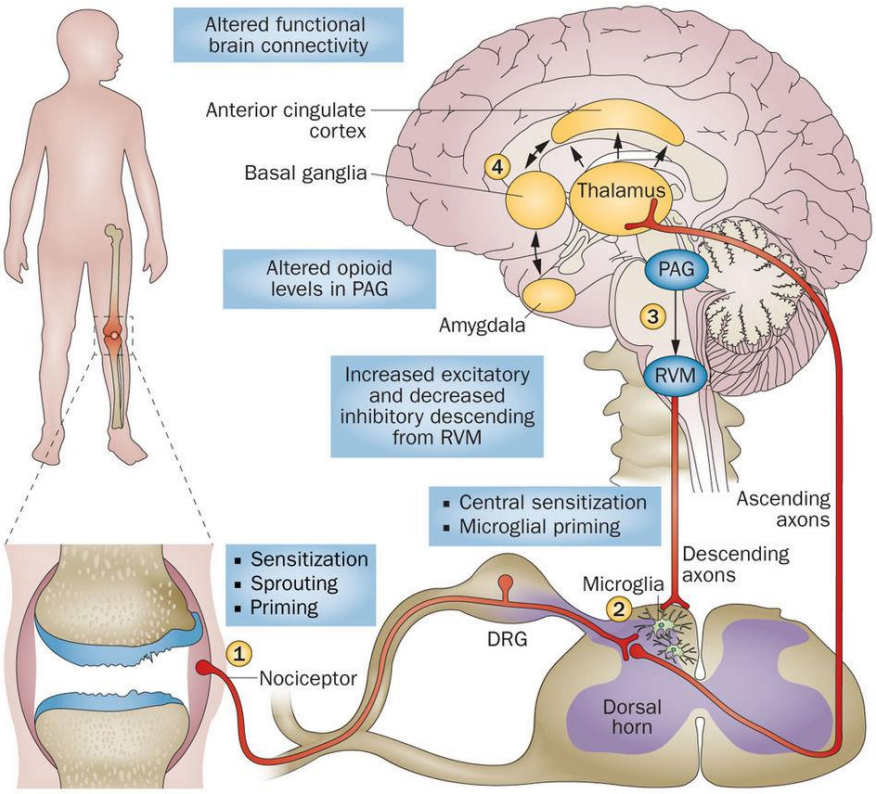

Figure 1.1: Basic representation of nociceptive pathways

Pictorial representation of nociceptive pathways with reference to joint pain. (1) Nociceptor sensitisation and spouting may occur within the implicated anatomy followed by (2) the development of central sensitisation. (3) Changes in descending activity may also occur with increased excitatory and reduced inhibitor input from the RVM, alongside (4) alterations in functional brain connectivity within regions associated with pain processing, such as the amygdala and basal ganglia. Abbreviations: DRG, dorsal root ganglion; PAG, periaqueductal grey; RVM, rostroventral medulla. Image adapted with permissions (de Lalouviere *et al.* 2014).

1.4 Neuropathic pain

1.4.1 Definition

The unique symptoms associated with neuropathic pain have long-since been differentiated from nociceptive pain. In 1872 Silas Weir Mitchell detailed the chronicity and psychological implications of nerve pain, describing causalgia as “the most terrible of all tortures, which a nerve wound may inflict” (Mitchell 1872). Our understanding of the mechanisms underlying neuropathic pain have progressed significantly more recently, but a clear consensus on an appropriate definition demonstrates that there is much to be determined. Neuropathic pain was described by the IASP in 1997 as ‘pain initiated or caused by a primary lesion or dysfunction of the nervous system’ (Merskey *et al.* 1997). This was modified by the Neuropathic Pain Special Interest Group

(NeuPSIG) which the replaced the terms ‘dysfunction’ and ‘nervous system’ with ‘disease’ and ‘somatosensory system’, respectively, to read ‘pain arising as a direct consequence of a lesion or disease affecting the somatosensory system’ (Treede *et al.* 2008). This required a specific lesion of the somatosensory system to be identified as the underlying aetiology of neuropathic pain, rather than generalised dysfunction (Cruccu *et al.* 2010). The definition was again adjusted by IASP to define neuropathic pain as ‘pain caused by a lesion or disease of the somatosensory nervous system’ (Jensen *et al.* 2011), though this has been considered to be somewhat limited in scope considering the variable aetiologies associated with CNP. Indeed, there are arguments both for and against the overarching classification of neuropathic pain (Bennett 2011).

1.4.2 Epidemiology

The population prevalence of neuropathic pain syndromes is difficult to precisely ascertain. This is primarily due to obstacles facing patient presentation in primary care and the subsequent challenges encountered by primary care physicians in achieving an accurate differential diagnosis when using the case identification tools (section 1.4.6.2) and clinical standards currently available (Bennett 2011). Epidemiological studies seeking to determine the frequency of painful neuropathies have thus far provided a range of approximations, but such studies are not only constrained by the aforementioned limitations, there is also significant methodological heterogeneity between the studies, rendering meta-analysis impractical (van Hecke *et al.* 2014). Moreover, multiple studies are also limited in their potential to reflect the prevalence of CNP in the general population, as they were conducted on a specific subpopulation, such as those in secondary care or were inclusive of persons with specific occupations (van Hecke *et al.* 2014). Several studies have sought to determine the incidence of disease-associated CNP in primary care using general medical practice records. This benefits from not requiring direct patient involvement and as such, these studies tend to consist of comparatively large sample sizes. Two epidemiological studies undertaken in the UK using primary care records, which encompassed an accumulative sample size of approximately 10 million patients, sought to determine the incidence of postherpetic neuralgia (PHN), trigeminal neuralgia (TGN), phantom limb pain and painful diabetic peripheral neuropathy, with notable variation (Hall *et al.* 2006, Hall *et al.* 2008). Despite methodological consistencies between the two studies, nearly two fold disparity was observed in the population incidence of phantom limb pain (0.8-1.5 cases per 100,000 patient years) and painful diabetic peripheral neuropathy (15.3-26.7 cases per 100,000 patient years). This does, however, depict relative agreement when compared to a separate postulated incidence rate for painful diabetic

peripheral neuropathy of 72.3 cases per 100,000 patient years (Dieleman *et al.* 2008). When considering studies with comparable methodology, wide variation remains evident with regards to post herpetic neuralgia (3.9 to 42.0 per 100,000 person years) (Jih *et al.* 2009, Koopman *et al.* 2009) and TGN (12.6 to 28.9 per 100,000 patient years) (Koopman *et al.* 2009, van Hecke *et al.* 2014).

The general population prevalence of CNP, or chronic pain with a neuropathic component, regardless of causation or specific pain diagnosis (e.g. TGN), has also been the subject of several epidemiological studies. Many of these studies utilised self-reporting questionnaires which clearly benefits from the inclusion of 'silent sufferers', or those who have not sought medical attention, which is thought to account for a reputed 22.4% of people with chronic pain (Watkins *et al.* 2006). One of the main limitations of self-reporting screening tools is the potential for misinterpretation. The sensitivity and specificity of screening tools, such as the self-reporting version of the Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS), is greater when used in interview format than unaided completion (Bennett *et al.* 2005), with both scenarios exhibiting a compromise from the 85% sensitivity and 80% specificity observed with the LANSS tool (Bennett 2001). Two postal surveys, conducted in France and the UK, deduced the prevalence of chronic pain with neuropathic characteristics to be 6.9% and 8.2%, using the self-administered *Douleur neuropathique en 4 questions* (DN4) and S-LANSS screening tools, respectively (Torrance *et al.* 2006, Bouhassira *et al.* 2008). In contrast, separate studies undertaken in the Americas using the DN4 screening tool concluded the prevalence of signs suggestive of neuropathic pain to be 17.9% (Canadian population) (Toth *et al.* 2009) and when using the DN4 tool alongside physical examination, the prevalence of neuropathic pain was 10% (Brazilian population) (de Moraes Vieira *et al.* 2012). The Canadian study however, used relatively relaxed inclusion criteria, providing the DN4 screening tool to all respondents reporting "daily or near daily pain" (Toth *et al.* 2009, van Hecke *et al.* 2014). This was in contrast to an Austrian study which deduced a prevalence of 3.3% but included multiple strict exclusion criteria, including positive response to over-the-counter analgesia and patients with an undifferentiated cause to their pain (Gustorff *et al.* 2008, van Hecke *et al.* 2014). Two studies undertaken in the UK using medical records determined he presence of chronic pain with neuropathic characteristics to be 0.9% and 1.3% (Gore *et al.* 2007, Gajria *et al.* 2011). However, the use of medical records necessitates accurate diagnosis followed by the accurate recording of information using a recognised medical coding system, such as Read and OXMIS codes (Hammad *et al.* 2008), in addition to the fundamental prerequisite of

patients needing to report their neuropathic symptoms in primary care. The limitations and variability between screening methodology was exemplified by an epidemiological and screening tool analysis undertaken in the USA, which showed that, of 64.4% of responders claiming chronic pain, 13.7% were determined neuropathic by S-LANSS score (≥12), which contrasted with 9.8% by clinical examination, 3% by Berger criteria (medical records) and 12.4% by self-reporting (Yawn *et al.* 2009).

Taken together, epidemiological studies provide variable estimates for the prevalence of CNP, ranging from 0.9 to 17.9% (Gore *et al.* 2007, Toth *et al.* 2009). When considering only studies utilising screening methods designed and validated for the way in which they were used (van Hecke *et al.* 2014), the prevalence estimates narrows to more generally accepted estimates of 6.9-10% (de Moraes Vieira *et al*. 2012, Yawn *et al*. 2009). The use of standardised screening methodology would undoubtedly facilitate improvements in epidemiological research. The current lack of consistent and comparable epidemiological data is perhaps an indictment of the current diagnostic criteria and tools available to clinicians, with a clear reliance on questionnaire based case identification tools. The identification of novel, reliable and easily accessible diagnostic biomarkers would undoubtedly provide the necessary foundation for comprehensive epidemiological studies.

1.4.3 Mechanisms of neuropathic pain

Neuropathic pain is maladaptive and often arises through abnormal activation of pain pathways resulting from injury to the peripheral nervous system or brain and spine. Peripheral nerve injury, which may, for instance, occur after crush injury, can lead to nociceptor sensitisation, thereby lowering transmission thresholds in the injured nerve and DRG to various stimuli, including cold, heat and mechanical force. Such ectopic discharge is somewhat underpinned by injury-induced sodium channel accumulation (England *et al.* 1996). This accumulation is often localised to the neuroma, a gathering of regenerative nerve spouts which arises at the proximal nerve stump after injury. Ephaptic cross-talk between damaged and undamaged neurons can also occur due to sprouting-related increases in the receptive field, leading to synchronous neurotransmission of damaged and undamaged nociceptors, thereby increasing afferent input (Bennett 2011).

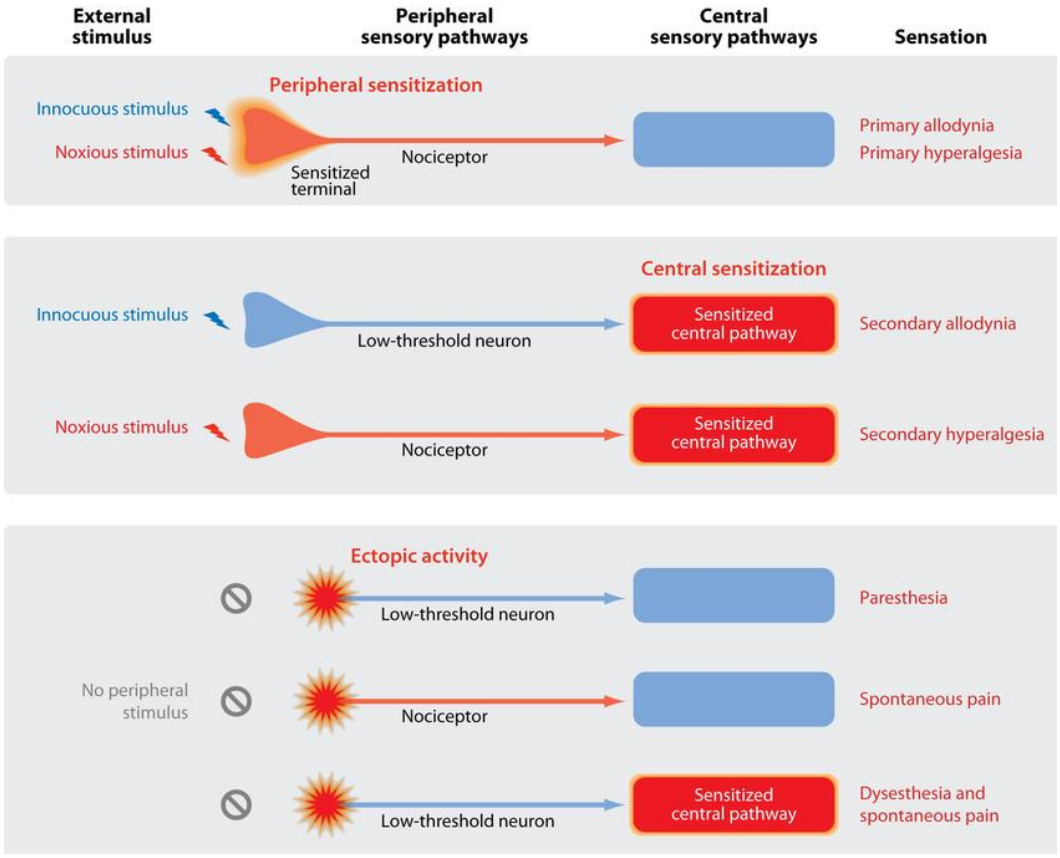

Figure 1.2: Representation of the mechanisms by which allodynia and hyperalgesia occur after injury

After injury peripheral sensitisation may occur. Subsequent innocuous and noxious stimuli lead to the development of primary allodynia and hyperalgesia, which is mediated by nociceptors (Aδ and C fibres). The development of central sensitisation leads to allodynia resulting from innocuous stimulus and is mediated by low-threshold neurons (e.g. Aβ fibres). Secondary hyperalgesia is often present. Pain may also occur in the absence of external input due to ectopic activity, leading to parasthesia, dysesthesia and spontaneous pain. Image adapted (Costigan *et al.* 2009b).

Another source of change contributing to development and maintenance of CNP is central sensitisation, which is underpinned by CNS plasticity. Central sensitisation may be triggered by repeated high intensity afferent input from sensitised peripheral nociceptors (Figure 1.2), or a reduction in inhibitory pathways (Woolf 1983). Moreover, in the non-injured DH, low threshold (Aβ) mechanoreceptors terminate within laminae III and IV, whilst high-threshold nociceptors, including C-fibres and Aδ-fibres, terminate within laminae I and II. However, after peripheral nerve injury, Aβ spouting towards laminae II may occur (Woolf *et al.* 1992). Such innervation is thought to contribute to painful sensations mediation by Aβ transmission. On a molecular level,

multiple complex changes occur after nerve injury, some of which are strongly mediated through immune cell migration and microglial activation (Figure 1.3).

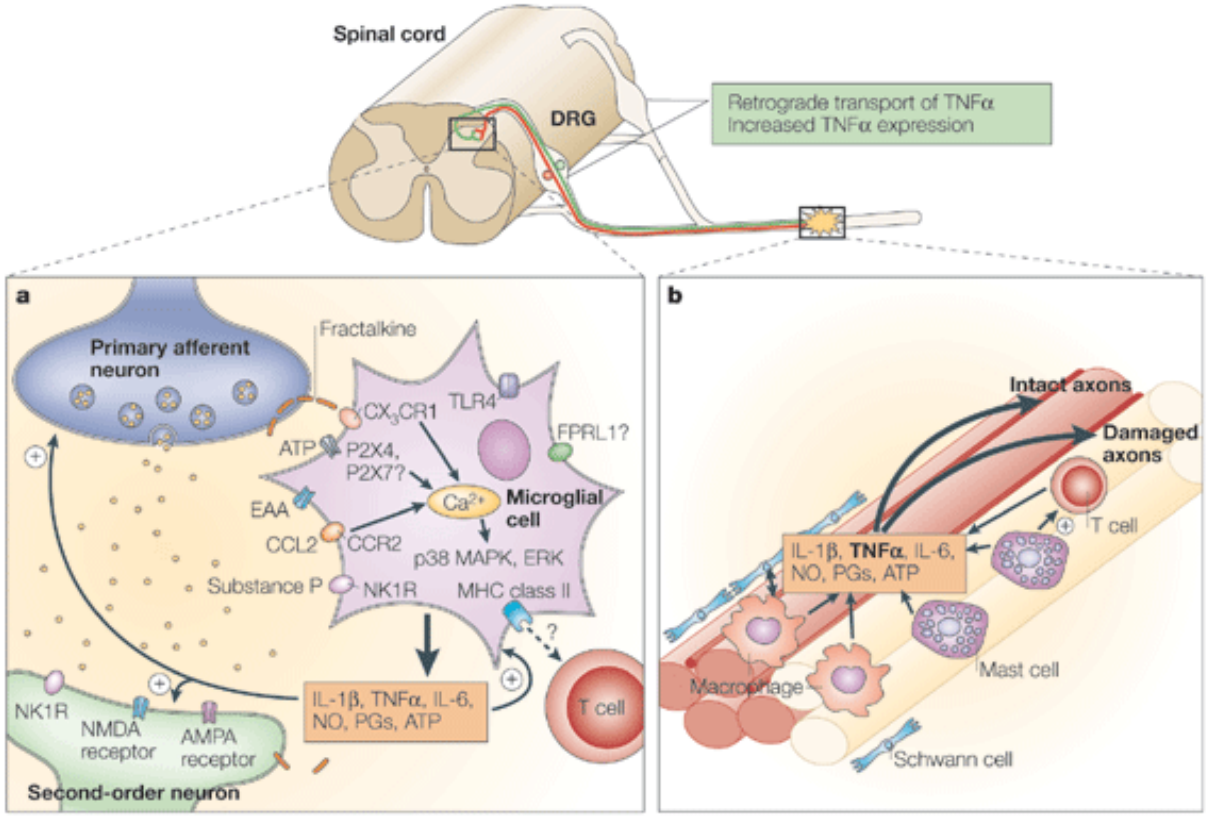

Figure 1.3: Activation of multiple convergent pathways in the injured nerve and dorsal horn

(A) The development of neuropathic pain is synonymous with microglial activation, resulting in the release of cytokines, chemokines and other pain-modulating agents, which increases pain through presynaptic and postsynaptic mechanisms. The release of these agents also results in a degree of positive feedback, leading to p38 MAPK activation. (B) At the site of injury the recruitment and proliferation of several cells, including macrophages, T cells and Schwann cells, are a common feature. These cells release multiple cytokines, chemokines, prostaglandins (PGs) and nitric oxide (NO), which contributes to sensory abnormalities. Retrograde transport may also contribute to changes in the dorsal root ganglion. AMPA, amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CCR2, CCL2 receptor; CX3CR1, fractalkine receptor; EAA, excitatory amino acids; ERK, extracellular signal-regulated kinase; FPRL1, formyl peptide receptor-like 1; MHC, major histocompatibility complex; NGF, nerve growth factor; NK1R, neurokinin-1 receptor; NMDA, N-methyl-D-aspartate; P2X4, P2X7, ionotropic purinoceptors; p38 MAPK, p38 mitogen-activated protein kinase; TLR4, Toll-like receptor 4. Image and legend adapted with permission (Marchand *et al.* 2005).

1.4.4 Common causes of CNP

The underlying causes of nerve damage triggering the development of CNP are both numerous and diverse (Table 1.1), and are often categorised as either resulting from central or peripheral neuropathology. One of the most common causes of peripheral neuropathic pain is diabetes, which affects around 415 million people worldwide (Federation 2015). It is estimated that 15-20% of people with diabetes will develop painful distal symmetrical polyneuropathy (Tesfaye *et al.* 2013), which generally affects the toes and foot, but may gradually progress proximally though the limb (Schreiber *et al.* 2015). The prevalence of painful diabetic neuropathy is perhaps only superseded by that of CNP associated with the lower back (Galluzzi 2005). Other relatively common causes include trauma or injury, often as a consequence of crush injury or amputation, and PHN, a long-term complication of herpes zoster. Central CNP may emerge after stroke, spinal cord injury or as a consequence of the pathological processes of neurological diseases such as multiple sclerosis, including neuroinflammation, demyelination, and axonal damage (Khan *et al.* 2014)

Table 1.1: Various peripheral and central causes of neuropathic pain

Peripheral nervous system	Central nervous system
Chemotherapy-induced neuropathy	Central post-stroke pain
Complex regional pain syndrome	Multiple sclerosis
Low back pain with nerve root involvement	Spinal cord injury
HIV	Spinal cord ischaemia
Neuroma	
Painful diabetic neuropathy	
Postherpetic neuralgia	
Post-surgical pain (e.g. phantom limb pain)	
Trigeminal neuralgia	
Tumour infiltration	

Table adapted and modified (Bennett 2011).

1.4.5 Common symptoms

The symptoms associated with neuropathic pain are relatively diverse in their presentation. Pain tends to be either paroxysmal or persistent and is generally categorised into either positive or negative symptoms. Negative symptoms include sensory deficits often presented as hypoesthesia (loss of sensation to sensory stimuli). Positive symptoms include allodynia (pain in response to innocuous stimulus), hyperalgesia (heightened pain sensation in response to a modestly painful stimulus) and dysesthesias (often presenting as incapacitating burning or lancinating pain in response to touch).

1.4.6 Diagnosis

1.4.6.1 Clinical Examination

The purpose of clinical examination when neuropathic pain is suspected, is to determine altered function of the nervous system in order to obtain supportive evidence, such as that pertaining to a lesion of the somatosensory system, rather than to reach a definitive diagnosis (Haanpaa *et al.* 2011). Neuropathic pain is best described as a syndrome of multiple signs and symptoms representative of a wide range of underlying aetiologies. This emphasises the importance of undertaking the necessary tests, including a comprehensive neurological history and examination, to reach a reliable conclusion. A grading system for neuropathic pain has been suggested with three categories; possible, probable or definite neuropathic pain (Treede *et al.* 2008).

The process of clinical examination is essential and should not be sacrificed for the use of alternative methods such as screening tools. Only clinical examination has the potential to elucidate the underlying pathological cause of the presenting painful complaint. Such diagnostic methodology may also elucidate other symptoms, such as altered muscle tone, which may be overlooked by other diagnostic methods (Haanpaa *et al.* 2011).

Sensory testing involves the use of relatively simple utensils for the assessment of several elements of sensation, including touch, vibration, pinprick and thermal perception (Haanpaa *et al.* 2011). Thus, sensory abnormalities pertaining to allodynia can be determined by light contact of the affected area with cotton wool, which in comparison to a normal area, will cause significant discomfort. Hyperalgesia can be assessed by using equipment as simplistic as a cocktail stick, but standardisation of this technique may be improved by using a 23G needle (Callin *et al.* 2008). Surveying of whole areas implicated with somatosensory aberrations is considered mandatory, as the bordering regions of the implicated area may be reflective of the associated peripheral nerve structure (Haanpaa *et al.* 2011). In unilateral presentations, the observations accrued from sensory tests in the painful area may be compared to the contralateral side. However, it should be noted that sensory tests provide information regarding sensory abnormalities, but they are of limited power for the differentiation of non-neuropathic and neuropathic pain (Rasmussen *et al.* 2004). For instance, both allodynia and hyperalgesia are common features of neuropathic pain and are present in up to 50% of patients (Jensen *et al.* 2014), yet these features are also observed in patients with a predominantly inflammatory component to their pain (Ren *et al.* 1999).

1.4.6.2 Screening tools

The use of screening tools for the differentiation of neuropathic and nociceptive symptoms is underpinned by the observation that verbal descriptors of pain are sufficiently discriminative between neuropathic and non-neuropathic pain (Dubuisson *et al.* 1976, Boureau *et al.* 1990). This, in particular, relates to burning, electric shock and tingling sensations, which feature prominently on several currently validated neuropathic pain screening tools (Bouhassira *et al.* 2005). The NeuPSIG of the IASP has recommended five screening tools for the identification of neuropathic pain (Haanpaa *et al.* 2011).

1.4.6.2.1 S-LANSS

The availability of validated screening tools for the differentiation of neuropathic and nociceptive pain has notably increased over the last decade. The 7-item LANSS screening tool was one of the first such tools and used a combination of five questions describing pain traits and a further two pertaining to sensory testing, including allodynia and assessment of pin-prick threshold (Bennett 2001). The LANSS test was initially deployed and validated in two cohorts of nociceptive and neuropathic patients, with reasonable sensitivity and specificity. A self-reporting version, the S-LANSS tool, was developed in order to facilitate screening of neuropathic signs and symptoms outside of the clinic, thereby facilitating clinical research and epidemiological studies. It differs from the LANSS tool in that the sensory testing section has been adapted for self-assessment, and the other items were also modified to improve clarity, but the crux of the questions remained unchanged. The S-LANSS tool identifies pain of predominantly neuropathic origin, and was initially developed in a cohort of patients with nociceptive or neuropathic pain of various aetiologies. The use of the S-LANSS tool has shown that varying the cut-off score provides different degrees of sensitivity and specificity when the questionnaire is completed in both unaided and interview situations. A score of greater than 12 is generally considered indicative of pain with a considerable neuropathic component (Bennett *et al.* 2005). Although unintended for use as a measure of treatment efficacy, the LANSS screening tool has also been shown to be responsive to pharmacological intervention (Khedr *et al.* 2005, Mercadante *et al.* 2009), which may reflect a notable shortcoming of such tools when used in clinical research to differentiate pain types. Critique of the LANSS scale has also been made in reference to the ‘openness’ of questions and the high weighting placed on a question relating to the change of skin colour at the painful area, which is not a considered essential for the diagnosis of pain with a definite neurological lesion (Bouhassira *et al.* 2005).

1.4.6.2.2 DN4

The 10-item DN4 screening tool is designed for use within the clinical setting and consists of a simplified scoring system with 3 items relating to clinical examination (Bouhassira *et al.* 2005). Unlike the LANSS/S-LANSS screening tools, the items within then DN4 are of equal weighting, and a score of 4 (out of 10) is sufficient to indicate neuropathic pain. It has been demonstrated that the use of discriminate pain descriptors, particularly in relation to paraesthesia and dysesthesia, were of particular value (Bouhassira *et al.* 2005). A shortened version, the 7-item DN4-interview, demonstrated a relatively small reduction in sensitivity and specificity, and may be considered for alternative purposes, such as epidemiological research (Bouhassira *et al.* 2005). A comparative assessment between the DN4 and S-LANSS found moderate agreement regarding pain classification and pain scores, but determined that the respective cut-off scores may not be comparable (Walsh *et al.* 2012).

1.4.6.2.3 NPQ

The Neuropathic Pain Questionnaire (NPQ) is a 12-item screening tool that incorporates 10 items pertaining to sensation or sensory response and 2 items relating to affect (Krause *et al.* 2003). In contrast to other screening tools such as the DN4 and LANSS, the NPQ tools features a comparatively complex scoring system. Selectivity and specificity is thought to be hampered by the inclusion of questions implicating the affective dimension of pain, including the rating of unpleasantness and to what degree is the pain overwhelming, which, in addition to the effect of meteorological changes on pain severity, are considered inadequately discriminative (Bouhassira *et al.* 2005).

1.4.6.2.4 ID PAIN

The ID-Pain screening tool, designed in order to detect a neuropathic component to pain, comprises of 6-items, including one item pertaining to whether pain is located within the joint (in order to identify nociceptive pain) and does not require clinical examination (Portenoy 2006, Bennett *et al.* 2007b). Using a cut-off score of 3, the tool was initially used in 3 groups of patients; those with nociceptive pain, mixed pain and neuropathic pain. The use of the ID-Pain tool suggested a neuropathic components in 22%, 39% and 58% of patients within these groups, respectively (Portenoy 2006).

1.4.6.2.5 painDETECT

The painDETECT questionnaire is a 9-item self-reporting screening tool that does not require clinical examination. It includes 7 items pertaining to sensory descriptors and 2 items relating to the spatial and temporal characteristics of pain (Freynhagen *et al.* 2006, Gauffin *et al.* 2013). The tool was initially developed in Germany using patients with back pain. In a cohort of approximately 8000 patients with lower back pain, the screening tool achieved a sensitivity of 85% and specificity of 80% (Freynhagen *et al.* 2006).

1.4.6.3 Usefulness of screening tools

A systematic review of neuropathic pain screening tools deduced that in many cases, the level of evidence supporting the use of a given tool was inadequate. The DN4 and NPQ screening tools possessed the greatest evidence for their measurement properties (Mathieson *et al.* 2015), though preference of screening tool should be given to one which has been validated in the required language (Haanpaa *et al.* 2011). Overall, both the LANSS and DN4 screening tools exhibit the greatest sensitivity and specificity, which has been attributed to the inclusion of items requiring physical examination (Cruccu *et al.* 2009). The Standardized Evaluation of Pain (StEP) assessment tool, which comprises of six interview questions and ten physical tests, again emphasises the power of physical examination to provide an accurate assessment. The StEP tool was successfully used in patients with radicular or axial (non-neuropathic) lower back pain. Patients with radicular pain were identified with a sensitivity and specificity of 92% and 97%, respectively (Scholz *et al.* 2009).

It should also be noted that, in general, screening tools clearly fail to identify around 10-20% of patients when compared to a clinician's diagnosis, so their use should be confined to that of a supplementary method used to further evaluate and inform diagnosis and treatment strategies, rather than replacing clinical judgement (Haanpaa *et al.* 2011, Mathieson *et al.* 2015). Limitations of screening tools, including a lack of discriminatory capability and quite possibly, inconsistent or inadequately comprehensive questions, are potential explanations for the discrepancy in neuropathic case identification rates.

1.4.6.4 Alternative diagnostic methods

There are several other techniques are available to assist in the characterisation of CNP, although their use in the clinic is sporadic. Microneurography is a minimally invasive technique used to assess single fibre action potentials from peripheral nerves. The inception and subsequent optimisation of microneurography has provided a degree of insight into nociceptor

neurophysiology (Hagbarth 2002). It is considered the sole available method of recording and quantifying sensory phenomena resulting from large myelinated, small myelinated and unmyelinated fibres (Cruccu *et al.* 2010). However, microneurography is relatively time consuming and requires both a specialist investigator and collaborative patient, with few centres providing the technique worldwide (Serra 2009, Cruccu *et al.* 2010).

A punch biopsy of the skin may also be considered as a method of determining epidermal nerve fibre density. Skin biopsies were able to highlight loss of small diameter nerve fibres in patients with painful or burning feet despite normal sensory-nerve conduction results (Holland *et al.* 1998). However, clarity regarding the diagnostic value of skin biopsy in the diagnosis of peripheral neuropathies is lacking (Sommer *et al.* 2007). Imaging studies, using positron emission tomography (PET) or functional magnetic resonance imaging (fMRI), have demonstrated observable variations in both cerebral blood flow and CNS activity patterns in patients with painful mononeuropathy, before and after regional nerve block (Hsieh *et al.* 1995). Similar observations have also been made in patients with nerve injury using brush-evoked allodynia (Schweinhardt *et al.* 2006, Witting *et al.* 2006) and in healthy volunteers with capsaicin-induced secondary hyperalgesia (Baron *et al.* 1999). Such methodology is however, largely experimental and has little use as a clinical utility for the routine diagnosis of CNP.

1.4.7 Neuropathic pain assessment tools

In addition to the basic pain rating tools, such as the visual analogue scale (VAS), verbal rating scale (VRS) and the numerical rating scale (NRS), there are specifically designated tools available which permit the assessment of neuropathic pain (Williamson *et al.* 2005), However, these tools are not designed to allow discrimination between neuropathic and nociceptive pain. The Neuropathic Pain Scale (NPS) (Galer *et al.* 1997) and Neuropathic Pain Symptom Inventory (NPSI) (Bouhassira *et al.* 2004) assess numerous parameters associated with pain. These include pain traits such as spontaneous, paroxysmal or evoked pain. The Pain Quality Assessment Scale was introduced in order to account for perceived limitations in the NPSI, which includes the omission of several pain qualities commonly observed by patients with nociceptive and neuropathic pain (Jensen *et al.* 2006).

1.4.8 Quantitative sensory testing

Quantitative sensory testing (QST) is a non-invasive standardised extension of the bedside examination with the inclusion of calibrated equipment to provide quantitative assessment, but remains fundamentally subjective. The purpose of QST is to assess pain perception and thresholds,

thereby providing evidence for positive and negative sensory signs (Mucke *et al.* 2016). It is achieved by the application of mechanical and thermal stimuli to examine the function of large (Aβ) and small diameter (Aδ and C) nerve fibres, and the related central pathways (Krumova *et al.* 2012). The magnitude of the applied stimulus is often sequentially increased to determine thresholds and tolerance. There are a wide range of relatively simple methods available for QST including von Frey hairs (mechanical sensitivity), thermal probes (thermal pain perception) and weighted needles (pinprick sensitivity) (Cruccu *et al.* 2010). QST is frequently used in clinical trials and it remains a useful tool in clinical practice to facilitate assessment of the somatosensory system (Mucke *et al.* 2016). It has demonstrated particular benefit in the early diagnosis of small fibre neuropathies, such as those associated with diabetes (Association 1993), and in the evaluation of treatment efficacy on the different modalities of pain, including hyperalgesia and allodynia (Cruccu *et al.* 2010). However, since changes in QST parameters are also associated with non-neuropathic pain (Pavlakovic *et al.* 2010), it has little discriminative value in the differential diagnosis of neuropathic pain (Cruccu *et al.* 2010).

1.4.9 Treatment

The treatment of CNP predominantly entails the use of pharmacotherapy to reduce pain intensity, rather than treatment directed at eradicating the root cause of pain (Bennett *et al.* 2007b). However, the pharmacological management of CNP is comparatively distinct from that of non-neuropathic pain. Analgesics, such as paracetamol and the non-steroidal anti-inflammatory drugs, have insufficient supportive evidence in the treatment of CNP. The necessity for alternative analgesics, which themselves are not entirely effective, emphasises the crucial need for accurate diagnosis and timely selection of clinically appropriate treatment. Indeed, efforts are ongoing to clarify a role for specific sodium channel antagonists, such as $Na_v1.7$ inhibitors (Emery *et al.* 2016), although such treatments are yet to receive regulatory approval. Non-pharmacological methods for amelioration of CNP include acupuncture and transcutaneous electrical nerve stimulation (TENS).

1.4.9.1 Current pharmacotherapy

1.4.9.1.1 Tricyclic antidepressents

Shortly following the serendipitous discovery that an iminodibenzyl derivative, now marketed as imipramine, caused hypomania and amelioration of depressive symptoms in hospitalised schizophrenic patients, the newly discovered tricyclic antidepressant (TCA) was considered for its use as an analgesic (Paoli *et al.* 1960). Amitriptyline and fluphenazine, also TCAs, subsequently

demonstrated efficacy in a small cohort of patients with refractory painful diabetic neuropathy (Davis *et al.* 1977). Several randomised, double-blind, placebo-controlled trials, have since cemented the role of TCAs in the treatment of CNP associated with several causative diseases, including diabetic neuropathy (Max *et al.* 1987), central post-stoke pain (Leijon *et al.* 1989) and PHN (Raja *et al.* 2002). TCAs are relatively diverse in their differential inhibition of monoamine reuptake. Amitriptyline, imipramine and clomipramine all exhibit greater tendency for inhibition of the serotonin transporter (SERT), whilst their respective metabolites, nortriptyline, desipramine and desmethylclomipramine, exhibit comparatively greater inhibition of the noradrenaline transporter (NAT) relative to SERT (Sindrup *et al.* 2005, Gillman 2007). In addition to inhibition of presynaptic reuptake of serotonin and noradrenaline, TCAs inhibit the N-methyl-d-aspartate (NMDA) receptor (Sindrup *et al.* 2005) and the inactivated $Na_v1.7$ channel (Dick *et al.* 2007). Sodium channel blockade is anticipated at concentrations typical of those present in plasma at therapeutic doses (Sindrup *et al.* 2005). TCAs also possess adrenergic, cholinergic and histaminergic antagonism (Lynch *et al.* 2006). This broad spectrum of pharmacological activity underpins both the relative efficacy and the profound side effect profile of TCAs. They are generally poorly tolerated in the elderly, pro-arrhythmogenic, epileptogenic and exhibit classical adverse anticholinergic side effects, including xerostomia, constipation and urinary retention.

1.4.9.1.2 SSRIs/SNRIs

Subsequent to the establishment of TCAs as a prominent pharmacological means for the treatment of major depressive disorder, rational based drug design led to the discovery of zimeldine, a first-in-class selective serotonin reuptake inhibitor (SSRI). Although zimelidine was withdrawn due to incidences of Guillain–Barré syndrome (Mulinari 2015), multiple SSRIs were subsequently introduced, including fluoxetine, paroxetine and citalopram. The side effect profile of SSRIs is comparatively favorable to TCAs, and reflects the selectivity of these compounds for SERT inhibition. These include incoordination, sexual dysfunction, and occasionally, serotonin syndrome. However, evidence supporting the efficacy of SSRIs in the treatment of CNP is relatively sparse. Following their introduction, several comparative trials of TCAs and SSRIs were undertaken, showing zimelidine to be inferior to amitriptyline for PHN (Watson *et al.* 1985) and in painful diabetic neuropathy, paroxetine was less effective than imipramine (Sindrup *et al.* 1990). Fluoxetine, which failed to alleviate hyperalgesia and allodynia in rat pain models (Jett *et al.* 1997, Pal Singh *et al.* 2001), subsequently exhibited no greater efficacy than placebo (Max *et al.* 1992).

Shortly following the introduction of SSRIs, however, commenced the development of serotonin noradrenaline reuptake inhibitors, which permit the simultaneous inhibition of SERT and NAT, with a considerably improved side effect profile in comparison to TCAs. At low doses, venlafaxine functions predominantly to inhibit SERT, but at higher doses, inhibition of NAT occurs (Debonnel *et al.* 2007). The importance of NAT inhibition, which contrasts SSRIs and SNRIs, may be reflected in that high doses of venlafaxine provided a similar number needed to treat (NNT) to TCAs, yet low doses were only marginally more effective than placebo (Rowbotham *et al.* 2004). Duloxetine is also effective in the treatment of painful diabetic neuropathy (Goldstein *et al.* 2005, Lunn *et al.* 2014), and possesses regulatory approval for the treatment of this condition (Shi *et al.* 2012).

1.4.9.1.3 Anticonvulsants

Evidence supporting the use of anticonvulsants in the treatment of CNP is underpinned by pathophysiological similarities observed in animal models of epilepsy and neuropathic pain (Tremont-Lukats *et al.* 2000). Carbamazepine in structurally related to the TCAs, elicits pronounced antiepileptogenic effects and has been considered in the treatment of TGN for over half a century (Blom 1962). Initial trials demonstrated that carbamazepine was superior to placebo for TGN (Campbell *et al.* 1966) and painful diabetic peripheral neuropathy (Rull *et al.* 1969), but adverse events were prominent, occasionally requiring withdrawal of treatment. In fact, carbamazepine remains the currently accepted first-line therapy for TGN (Obermann 2010), although the evidence for its use in other neuropathies is somewhat limited (Tremont-Lukats *et al.* 2000). After peripheral nerve injury, maladaptive responses, including abnormal sodium channel expression and accumulation, underpins the use of carbamazepine in CNP. The mechanism of action is commonly attributed to frequency-dependant blockade of voltage-sensitive sodium channels, leading to reduced ectopic discharge, and inhibition of impulse firing and propagation (Obermann 2010). Carbamazepine, however, is also associated with associated severe haematological, dermatological and hepatic adverse reactions. The Food and Drug Administration advocates genotyping of Asians for the *HLA* allele *B*1502* due to increased risk of carbamazepine-associated Stevens–Johnson syndrome and toxic epidermal necrolysis (Ferrell *et al.* 2008).

Phenytoin, aside from its established use as an anticonvulsant, was first used in the 1940s in the treatment of TGN (Ryder *et al.* 2005). However, its current use is restricted due to questionable evidence (Birse *et al.* 2012) and pronounced side effects exacerbated by non-linear

pharmacokinetics. A systematic review found no evidence of sufficient robustness to advocate the use of phenytoin in CNP (Birse *et al.* 2012), with modest evidence existing for its use as a co-analgesic (Yajnik *et al.* 1992). Its use, or that of its prodrug fosphenytoin, is now generally restricted to intravenous administration in acute flare-ups, particularly in relation to acute TGN crisis (McCleane 1999, Cheshire 2001). Several alternative antiepileptic drugs do not possess sufficiently robust evidence supporting their use in CNP, including sodium valproate (Gill *et al.* 2011), levetiracetam (Wiffen *et al.* 2014) and topiramate (Wiffen *et al.* 2013).

The use of the aforementioned antiepileptic drugs, in many instances, has been superseded by the introduction of gabapentin and pregabalin. They are similar in structure to γ-aminobutyric acid (GABA), but contrary to original reports (Taylor 1997), their mechanism of action is not thought to be related to modulation of GABA. It is now considered that selective inhibition of voltage-gated calcium channels containing the α2δ1 subunit is responsible for their efficacy (Sills 2006). As second generation antiepileptics, gabapentin and pregabalin are frequently prescribed in the treatment of CNP, and tend to exhibit comparatively favourable tolerability and side effect profiles (Maizels *et al.* 2005). However, the NNT for gabapentin in polyneuropathy was comparatively larger than the antiepileptic sodium channel blockers and TCAs (Sindrup *et al.* 2000), suggesting that the improved safety profile may not be coupled with improved efficacy. Current evidence suggests a role for gabapentin and pregabalin in PHN and painful diabetic neuropathy, yet in general, only one third of patients taking gabapentin will experience a 50% reduction in pain intensity, and over 50% will not benefit (Moore *et al.* 2014).

1.4.9.1.4 Opioids

The endogenous opioid peptide neurotransmitters, β-endorphins, enkephalins and dynorphins, provide rapid onset analgesia by exhibiting agonistic action at the three predominant opioid g protein-coupled receptors, mu, kappa and delta. The exogenous opiates, together with the opioids, provide the mainstay of treatment in moderate to severe somatic or visceral (nociceptive) pain. Their efficacy in the treatment of CNP is however, less convincing (Dickenson *et al.* 2005). The development of neuropathic pain after peripheral or spinal nerve injury results in adaptations within the opioid system, with particular regards to endogenous opioid synthesis and receptor density, which confers reduced responsiveness to narcotic analgesics (Przewlocki *et al.* 2005). The efficacy of opioids in the treatment of CNP has demonstrated contradictory outcomes. A systematic review determined opioids to be of uncertain value in CNP with only half of the

included studies able to conclude that opioids provided greater perceived analgesia than placebo (McNicol *et al.* 2013).

In addition to the equivocal evidence regarding the efficacy of opioids in CNP, prolonged administration often leads to the development of tolerance and the resultant demand for dose escalation (Morgan *et al.* 2011). For many patients with chronic pain, opioid related side effects become intolerable, and include cognitive deficits, hyperalgesia, sedation and in excessive doses, respiratory depression. The opioid analgesics with a secondary mechanism of action, namely tramadol (with serotonergic and noradrenergic pathway enhancement) and methadone (which exhibits NMDA antagonism) may be of greater value in the treatment of CNP, but are not without significant side effects.

1.4.9.1.5 Other pharmacological targets

Capsaicin, the predominant capsaicinoid derived from the *Capsicum* genus, is a transient receptor potential vanilloid 1 (TRPV1) agonist used topically for the treatment of PHN and painful diabetic peripheral neuropathy. A previous systemic review has highlighted a significant benefit for topical capsaicin when compared to placebo (Jorge *et al.* 2011). Depletion of substance P was traditionally attributed as the mechanism of action of capsaicin, but it is now considered that capsaicin functions through defunctionalisation of nociceptor fibres, including a reduction in epidermal nerve fibre density (Anand *et al.* 2011a).

Topical lidocaine, which selectively blocks sodium channels resulting in reduced peripheral nociceptor sensitisation (Argoff 2000), can be applied to the skin in form of plasters. This may be of benefit in the treatment of CNP localised to distinct areas, such as that observed with PHN. However, the use of systemic lidocaine is severely limited by side effects. Lidocaine congeners, such as mexiletine, are available and have evidence for analgesic properties, but are similarly limited by intolerable side effects (Carroll *et al.* 2008).

1.4.10 Prognosis

Although CNP may originate from various aetiologies, it is generally considered that the prognosis is considerably worse than that associated with non-neuropathic pain. CNP generally fails to relent over time (Cohen *et al.* 2014) and is often refractory to conventional pharmacotherapy. In comparison to the prognosis associated with a causative disease, such as cancer, diabetes or HIV, the prognosis pertaining to CNP is largely considered of little clinical concern and may be somewhat disregarded. Aside from the potential for the detrimental physiological changes

previously described (section 1.2), consideration should also be given to the psychological implications of CNP. Evidence exists that patients with widespread pain are more susceptible to mortality by non-diseases related incidences, such as violence and suicide (Macfarlane *et al.* 2001). A pertinent summary highlighting the current deficit in pain management, concluding that 'although few people die of pain, many die in pain, and even more live in pain' (Niv *et al.* 2001).

1.5 Biomarkers

1.5.1 Definition, scope and methods

A biomarker, or biological marker, is defined as a 'characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' (Group 2001). Other definitions have been proposed over time, but there remains a strong consensus (Hulka *et al.* 1988). Disease related biomarkers have the potential to cover a variety of clinical functions, including the prediction of future disease development, the diagnosis of emerging or current disease and in prognostics, to predict the course or outcome of a disease. The use of biomarkers is not a modern concept, and can be traced back to the inception of medical practice, although the modern concept of a biomarker detected by analytical methods in the laboratory emerged in the mid-19th century. The regular clinical utilisation of biomarkers subsequently accelerated considerably throughout the latter half of the 20th century (Jain 2010). Activities incorporating biomarkers in current medical practice range from the simple recording of blood pressure as an indicator of hypertension and stroke risk, to genetic testing in order to facilitate the diagnosis of a disease, such as cystic fibrosis, and to predict drug suitability/response (Novelli *et al.* 2008). Indeed, the current clinical landscape now utilises many of the methods akin to those considered in this thesis. Microarray technology (Lagraulet 2010) and more recently, RNAseq (Byron *et al.* 2016), have both been considered for their value as clinical utilities to aid diagnostics and therapeutics. Such advances have revolutionised molecular capabilities, enabling the identification and diagnosis of rare genetic diseases (Cummings *et al.* 2017). In addition to established PCR-based methods and ELISAs, advancements in proteomic and metabolomic based biomarker discovery have also been facilitated by progressive developments in mass spectrometry and associated techniques (Crutchfield *et al.* 2016).

1.5.2 Blood as a source of nervous system biomarkers

The use of blood as a source of accessible biomarkers is routinely used in clinical practice, covering a range of medical disciplines. These include troponin as a marker of cardiac injury (Babuin *et al.* 2005), prostate specific antigen in prostate cancer screening (Catalona *et al.* 1991) and various, largely immunological biomarkers, for the identification and monitoring of patients with HIV (Kanekar 2010). For many diseases, the discovery and validation of a sensitive and specific biomarker to aid diagnosis, inform prognosis and assess treatment efficacy, remains somewhat elusive. This is of particular relevance when considering diseases of the nervous system, wherein differential diagnosis is often protracted, and may require relatively invasive procedures. For instance, the diagnosis of multiple sclerosis remains notoriously challenging. Diagnostic methods and techniques such as MRI and lumbar puncture are both highly unpleasant for the patient and may fail to differentiate multiple sclerosis from other relatively rare diseases, such as neuromyelitis optica and transverse myelitis (Lalan *et al.* 2012). Indeed, blood as a source of multiple sclerosis biomarkers has shown significant progress (Sondergaard *et al.* 2013, Dickens *et al.* 2014, Honardoost *et al.* 2014, Huber *et al.* 2014, Naghavian *et al.* 2015) in comparison to urine (Bielekova *et al.* 2004, Dobson 2012) and CSF (Giovannoni 2006, Fitzner *et al.* 2015). Similarly, putative blood biomarkers have been recently described for other diseases with nervous system involvement, including Alzheimer's (Doecke *et al.* 2012, Olsson *et al.* 2016) (Delaby *et al.* 2015), epilepsy (Chang *et al.* 2012) and Huntington's (Mastrokolias *et al.* 2015). The suggestion of blood biomarkers of neuropsychiatric disorders, such as depression (Bilello *et al.* 2015, Gottschalk *et al.* 2015) and attention deficit hyperactivity disorder (Sasaki *et al.* 2015), also demonstrates considerable potential for blood to herald biomarkers for a diverse range of nervous system pathologies.

1.5.3 Current status of pain biomarkers in humans

The use of animal models and *in vitro* studies have led to significant advances in our understanding of transcriptomic changes caused by inflammation and related nerve injury (Young *et al.* 2012). Several of these genes have been shown to influence pain sensitivity (Fillingim *et al.* 2008, Young *et al.* 2012) and of which, GTP cyclohydrolase is extensively reviewed (section 3.1.1). However, the search for biomarkers of pain has largely followed a targeted rather than exploratory approach with the analysis of pre-defined groups of cytokines in blood and more frequently, CSF, with mixed results. Increased levels of IL-8 in CSF (Kotani *et al.* 2004) and IL-6 in blood (Zhu *et al.* 2009) have both been suggested as indicators for propensity to develop PHN. However,

quantification of markers pertaining to various serum Th1 and Th2 cytokines, antibody titres to varicella-zoster and biopsies of zoster lesions found limited evidence for a reliable marker to predict the emergence of PHN (Zak-Prelich *et al.* 2003).

Similarly, Uçeyler *et al* (2007) sought to determine a proinflammatory cytokine profile to differentiate patients with painful neuropathy and healthy controls, but also included patients with non-painful neuropathies. Blood mRNA and protein levels of IL-2 and TNF-α were elevated in painful neuropathies, which contrasted to both patients with non-painful neuropathies and healthy controls. Furthermore, levels of the anti-inflammatory cytokine IL-10 were found to be higher in patients with painless neuropathy than in those with painful neuropathy and controls, whilst IL-4 protein levels were also notably higher in all patients with neuropathy, indicating that IL-4 may function as a non-specific marker of peripheral neuropathy (Uceyler *et al.* 2007). Another study, which compared nerve biopsies from patients with painful and non-painful neuropathies, showed that patients with painful neuropathies exhibited greater TNF-α immunoreactivity in myelinating Schwann cells, whilst elevated serum soluble TNF-α receptor was observed in patients exhibiting mechanical allodynia (Empl *et al.* 2001).

It has also been reported that levels of serum biomarkers correlated with lower back pain and subsequent functional impairment (Sowa *et al.* 2014), whilst others have observed that the severity of polyneuropathy is associated with elevated TNF-α and IL-6 (Ludwig *et al.* 2008). Several cytokines in the serum and CSF of patients with lumbar disc herniation and sciatica have been studied, which found that only IL-8 levels in CSF was increased, albeit not consistently. However, no clear association was found between blood cytokine levels in patients with lumbar disc herniation and sciatica (Brisby *et al.* 2002).

The case of cystatin C is perhaps a further indictment of the current state of pain biomarkers. A cysteine protease inhibitor, cystatin C, was initially shown to be upregulated in the DH by persistent noxious input in a carrageenan-induced animal model of peripheral inflammation (Yang *et al.* 2001). It was hypothesised that, as cystatin is secreted, its levels in CSF may be representative of a nociceptive state. Levels of cystatin C in the CSF were indeed higher in persons experiencing labour prior to anaesthesia for caesarean section than those with elective caesarean sections (Mannes *et al.* 2003). The validity of cystatin C as a biomarker was subsequently analysed further in healthy controls, women with severe labour pain, women undergoing elective caesarean without pain, and in patients with CNP. Cystatin C levels were elevated in both pregnancy cohorts

regardless of pain, and did not differ between controls and CNP patients (Eisenach *et al.* 2004). The suggestion that cystatin C may function as pain marker (Mannes *et al.* 2003), and the methodology used by Eisenach *et al* (Eisenach *et al.* 2004), has been the subject of considerable criticism (Kalso 2004).

1.5.4 Genes association with neuropathic pain

Genes previously associated with pain in human subjects are numerous, although their value as CNP biomarkers is largely uncomfirmed. A total of 23 genes were previously summarised with associations to experimental pain, clinical pain or analgesia, although there is often contradictory evidence to conclusively attribute a specific gene to changes in pain sensitivity or susceptibility (Lacroix-Fralish *et al.* 2009). A further systemic review highlighted a similar number of genetic associations with CNP (van Hecke *et al.* 2015). Indeed, animal studies have elucidated a plethora of differentially regulated genes in animal models of pain, but few have been confirmed to be of direct relevance to pain sensitivity, or are involved in the propensity of CNP development in humans (Diatchenko *et al.* 2005).

Poignant examples of genes with associations to pain phenotype include catecholamine-O-methyltransferase (*COMT*), melanocortin 1 receptor (*MC1R*), and GTP cyclohydrolase 1 (*GCH1*). COMT has been studied extensively in relation to the association of *COMT* polymorphisms and pain sensitivity. A common non-synonymous SNP within *COMT* (rs4680; *Val158Met*) has been shown to result in a functional protein with similar catalytic activity, but exhibits thermolability at physiological temperatures (Lotta *et al.* 1995), resulting in a 3 to 4 fold variation in COMT activity (Mannisto *et al.* 1999). The proposed influence of MC1R on pain sensitivity has been discussed in detail (5.4.2.2).

1.6 Thesis rationale

Chronic neuropathic pain is protracted, often debilitating, and has considerable socioeconomic implications. Persons with CNP are often diagnosed with major depression and suffer considerable deterioration in quality of life. In contrast to many similarly common diseases, the sensitivity and specificity of diagnostic methodology and the subsequent efficacy of pharmacotherapy is severely limited. Indeed, advocating for a ‘possible, probable and definite’ case categorisation (Treede *et al.* 2008) is perhaps an indictment of the current state of diagnostic capability. It should also be considered that the currently available screening tools fail to identify approximately 20-25% of cases previously identified by clinician diagnosis, though with limited diagnostic methodology

available, the broad reliability of this ‘gold standard’ of CNP diagnosis is also questionable, particularly in primary care. Importantly, both screening tools and clinician diagnosis rely heavily upon accurate responses to verbal descriptors of pain, which may be of negligible value in certain patient groups, particularly in those with neurological co-morbidities such as dementia, which are of continuously increasing prevalence. This, in addition to the lack of efficacious treatments and absence of novel pharmacotherapy targeting the distinct pathophysiological changes associated with CNP, are the prevailing factors providing the impetus for the identification of diagnostic biomarkers of CNP, which in turn, may assist in the identification of novel drug targets.

1.7 Core thesis aims

- To determine whether tetrahydrobiopterin related genes and molecules are differentially regulated in the blood of CNP patients
- Improve our understanding of how the *GCH1* pain protective haplotype may confer reduced sensitivity to pain
- Determine gene expression changes in the blood from two distinct cohorts of patients with CNP
- Use bioinformatic resources to rationalise changes in gene expression, considering literature evidence and elucidate groups of candidate biomarkers for future validation, including a consideration for their potential as pharmacological targets for amelioration of CNP
- Determine whether the candidate biomarker genes function as potential translational biomarkers by analysis of their expression in the rat DH in a common animal model of neuropathic pain

Chapter 2 Methods & Materials

2.1 Cohort specific methods

2.1.1 Discovery cohort

2.1.1.1 Sample acquisition

Blood from 10 individuals with CNP of the back or lower back (CNBP) lasting for more than 6 months was obtained through ProteoGenex tissue procurement services (Culver City, CA), alongside a further 10 age and gender matched controls, also acquired through ProteoGenex tissue procurement services. Patients were recruited after clinical assessment of their pain symptoms, including computed tomography (CT) and MRI scans, electroneuromyography, microneurography and assessment of the nociceptive flexion reflex. Pain intensities were determined using the VRS. All patients were non-responsive to non-narcotic and anti-inflammatory analgesics. Plasma was obtained using BD Vacutainer K2-EDTA tubes with centrifugation at 1000 x *g* for 10 minutes and immediate storage of the plasma at -80ºC, alongside isolation of buffy coat (for gDNA extraction). Sample acquisition was also undertaken using PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Switzerland). Patients with major psychiatric disorders, cancer or diabetes were excluded from this study. Donor consent was obtained through ProteoGenex under Protocol PG-ONG2003/1, titled: Collection of Tissue, Blood and Bone Marrow. Herein this cohort will be termed the discovery cohort. Demographics and details pertaining to all study participants are detailed (Appendix 1).

Plasma was also obtained from 12 patients with chronic inflammatory back pain (CIBP) in order to perform additional analysis pertaining to TIMP1 in plasma (section 2.2.6). Ethics approval was obtained from Galway University Hospitals *(Ref: C.A. 1037)* and National University of Ireland, Galway. The absence of CNP was determined by clinical assessment, and an S-LANSS score of less than 12. Pain severity was determined using the Chronic Pain Grade questionnaire. Demographics and details pertaining to these participants are detailed (Appendix 1).

2.1.1.2 Extraction of RNA (discovery cohort)

Total RNA was isolated from the PAXgene Blood RNA Tubes using the Preserved Blood RNA Purification Kit II (Norgen, Canada). The PAXgene tube was allowed to thaw at room temperature before centrifugation at 3000 x *g* for 10 minutes. The resulting supernatant was discarded and 4 mL of NPX1 reagent added to the pellet, vortexed until dissolved, and centrifuged at 3000 x *g* for 10 minutes. The supernatant was discarded and 600 µL of NPX2 mixed with the pellet by

vortexing until dissolved, followed by centrifugation of the lysate at 14000 x *g* for 1 minute. The supernatant was then transferred to a sterile 1.5 mL microcentrifuge tube. Three hundred microliters of ethanol was added and vortexed before the addition of 600 µL of the resulting lysate-ethanol mix to the column. The column was centrifuged at 3500 x *g* for 1 minute. The column was then washed by the addition of 400 µL of NPX3 and centrifuged at 14000 x *g* for 1 minute. For the removal of gDNA, 15 µL of DNase I was combined with 100 µL of NPX4, mixed gently by inversion, and applied to the column followed by centrifugation at 14000 x *g* for 1 minute. The eluate containing DNase I was then reapplied to the column and incubated at 25^{o}C for 15 minutes. Four hundred microlitres of NPX4 was added to the column and centrifuged at 14000 x *g* for 1 minute. This step was repeated for a total of 2 washes followed by centrifugation at 14000 x *g* for 2 minutes to remove residual solution from the column. The column was transferred to a sterile 1.5 mL microcentrifuge tube and RNA eluted by the addition of 50 µL of NPX5 to the column. Centrifugation was then carried out at 200 x *g* for 2 minutes and 14000 x *g* for 1 minute. The eluate containing the RNA was stored at -80^{o}C. RNA concentration was measured using a NanoDrop ND2000 ultraviolet–visible spectrophotometer (Labtech International Ltd, UK) and electrophoresed as described (section 2.2.1). RNA integrity was analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands).

2.1.1.3 Affymetrix microarray and data analysis

Total RNA was labelled using an Ambion WT Expression kit (Life Technologies, The Netherlands) and hybridized to Affymetrix Human Gene 1.0 ST expression arrays (Affymetrix, USA). Sample labelling, hybridisation to chips, and image scanning were performed according to the manufacturer's instructions on an Affymetrix GeneTitan instrument by SourceBiosciences (Germany). Quality control was performed using Affymetrix Expression Console and interpretation of data was facilitated by Affymetrix Transcriptome Analysis Console 2.0 (TAC 2.0). Transcripts exhibiting a fold change of ≥1.2 and a *p* value of ≤0.05 (ANOVA) were considered differentially expressed and suitable for further analysis. Microarray files pertaining to this cohort are available in the electronic supplementary material.

2.1.1.4 Analysis of gene-gene correlations

A total of 3,900 human 2-color microarray experiments were downloaded from NCBI's Gene Expression Omnibus (GEO) and normalised as described previously (Wren 2009) . Two-color arrays were chosen because they reflect how gene expression differs between two conditions, usually experimental and control, which emphasises how genes are correlated in their response.

Gene-gene Pearson's correlation coefficients were calculated using only the experiments where the two genes were present on the same microarray. This work was conducted in collaboration with Dr J. Wren, an affiliate of the Oklahoma Medical Research Foundation, and enables the identification of gene expression patterns which differ from those normally observed.

2.1.1.5 Analysis of biopterins using HPLC

After collection of blood using EDTA tubes, a proportion of the sample was cooled on ice for 5 minutes. Separation of plasma from blood cells was achieved by centrifugation at 4°C for 10 minutes at 1000 x *g*. A total of 90 µL of blood plasma was then transferred into a new, cooled 1.5 mL microcentrifuge tube containing 10 µL of 10 mM dithioerythritol (DTE) in phosphate buffer saline (PBS), and stored at -80°C. Plasma samples were then diluted 1:10 in ice-cold resuspension buffer. To 180 µL of all samples and standards, 20 µL of 10x precipitation buffer was added, followed by centrifugation at 4ºC for 5 minutes at 16000 x *g*. A total of 100 µL of the supernatant was injected into an isocratic HPLC system and quantified using sequential electrochemical (Coulochem III, ESA Inc., UK) and fluorescence (Jasco, UK) detection. HPLC separation was performed using a 250 mm, ACE C-18 column (Hichrom, UK) and mobile phase comprising 50 mM sodium acetate, 5 mM citric acid, 48 µM EDTA, and 160 µM DTE (pH 5.2) (all ultrapure electrochemical HPLC grade) at a flow rate of 1.3 mL/min. Quantification of BH_4 was made directly by electrochemical detection (background currents of +500 nA and -50 nA were used for the detection of BH_4 on electrochemical cells E1 and E2, respectively). Biopterin and BH_2 were measured as separate chromatographic picks in the same sample using a Jasco FP2020 fluorescence detector, serially connected to the electrochemical detector. Electrochemical detection of biopterins in the discovery cohort was conducted through the Division of Cardiovascular Medicine at the University of Oxford, as described here and as similarly as previously described (Crabtree *et al.* 2009b).

2.1.2 Discovery/validation methods

2.1.2.1 Sample acquisition

Samples pertaining to the discovery/validation cohort were collected at the University of Huddersfield (control subjects) and through the Pain Management Services at Seacroft Hospital (patients). A total of 24 subjects in each group were included within this thesis. Control and patient details are described (Appendix 2). The screening tool and patient questionnaires (including the S-LANSS test and Chronic Pain Grade tool) are included within the electronic supplementary materials, in addition to the study protocol details (including inclusion and exclusion criteria).

Venous blood was obtained from the antecubital fossa using standard phlebotomy technique. The BD Vacutainer Safety-Lok™ blood collection set was used to draw a total of 30 mL of blood from each volunteer. Ten millilitre BD Vacutainer K2EDTA tubes, 2.5 mL PAXgene Blood DNA tubes Tubes (PreAnalytiX GmbH, Switzerland) and 2.5 mL PAXgene Blood RNA tubes were used, in the order described, to collect blood. All PAXgene tubes were stored at -20ºC for >24 hours before transfer to -80ºC for long-term storage. BD Vacutainer tubes were immediately centrifuged at 1000 x *g* for 10 minutes at 4ºC and the resulting plasma was immediately frozen on dry ice followed by long-term storage at -80ºC. Herein this cohort will be termed the discovery/validation cohort.

2.1.2.2 Extraction of RNA (disocovery/validation cohort)

RNA was extracted using the PAXgene Blood RNA Kit (PreAnalytiX GmbH, Switzerland). PAXgene Blood RNA tubes were removed from -80ºC storage, equilibrated to room temperature, and incubated for 2 hours to ensure complete cell lysis. The tubes were then centrifuged for 10 minutes at 4000 x *g*. The supernatant was removed by decanting before the addition of 4 mL of RNase-free water to each tube and the application of a fresh secondary BD Hemogard closure. The pellet was dissolved by vortexing, and centrifuged for 10 minutes at 4000 x *g*. After decanting of the supernatant, 350 µL of resuspension buffer was added to the pellet and vortexed until dissolved. The sample was then transferred to a 1.5 mL microcentrifuge tube with the addition of 300 µL of binding buffer and 40 µL of proteinase K followed by vortexing for 5 seconds and incubation for 10 minutes at 55ºC with shaking (700 rpm). The sample was then transferred to a PAXgene Shredder spin column and centrifuged for 3 minutes at 16160 x *g*. The supernatant of the flow-through was then transferred to a fresh 1.5 mL microcentrifuge tube, followed by the addition of 350 µL of ethanol, vortexed briefly and centrifuged for 1-2 seconds at 1000 x *g*. Seven hundred microliters of the sample was transferred to a PAXgene RNA spin column and centrifuged for 1 minute at 16160 x *g*, followed by the addition of any remaining sample and centrifugation with identical parameters. Three hundred and fifty microliters of Wash Buffer 1 was added to the PAXgene RNA spin column followed by centrifugation for 1 minute at 16160 x *g*. After replacing the processing tube containing the flow-through, 10 µL of DNase I solution was mixed with 70 µL of DNA Digestion Buffer, per sample, in a 1.5 mL microcentrifuge tube and added to the column membrane. The spin columns were then incubated at room temperature for 15 minutes. Three hundred and fifty microliters of Wash Buffer was added, followed by a further 500 µL of Wash Buffer. Centrifugation steps for 1 minute at 16160 x *g* were carried out, followed by replacement of the processing tube after each addition of Wash Buffer. Another 500 µL of wash

buffer 2 was then added, and centrifuged at the same speed for 3 minutes, again replacing the processing tube. The spin column was then centrifuged for 1 minute at 16160 x *g*. After discarding the eluate, the spin column was placed in a clean 1.5 mL microcentrifuge tube and 40 µL of elution buffer was added directly onto the membrane followed by centrifugation at 16160 x *g* for 1 minute. This elution step was repeated using the same parameters and the same collection tube containing the previous eluate. The complete RNA-containing eluate was then incubated for 5 minutes at 65^{o}C, chilled on ice and then stored at -80^{o}C until use. RNA was quantified using a NanoDrop 2000 UV-Vis spectrophotometer (Labtech International Ltd, UK). Qualitative analysis was performed by gel electrophoresis to confirm intact 28S and 18S ribosomal RNA (section 2.2.1).

2.1.2.3 Affymetrix microarray and data analysis

Analysis of gene expression by microarray was conducted by AROS Applied Biotechnology (Aarhus N, Denmark) using the GeneChip Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, CA, USA). All methodology pertaining to sample processing is included as electronic supplementary material, entitled 'discovery validation microarray methodology'. Quality control was performed using Affymetrix Expression Console and interpretation of data was facilitated by Affymetrix Transcriptome Analysis Console 2.0 (TAC 2.0). Transcripts exhibiting a fold change of ≥1.2 and a *p* value of ≤0.05 (ANOVA) were considered differentially expressed and suitable for further analysis. Microarray files pertaining to this cohort are available in the electronic supplementary material.

2.1.2.4 cDNA synthesis

Synthesis of cDNA was performed using 300 ng of RNA, initially diluted to 11 µL with nuclease free water. Using the Verso cDNA synthesis kit (Thermo Scientific, UK) a total of 9 µL of reaction mix was added to the diluted RNA (after DNase treatment where applicable), consisting of 4 µL cDNA synthesis buffer, 2 µL dNTP Mix, 0.75 µL random hexamers, 0.25 µL oligo dT, 1 µL RT Enhancer and 1 µL Verso Enzyme Mix (no template controls and reverse transcription controls were included). For clinical samples, prior DNAse treatment was not necessary as this was complete on-column during the extraction process. The reaction was then incubated at 42^{o}C for 1 hour followed by 95^{o}C for 2 minutes. Any cDNA not diluted and processed immediately was subject to short-term storage at -20^{o}C.

2.1.2.5 qRT-PCR

Synthesised cDNA was initially diluted 1:5 (to 100 µL). Aliquots were then further diluted 1:10 (giving a final cDNA dilution of 1:50) prior to qRT-PCR analysis. Genes were analysed in

triplicate using a CFX96 instrument (Bio-Rad, UK), with each 12 µL reaction consisting of 6 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, UK), 300 nM of each forward and reverse primer, nuclease free water and 5 µL of diluted cDNA. Assays incorporating primers supplied by Primerdesign Ltd were used at a combined volume of 0.6 µL per 12 µL reaction. Incubation consisted of polymerase activation and initial DNA denaturation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 5 seconds with annealing and extension at 60°C (unless otherwise stated) for 30 seconds (unless otherwise stated) followed by fluorescence detection. Upon completion of thermal cycling, melt-curve analysis (and gel electrophoresis) was performed to confirm reaction specificity. Quality control, baseline subtraction and determination of the threshold cycle (C_q) were performed using Bio-Rad CFX Manager 3.1 (Bio-Rad, UK). Data was subsequently analysed with qbase+ software (Biogazelle, Belgium) using an unpaired t-test. Primer details and any deviation from the described PCR cycling conditions are also presented (Appendix 4).

2.1.2.6 BRB Array Tools

In order to determine gene expression correlations with self-reported parameters, the BRB Array Tools (v4.5.0) quantitative trait analysis tool was used within Microsoft Excel after uploading of the array files. All genes initially uploaded to BRB Array Tools were subject to filtering, which excluded genes wherein less than 20% of the signals were greater than 1.2 fold differentially expression from the median. Pearson's correlation values between gene expression and either S-LANSS score or modified Chronic Pain Grade result, were then obtained. The default significance threshold was used ($p = 0.001$).

Given that all patients included in the array analysis obtained a minimum S-LANSS score of 12, analysis of Pearson's correlations between S-LANSS score and microarray based gene expression values could only be achieved using patients with a neuropathic component to their pain. Gene expression correlation with S-LANSS scores therefore seeks to determine genes which correlate with the weighting of the neuropathic component to their pain, which may be reflected by a higher S-LANSS score.

In order to determined genes correlating with self-reported measures of pain severity, scores pertaining to three measures of the Chronic Pain Grade questionnaire (questions 3, 4 and 5), in addition to two further questions (1 and 2), were combined. This was done to provide a balanced overview of pain severity which considered various time-points, ranging from the present moment

to the previous three months. Other measures were excluded as they related to the affective aspects of pain and sought to grade the level of disability and restriction of day-to-day activities. Both the S-LANSS tool and Chronic Pain Grade questionnaire can be found with the file name 'Control and patient questionnaire' in the attached supplementary electronic material.

2.2 Non-specific cohort methods

2.2.1 Agarose gel electrophoresis (RNA)

A 1.5% agarose gel was made by melting 1.5 g of agarose (Thermo Scientific, UK) in 100 mL of 1x TBE, followed by the addition of 1 μL of GreenView DNA Gel Stain (GeneCopedia, USA) or 3 μL of ethidium bromide solution, and cast into a dedicated RNA electrophoresis system. RNA samples to be electrophoresed were thawed on ice. One microlitre of RNA sample was added to a 0.2 mL tube per 0.2 μL of Gel Loading Dye (NEB, UK), loaded on to the gel, and electrophoresed at 60 V for 30 minutes. The gel was visualised and photographed with a ChemiDoc MP Imaging System (Bio-Rad, UK).

2.2.2 Literature refinement of microarray data

In order to determine genes with the greatest evidence for involvement in CNP, refinement of gene expression data was undertaken with specific criteria, which includes a greater statistical stringency, the presence of a gene within correlation analysis, and finally, whether there is a body of literature pertaining to the role of the molecule in established pain pathways. Literature was searched to include all publications available up to, and including, February 2017, using both PubMed and general electronic information databases with the gene name or symbol, along with the terms 'pain', 'neuropathic' or 'neuropathic pain'.

2.2.3 Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) (Qiagen, USA) was used to identify pathways and molecular interactions between differentially regulated genes. Initially the data set, directly uploaded after extraction from the Affymetrix TAC2.0, was processed by IPA and genes corresponding to those within the IPA database were successfully mapped by the software. All genes mapped to the IPA database were then subject to the insertion of direct and indirect molecular interactions using the 'connect tool' with default parameters. Interaction networks consisting of fewer than five molecules were disregarded. Data pertaining to fold change regulation was then overlaid on the network to illustrate changes in gene expression.

In order to visualise potential interactions between the molecules identified within this study, and to those assigned within the IPA database as particularly pertinent to neuropathic pain, the mapped differentially regulated genes were combined with the 89 molecules held under the 'neuropathic pain' section of the 'Disease & Function' tool. To reduce network size, only direct interactions were sought and all xenobiotics removed prior to analysis.

2.2.4 Extraction of genomic DNA from blood

In order to extract gDNA from peripheral blood mononuclear cells, either 750 µL of whole blood (discovery/validation cohort) or buffy coat (discovery cohort) was mixed with 12 mL of reagent A (red blood cell lysis solution) and placed on a rotating mixer for 4 minutes at room temperature followed by centrifugation at 3000 x *g* for 5 minutes and discarding of the supernatant. To the pellet, 1 mL of reagent B (cell lysis solution) was added and vortexed briefly to resuspend. Two hundred and fifty microliters of 5 M sodium perchlorate was then added to the resuspended pellet and mixed by multiple inversions. The solution was then incubated in a water bath for 15 minutes at 65ºC. After allowing to cool to room temperature, 2 mL of ice-cold chloroform was added and placed on a rotating mixer for 60 minutes, followed by centrifugation at 2400 x *g* for 2 minutes. The upper phase was transferred to new tube followed by the addition of 3 mL of ice-cold ethanol and gentle inversion to precipitate DNA. Precipitated DNA was then transferred to a 1.5 mL microcentrifuge tube, briefly allowed to air dry, and resuspended in 200 µL of TE buffer. This method was adapted from a protocol described elsewhere (Bartlett *et al.* 2014).

2.2.5 Genotyping of *GCH1*

The *GCH1* genotype pertaining to the pain protective haplotype (rs8007267, rs3783641, rs10483639) was determined for both the discovery (n = 20) and discovery/validation (n = 47) cohorts using the PCR-restriction fragment length polymorphism (RFLP) method. DNA was extracted as described (section 2.2.4) and diluted to a working concentration of 5 ng/µL using nuclease free water. Each 10 µL PCR consisted of 3.92 µL water, 2 µL 5x Phire Reaction Buffer, 1 µL dNTPs (2 mM), 0.5 µM of forward and reverse primer, 2 µL of gDNA and 0.08 µL Phire Hot Start II DNA Polymerase (Thermo Scientific, UK). The annealing temperature for each reaction was initially optimised by gradient PCR. Cycling conditions for all reactions consisted of 98ºC for 30 seconds followed by 35 cycles of 98ºC for 5 seconds, 59.8ºC for 5 seconds and 72ºC for 10 seconds, terminating with 1 cycle of 72ºC for 1 minute. Restriction digest was undertaken directly after PCR by the addition of 20 µL consisting of 2 µL of 10x CutSmart Buffer and 2 U of either BcoDI (rs10483639), BstZ17I (rs8007267) or TspRI (rs3783641) (NEB, UK) followed by

incubation at 37ºC (BcoDI and BstZ17I) or 65ºC (TspRI) for 3 hours. After incubation, 6 µL of 6x loading dye was added and the sample electrophoresed in a 3% agarose gel at 90 V for 1 hour (section 2.4.8) followed by imaging using a ChemiDoc MP Imaging System (Bio-Rad, UK).

2.2.6 Plasma TIMP1 ELISA

In order to determine if circulating levels of tissue inhibitor of matrix metalloproteinase-1 (TIMP1) varied between patients with CNBP (*n* = 10), CIBP (*n* = 12) and healthy controls (*n* = 10), a total of 32 plasma samples from the discovery cohort were subject to a TIMP1 enzyme-linked immunosorbent assay (ELISA) (Invitrogen, UK). This was subsequently repeated with the discovery/validation cohort, comprising of patients with CNP (*n* = 24) and controls (*n* = 24). The assay was performed according to manufacturer's instructions. In total 10 µL of plasma was diluted to 200 µL prior to the procedure using the Standard Diluent Buffer, and 50 µL of a sample, standard (0-25 ng/mL) or blank (Standard Diluent Buffer), was applied to a 96-well plate in duplicate. Fifty microlitres of human TIMP1 (biotin conjugate) was added to all liquid-containing wells and incubated at room temperature for 2 hours. The solution was then decanted from the wells and the wells washed 4 times with wash buffer. One hundred microlitres of Streptavidin-HRP was then added to all wells originally allocated as sample, standard or blank and incubated at room temperature for 30 minutes. The solution was then decanted and the wash step repeated. One hundred microlitres of Stabilised Chromogen was added to all wells, including 2 wells without prior sample or standard addition. The plate was incubated at room temperature for 20 minutes and 100 µL of Stop Solution added to each well. Absorbance data was obtained using an Infinite F50 microplate reader (Tecan, UK) at 450 nm. After subtraction of background absorbance and the chromagen blank, absorbance data was converted into plasma TIMP1 levels (correcting for the initial plasma dilution) and analysed with GraphPad Prism 6.0.

2.2.7 Griess assay

Total plasma nitrate, after reduction of nitrate to nitrite, was quantified using the Nitrite/Nitrate Colourmetric Assay Kit (Cayman Chemical, UK). Plasma from control subjects (*n* = 8) and CNBP patients (*n* = 8) in the discovery cohort, and control subjects (*n* = 23) and CNP patients (*n* = 23) from the discovery/validation cohort, were initially allowed to thaw. After which, 800 µL was immediately transferred to an Amicon Ultra-4 Centrifugal Filter Unit (10 kDa) (Merck Milllipore, USA). Samples were centrifuged at 3800 x *g* for 20 minutes. Each sample was analysed in duplicate with a total of 40 µL of filtrate, diluted to 80 µL with assay buffer, per 96-well. The plate was incubated for 3 hours at room temperature after the addition of 10 µL of nitrate reductase

cofactors and 10 µL of reconstituted nitrate reductase to all wells, except the blank which comprised of 200 µL of assay buffer. After incubation, 50 µL Griess Reagent R1 (sulphanilamide) and 50 µL of Griess Reagent R2 (N-1-napthylethylenediamine) was added to all samples and standards, incubated for 10 minutes, and absorbance was recorded at 570 nm using a Tecan Infinite F50 microplate reader (Tecan, UK). Data was analysed in GraphPad Prism 6.0 using an unpaired t-test.

2.2.8 Analysis of neopterin using HPLC

Plasma was initially allowed to thaw at room temperature, after which 300 µL of 5% (w/v) trichloroacetic acid (TCA) was added to 300 µL of plasma, vortexed for 5 seconds, and centrifuged at 12000 x *g* for 5 minutes. The supernatant was then processed through a 0.45 µM RC membrane (4 mm) syringe filter (Phenonomex, UK). HPLC was performing using a mobile phase consisting of 20 mM potassium dihydrogen phosphate pH 7.4 at a flow rate of 1 mL/min with a SphereClone™ 5µM ODS(2) 250x4.6mm column coupled to a SecurityGuard guard cartridge (Phenonomex, UK). Each sample was analysed in triplicate with an injection volume of 60 µL. An Agilent Technologies 1100 HPLC system was used, comprising of a degasser (G1322A), quaternary pump (G1311A), autosampler (G1329A) and a 1260 Infinity Fluorescence Detector (G1321B) with an excitation and emission of 353nm and 438nm respectively.

2.2.9 DAVID bioinformatics resource

The Database for Annotation, Visualization and Integrated Discovery (DAVID) is a web-based bioinformatics resource for functional annotation and classification of microarray data (Closs *et al.* 2007). Genes with a *p* value of ≤0.05 and a fold change of ≥1.2 were exported from the Affymetrix TAC software into DAVID (v6.7) using the probe ID or gene symbol. Default parameters for analysis in DAVID were maintained, including a minimum count of 2 genes and an EASE score threshold (modified Fisher Extract *p* value) of 0.1. A total of 421 and 189 differentially regulated genes were exported from Affymetrix TAC software, and of which, 354 and 126 were successfully mapped by DAVID, for the discovery and discovery/validation cohorts, respectively.

2.3 Translational biomarker discovery

2.3.1 Animal husbandry

Adult male Sprague Dawley (*n* = 18) and Wistar Kyoto (*n* = 18) rats (matched at 7-8 weeks of age upon delivery and 250-350 g at the time of experimentation; Harlan, UK) were housed singly, with

food and water available *ad libitum* and maintained at constant temperature (21 ± 2ºC) under 12 hour cycling of light-dark exposure (lights on at 07.00 h). The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, and carried out under license from the Department of Health in the Republic of Ireland and in accordance with EU Directive 2010/63. One week following delivery and acclimatisation to the animal unit, animals underwent surgery after allocation into either L5 spinal nerve ligation (SNL) 5 (n = 10) or sham (n = 8) groups for both rat strains. In brief, the rats were anaesthetised under isoflurane anaesthesia (3% induction, 1.5-2% maintenance in 0.5 L/min O_2) and upon exposure of the left L5 spinal nerve, a ligature was applied. Sham rats were treated identically, aside from the application of a ligature. Animals were maintained until 35 days post-surgery at which point euthanasia was performed by decapitation and tissue was harvested from the spinal cord DH ipsilateral to the site of nerve injury, snap-frozen on dry ice and stored at -80ºC. RNA was extracted from tissue using the NucleoSpin® RNA kit (Machery–Nagel, Germany) with on-column DNase treatment followed by storage at -80ºC. The work described here was undertaken byProf D. Finn of the National University of Ireland. Further work on the extracted RNA described herein was undertaken at the University of Huddersfield.

2.3.2 Whole transcriptome amplification

A total of 25 ng of RNA from each DH sample was used for reverse transcription and subsequent amplification using the QuantiTect Whole Transcriptome Kit (Qiagen, UK). An appropriate volume of RNA was made to a volume of 2.5 µL with nuclease free water. A combination of 2 µL of T-Script Buffer and 0.5 µL of T-script enzyme were added to the RNA for reverse transcription and incubated at 37ºC for 30 minutes then 95ºC for 5 minutes. Ligation of the cDNA was then performed by adding a combination of 3 µL of Ligation Buffer, 1 µL Ligation Reagent, 0.5 µL of Ligation Enzyme 1 and 0.5 µL of Ligation Enzyme 2, followed by incubation at 22ºC for 2 hours. Amplification of the ligated cDNA was then undertaken by adding a combination of 14.5 µL of REPLI-g Midi Reaction Buffer and 0.5 µL REPLI-g Midi DNA Polymerase, followed by incubation at 30ºC for 8 hours and 95ºC for 5 minutes. Amplified cDNA was the stored short-term at -20ºC until use.

2.3.3 qRT-PCR (animal pain model)

After serial dilution of the amplification product (1:2000) (section 2.3.2), qRT-PCR was performed using a CFX96 instrument (Bio-Rad, UK). Analysis of samples was performed in triplicate as previously described (section 2.1.2.5). Upon completion of thermal cycling, melt-

curve analysis (and gel electrophoresis) was performed to confirm reaction specificity. Baseline subtraction and determination of the threshold cycle (C_q) was performed using Bio-Rad CFX Manager 3.1 (Bio-Rad, UK). Data was subsequently analysed with qbase+ software (Biogazelle, Belgium) using an unpaired t-test.

2.3.4 Droplet digital PCR

A total of 20 ng of RNA was reverse transcribed using the Verso cDNA synthesis kit as previously described (section 2.1.2.3), with the exception that all reaction components were used at half the previously detailed volumes. The cDNA was subsequently diluted to 100 µL. Further dilutions were performed for the reference genes *Rpl13a* and *Ubc* to avoid saturation of the ddPCR system. Each 20 µL PCR consisted of 10 µL of QX200™ ddPCR™ Evagreen Supermix, 250 nM of forward and reverse primer, 5 µL of diluted cDNA and nuclease free water. This was loaded in to a DG8™ Cartridge with accompanying DG8™ Gasket and 70 µL of QX200™ Droplet Generation Oil for Evagreen for subsequent droplet generation using a QX200™ Droplet Generator. The 96-well plates were then sealed using pierceable foil plate seals with a PX1™ PCR plate sealer. A T100™ Thermal Cycler was used with the following cycling conditions; enzyme activation for 5 minutes at 95ºC, followed by 40 cycles of denaturation at 95ºC for 30 seconds and annealing/extension at 60ºC for 1 minute. Signal stabilisation was achieved by cooling to 4ºC for 5 minutes and heating to 90ºC for 5 minutes. A ramp rate of 2ºC/sec was required for each step in the PCR. Data was then obtained using a QX200™ Droplet Reader with ddPCR™ Droplet Reader Oil and QuantaSoft™ Software (v1.7). All equipment and reagents were provided by Bio-Rad (UK). Normalisation of data was performed by dividing the total number of transcript copies per 20 µL reaction, by the geometric mean of *Rpl13a* and *Ubc*. Data was analysed using GraphPad Prism 6.0 using an unpaired t-test.

2.4 Molecular cloning

2.4.1 PCR

Multiple PCRs were undertaken in order to enable cloning and subsequent reporter gene assays. The primer sequences, reaction constituents and PCR cycling conditions are detailed (Appendix 3). Sanger sequencing of purified PCR products and plasmids was undertaken through SourceBioscience (Cambridge, UK).

2.4.2 Post-PCR purification

After completion of PCR cycling and electrophoresis (section 2.4.8), the gel was briefly visualised on a UV Transilluminator 2000 (Bio-Rad, UK), and the agarose gel containing the band corresponding to the correct molecular weight was excised using a clean scalpel and placed in a 1.5 mL microcentrifuge tube. DNA was then extracted using the GeneJET Gel Extraction Kit (Thermo Scientific, UK) by the addition of 100 µL of Binding Buffer per 100 mg of excised agarose gel, and heated to 55ºC with intermittent inversion until the gel slice was fully melted. The homogenous solution was then transferred to a GeneJET purification column and centrifuged for 1 minute at 12000 x *g*. All further centrifugation steps were carried out using these parameters. After discarding of the eluate, 700 µL of wash solution was added to the column and centrifuged, followed by a further centrifugation step to remove residual wash solution. DNA was eluted from the column into a clean 1.5 mL microcentrifuge tube by the addition of 20 µL of ultrapure water to the membrane, incubation for 1 minute, and centrifugation. The elution step was repeated with a further 10-20 µL of ultrapure water if eluate from the first centrifugation step contained sufficient DNA (>30 ng/µL). DNA was then quantified using NanoDrop ND2000 ultraviolet–visible spectrophotometer (Labtech International Ltd, UK).

2.4.3 Restriction enzyme digests

A restriction enzyme digest of the purified PCR product and plasmid was performed to allow for ligation of the PCR product into the multiple cloning site of the plasmid. A total of 1-2 µg of plasmid DNA and 1-2 µg of PCR product was digested with the appropriate restriction enzyme(s) (20 U/µg DNA) (NEB, UK) and reaction buffer in separate 50-100 µL reaction (depending on the initial DNA concentration). The reaction was incubated at 37ºC overnight. If a single restriction enzyme was used, 1 U of shrimp alkaline phosphatase (rSAP) (NEB, UK) per 1 pmol of DNA ends was added directly to the plasmid restriction enzyme digest after overnight incubation (e.g. 2 µg of a 5.4 kb plasmid corresponds to 1.12 pmol DNA ends) in order to prevent recircularisation of the plasmid backbone. The dephosphorylation reaction was incubated at 37ºC for 1 hour followed by heat inactivation of rSAP at 65ºC for 5 minutes. An appropriate volume of 6x loading dye was added to the digested plasmid and PCR product, followed by electrophoresis and gel extraction as described. Quantification was undertaken using a NanoDrop ND2000 ultraviolet–visible spectrophotometer (Labtech International Ltd, UK). An additional quantification step was used if those obtained by spectrophotometry were low (~3 ng/µL), or lacked a defined peak at 260nm. In such cases, a proportion of the eluate (1-5 µL) was subject to gel electrophoresis

alongside 5 µL of MassRuler Express HR Forward DNA Ladder (Thermo Scientific, UK). Assessment of DNA concentration then was performed by densitometry analysis using a ChemiDoc MP Imaging System (Bio-Rad, UK).

2.4.4 Ligations

Ligation of the restriction enzyme digested and column purified plasmid and PCR product was carried out using T4 DNA ligase (NEB, UK). Each 10 µL reaction typically consisted of 50-100 ng of total DNA, comprising of 20-75 ng (typically 30 ng) of plasmid DNA at molar ratios (plasmid : PCR product) of 1:1, 1:2, 1:3 and 1:5. The remainder of the reaction consisted of 1 µL of 10x T4 DNA Ligase Buffer, 200 U of T4 DNA ligase and nuclease free water. Reactions were also included with the absence of insert (restriction-digested PCR product) or the lack of DNA ligase, in order to assess background ligation activity. In instances when ligations and subsequent transformation efficiency appeared to be limited by factors such as the size of the plasmid backbone and PCR product, 2000 U of Quick Ligase was used (NEB, UK). In both instances, incubation of the ligation was carried out overnight at ~16ºC.

2.4.5 Post ligation screening

After incubation of the ligation reaction overnight, 5 µL was added to 100 µL of DH5α™ competent cells (section 2.4.7) in a 0.2 mL tube followed by incubation on ice for 2.5 hours. The competent cells were then subject to heat shock by incubation at 42ºC for 45 seconds, followed by storing on ice for 5 minutes. The entire competent cell solution was then subject to 30 minutes outgrowth in 300 µL of LB, after which 350 µL was spread on pre-warmed LB (Luria-Bertani) agar selection (ampicillin) plates. For transformations limited in efficiency by construct size, the use of super optimal broth (SOB medium), rather than LB broth, seemingly improved recovery after transformation. In both instances, agar plates were subsequently incubated overnight, upside-down, at 37ºC.

Resulting colonies were inoculated into 5 mL of LB broth containing 5 µL of (1000x) ampicillin solution and incubated overnight at 37ºC with shaking (250 rpm). After centrifugation at 3000 x *g* for 5 minutes, the cell pellet was resuspended with 350 µL STET buffer and transferred to a 1.5 mL tube containing 25 µL of lysozyme solution. After incubation for 40 seconds in a 90-100ºC water bath, 10 µL of RNase A solution was added followed by centrifugation for 15 minutes at 16160 x *g*. The supernatant was mixed with 400 µL isopropanol by repeated inversion and further centrifuged at 16160 x *g* for 10 minutes to pellet DNA. After removal of the supernatant, 1 mL of 70% ethanol was added and centrifuged for 10 minutes at 7500 x *g*. The ethanol was completely

removed before dissolving of the pelleted DNA in 50 µL of ultrapure water. The plasmid DNA was then quantified using NanoDrop ND2000 ultraviolet–visible spectrophotometer (Labtech International Ltd, UK).

In order to screen for successful insertion of the restriction-digested PCR product into the linearised plasmid, a confirmatory restriction digest was performed. Each 30 µL reaction typically consisted of 200-400 ng of extracted DNA, 3 µL of the appropriate buffer (commonly 10x CutSmart Buffer), 5-10 U of restriction enzyme (commonly KpnI-HF and/or XhoI) (NEB, UK) and ultrapure water. The reaction was incubated at 37ºC for 3 hours followed by gel electrophoresis (section 2.4.8).

2.4.6 Mutagenesis

Mutagenesis of constructs was undertaken using two distinct methods. The procedures pertaining to mutagenesis (I) relate to the initial assessment of the pain protective haplotype, as described (section 2.4.6.1), and corresponds to reactions 1 to 3 (Appendix 5). An alternative method was used (section 2.4.6.2) for mutagenesis of the 15 kb construct annotated as reaction 4 (Appendix 5).

2.4.6.1 Mutagenesis (I)

Mutagenesis of plasmid constructs encompassed two simultaneous PCRs. For each plasmid, 25 µL reactions were prepared consisting of 14.5 µL nuclease free water, 2.5 µL of 10x reaction buffer, 5 µL GC Solution, 500 ng plasmid DNA, 0.25 µL Pwo DNA polymerase (Roche, UK) and 2.5 µM of either forward or reverse primer. Each pair of reactions were subject to 95ºC for 30 seconds followed by 18 cycles of 95ºC for 30 seconds, 55ºC for 1 minute and 72ºC for 7 minutes. Both reactions were then combined and incubated at 95ºC for 5 minutes, 90ºC for 1 minute, 80ºC for 1 minute, 70ºC for 30 seconds, 60ºC for 30 seconds, 50ºC for 30 seconds and finally, 40ºC for 30 seconds. After completion of cycling, 1.5 µL (30 U) of DpnI (NEB, UK) was added to the reaction and incubated overnight at 37ºC. Three microlitres of the reaction was added to 50 µL of competent DH5α cells and incubated on ice for 5 minutes followed by heat shock at 42ºC for 45 seconds and incubation on ice for 5 minutes. After incubation, the cells were added to 350 µL of LB broth and shaken (250 rpm) for 30 minutes. Three hundred and fifty microlitres were spread on a selection plate containing ampicillin and incubated at 37ºC overnight.

2.4.6.2 Mutagenesis (II)

A PCR was undertaken using the parameters described (Appendix 5) with the 15 kb *GCH1* promoter construct as a template. The resulting sample was then subject to gel electrophoresis and extracted as described (section 2.4.2). The eluate was then diluted to 67 ng/µL and processed with the Q5 Site-Directed Mutagenesis Kit (NEB, UK). A single 10 µL reaction was prepared consisting of 1 µL (67 ng) of amplified plasmid DNA, 5 µL of 2x reaction buffer, 1 µL of enzyme mix (kinase, ligase and DPNI) and 2 µL nuclease free water. The reaction was incubated at 22ºC overnight and transformed using the procedure described (section 2.4.5).

2.4.7 Competent cells

Competent cells were generated by inoculation of DH5α™ cells (NEB, UK) in 5 mL of LB broth in a 15 mL tube followed by shaking incubation (250 rpm) overnight at 37ºC. After which, 1 mL of the LB broth was transferred to 100 mL of LB broth in a conical flask, which was then subject to shaking incubation (250 rpm) at 37ºC for 2.5 hours. Fifty millilitres of the cell suspension was then transferred to each of two 50 mL tubes and centrifuged at 1500 x *g* for 5 minutes. After discarding the supernatant, the bacterial cell pellet was resuspended in 40 mL of 100 mM calcium chloride solution (ice cold) by repeated inversion and centrifuged at 1500 x *g* for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 8 mL of the same calcium chloride solution. Cells were then stored in ice, within a (2-8ºC) refrigerator, for up to one week.

2.4.8 Agarose Gel electrophoresis (DNA)

A 1.5% agarose gel (containing 1.5 µL of GreenView DNA Gel Stain or 5 µL of ethidium bromide solution) was made by melting 2.25 g agarose in 150 mL of 1xTBE or 1xTAE. The percentage of agarose was varied (1-3%) depending on the anticipated molecular weight of the electrophoresed DNA. An appropriate volume of 6x loading dye was added to the sample followed by electrophoresis at ~90V for ~1 hour. The gel was visualised and photographed using a ChemiDoc MP Imaging System (BioRad, UK).

2.5 Cell culture

2.5.1 Thawing

To thaw cells from liquid nitrogen storage the cryovial was placed in a 37ºC water bath for the minimum required time. Once thawed, the cells were transferred into 5 mL of warmed cell culture medium in a 15 mL tube and centrifuged at 500 x *g* for 5 minutes. After removal of the media, the

cells were resuspended in culture cell medium. The cell suspension was then transferred to a suitably sized cell culture flask and placed in a humidified 37ºC incubator for expansion.

2.5.2 Maintenance cell culture

Cell culture was undertaken using using several cell lines maintained in a humidified 37ºC cell culture incubator. Human embryonic kidney cells 293 (HEK293) and a mouse macrophage cell line (RAW264.7) were cultured in DMEM (Sigma, UK) containing 10% heat inactivated fetal calf serum (FCS) (Sigma, UK) and penicillin/streptomycin (Sigma, UK). Heat inactivation of FCS was carried out by increasing its temperature to 55ºC for 30 minutes, followed by gradual cooling. The neuroblastoma derived cell line, SH-SY5Y, was cultured in a base medium consisting of Ham's F-12 (Sigma, UK) and DMEM at a 1:1 ratio with 10% FCS and penicillin/streptomycin. Pooled human umbilical vein endothelial cells (HUVECs) (Cell Applications, USA) were cultured in Endothelial Cell Growth Medium (Cell Applications, USA). Population doublings of HUVECs were monitored and cells allowed to expand until the 15th doubling, after which they were not used for experimentation.

Cell culture media was replenished every 24-48 hours, depending upon confluency. When cell reached ~70% confluency or when required, cells were passaged as described (section 2.5.3), aside for RAW264.7 cells wherein a cell scraper was used to detach cells in PBS. After resuspension of the cell pellet in the desired media, ~20% of the cells were seeded for continuation of culture. The remainder was used as required and/or discarded. Cell counting was performed by the addition of 30 µL of trypan blue (Sigma, UK) to 30 µL of cell suspension in a 1.5 mL microcentrifuge tube. The resulting solution was transferred beneath 2 haemocytometer cover slips. Cells within a 1 mm^2 grid were counted. The average number of cells (x10^4/mL) from two counts was doubled to account for the initial trypan blue dilution.

2.5.3 Freezing

For long-term storage media was removed and cells washed with 5 mL of 1x PBS followed by the addition of 5 mL of 1x trypsin-EDTA solution (appropriately diluted with PBS) (Sigma, UK) and incubation for 3-5 minutes in a 37ºC incubator. After complete detachment, cells were transferred to a tube containing 10 mL of complete cell culture media. Cells were then centrifuged at 500-1000 x *g* for 5 minutes, the supernatant discarded and cells resuspended in ~5 mL of the appropriate freezing media (section 2.9). One millilitre was then transferred to each cryovial and placed at -80ºC in an isopropanol layered container enabling a controlled rate of cooling. The following day

the cryovials were transferred to liquid nitrogen for long-term storage. The volumes detailed here are applicable to a 75cm^2 cell culture flask.

2.6 Transfection

2.6.1 Extraction of DNA for transfection (HEK293 & SH-SY5Y cells)

In the event of successful ligation and mutagenesis, 5 µL of the plasmid DNA was transformed in to DH5α™ competent cells using the procedure described (section 2.4.5) except for a reduced incubation time of 30 minutes. Following overnight incubation, one colony was then inoculated into 20 mL of LB broth containing 20 µL ampicillin solution in a 50 mL tube, and incubated with shaking (250rpm) overnight at 37ºC. A stock for long-term storage was then made by mixing 750 µL of culture with 750 µL of 30% (v/v) sterile glycerol solution and stored at -80ºC. The remaining culture was then centrifuged at 3000 x *g* for 5 minutes and the supernatant discarded. Plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, UK). The cell pellet was resuspended in 350 µL of Resuspension Solution containing 100 µg/mL RNase A. After transfer to a 1.5 mL microcentrifuge tube, 250 µL of Lysis Solution was added and mixed thoroughly by repeated inversion, followed by 250 µL of Neutralisation Solution to precipitate cell debris. All centrifugation steps were carried out at 12000 x *g*. Cell debris was pelleted by centrifugation for 5 minutes and the supernatant (~800 µL) transferred to a GeneJET Spin Column. After centrifugation for 1 minute, the flow-through was discarded followed by two cycles of 500 µL of Wash Solution and centrifugation. Residual Wash Solution was then removed from the column by centrifugation for 1 minute. DNA was eluted from the silica membrane into a clean 1.5 mL microcentrifuge tube by the addition of 50 µL of ultrapure water to the membrane, incubation for 2 minutes and centrifugation for 2 minutes. The eluate was stored at -20ºC. DNA obtained using this extraction method was used to transfect HEK293 and SH-SY5Y cells.

2.6.2 Transfection and Dual-Glo Luciferase Assay

For the transfection of HEK293 and SH-SY5Y cells, plasmids were extracted using the GeneJET Plasmid Miniprep Kit as described (section 2.6.1). DNA was then quantified 5 times using a NanoDrop ND2000 ultraviolet–visible spectrophotometer (Labtech International Ltd, UK). The firefly (pGL4.26 [minimal promoter] backbone constructs) and *Renilla* (pRL-TK) luciferase encoding plasmids were then combined at a predetermined ratio (50:1) and diluted to a final concentration of 50 ng/µL. Cells were seeded by the addition of 100 µL of cell suspension onto a NUNC 96-well white cell culture plate at a density of 1x10^5 cells/mL and incubated for 24 hours.

After 24 hours the media was replaced with 90 µL of the same media. Forty microliters of transfection complex was then formed by the addition of 16 µL (800 ng) of DNA to 21.6 µL of (serum and antibiotics-free) DMEM (or DMEM and HAM's F-12), followed by the addition of 2.4 µL of Fugene 6 transfection reagent (Promega, USA). The transfection mix was gently agitated and allowed to complex at room temperature for 15 minutes. Transfections were then undertaken in triplicate (on three occasions) by the addition of 10 µL of complex (containing 200 ng DNA) to each well. After incubation for 24 hours, assessment of reporter gene expression was undertaken with the Dual-Glo Luciferase Assay (Promega, USA). All reagents were equilibrated to room temperature before use. Cells were lysed by the addition of an appropriate volume (equal to the culture media volume) of Dual-Glo Reagent and incubated for 60 minutes before recording of firefly luminescence using a FLUOstar OPTIMA Microplate Reader (BMG Labtech, Germany). Luminometer settings included a 0.5 second delay and a 9.5 second integration time. The Dual-Glo Stop & Glo Substrate was then diluted 1:100 in an appropriate volume of Dual-Glo Stop & Glo Buffer and a volume equal to the original cell culture media volume (100 µL) was added to each well. Renilla luminescence was then recorded after the same incubation time using the same luminometer settings. Data was then exported and background recordings (observed from wells treated as described with the exception of DNA in the transfection mix) subtracted. Firefly luciferase was then normalised to that of *Renilla* luciferase, followed by expression of data as a percentage of normalised pGL4.26 luminescence. Data was analysed in GraphPad Prism 6.0.

This method was subsequently modified with the following adaptations in order to permit efficient transfection of the 15 kb pGL4.20-*GCH1* construct into HEK293 cells. Twenty four hours prior to transfection, 55 µL (22000 cells) of cell suspension ($4x10^5$ cells/mL) was seeded in complete growth medium. The media was replaced immediately prior to transfection. The firefly (pGL4.20 backbone constructs) and *Renilla* (pRL-SV40) luciferase encoding plasmids were then combined at a predetermined ratio (10:1) and diluted to a final concentration of 100 ng/µL. A transfection complex was then formed consisting of 90 µL DMEM, 10 µL (1 µg) DNA and 2.5 µL of X-tremeGENE HP DNA Transfection Reagent (Roche, UK). After incubation at room temperature for 15 minutes, 20 µL of transfection complex (containing 200 ng DNA) was added to each of three wells. A total of 75 µL of each luciferase assay reagent was used and all other parameters remained as previously described within this subheading.

2.6.3 Extraction of DNA for transfection (HUVECs)

For the transfection of HUVECs, a previously detailed method for the transfection of RAW264.7 cells was adapted (Cheung *et al.* 2015). DNA was extracted in a 2-step process to enhance purity and minimise residual endotoxin. The first step used the PureLink HiPure Plasmid Midiprep Kit (Thermo Scientific, UK), followed by a second step of phenol-chloroform-isoamyl alcohol purification. After inoculation of 25 mL LB broth containing 25 µL of ampicillin solution and overnight incubation at 37ºC with shaking (250 rpm), the culture was centrifuged at 4000 x *g* for 10 minutes and the supernatant removed. The cell pellet was resuspended in 4 mL of Resuspension Buffer containing 150 µg/mL RNase A. An equal volume of Lysis Buffer was added followed by mixing by inversion and incubation for 5 minutes, after which 4 mL of Precipitation Buffer was added and again mixed by repeated inversion until homogenous. The resulting solution was centrifuged at 16000 x *g* for 10 minutes at 4ºC. The supernatant was transferred to a pre-equilibrated column (equilibrated by the application of 2 mL of Equilibration Buffer to the column and allowing the buffer to drain through) and allowed to drain by gravity flow, thus binding DNA to the column membrane. A total of 10 mL of Wash Buffer was added to the column, allowed to drain by gravity flow, and repeated for a total of 2 washes. A sterile 50 mL tube was placed under the column and 5 mL of Elution Buffer added to the column and allowed to drain by gravity flow. Precipitation was performed by the addition of 3.5 mL of isopropanol to the eluate and centrifugation at 16000 x *g* for 30 minutes at 4ºC. After removal of the supernatant, 3 mL of 70% ethanol was added to wash the pellet. Centrifugation was repeated at 16000 x *g* for 5 minutes at 4ºC. The supernatant was removed and the DNA pellet allowed to air dry, followed by the addition of 200 µL of TE buffer.

For the second phase of plasmid purification, 200 µL of phenol-chloroform-isoamyl alcohol (25:24:1, v/v) (Thermo Scientific, UK) was added to the DNA, followed by repeated mixing for 15 seconds and incubation for 5 minutes at room temperature. The mixture was centrifuged at 13000 x *g* for 10 minutes at room temperature. One hundred and forty microliters of the upper aqueous phase was transferred to a fresh 1.5 mL microcentrifuge tube. To the remaining lower organic phase, 140 µL of TE buffer was added with mixing, incubation and centrifugation as previous. The subsequent upper aqueous phase (140 µL) was combined with the initial 140 µL of upper aqueous phase containing the DNA. Precipitation of DNA was carried out by the addition of 28 µL of 3 M sodium acetate (pH 5.2) and 280 µL of isopropanol. The sample was mixed by repeated inversion and incubated at room temperature for 10 minutes, followed by centrifugation

for 10 minutes at room temperature. The supernatant was removed and 200 µL of 75% ethanol was added to the DNA pellet, vortexed, and centrifuged at 5000 x *g* for 5 minutes at room temperature. The supernatant was removed, and the pellet allowed to air dry. One hundred microliters of TE buffer was added to dissolve the DNA. Dissolution of the pelleted DNA was facilitated by heating to 50ºC for 10 minutes. DNA was quantified using a NanoDrop 2000 UV-Vis spectrophotometer (Labtech International Ltd, UK).

2.6.4 Transfection of HUVECs

Transfection of HUVECs was carried out using the Cytofect HUVEC Transfection Kit (Cell Applications, USA). Cells were cultured until ~75% confluency in a T75 cell culture flask (population doubling <10). Endothelial Cell Growth Medium (Cell Applications, USA) was removed, cells washed with PBS, trypsinised and pelleted as per standard protocol (section 2.5). The cells were resuspended in a small volume (~2 mL) of antibiotics-free Endothelial Cell Growth Medium and diluted further in the same medium to a concentration of $1x10^5$ cells/mL. One hundred microlitres of the resulting cell suspension ($1x10^4$ cells) was distributed per well of a 96-well cell culture plate and incubated for 20-24 hours. The firefly (pGL4.20/pGL4.26 backbone constructs) and *Renilla* (pRL-SV40) luciferase encoding constructs, quantified as described (section 2.6.2), were then combined at a predetermined ratio (50:1) and diluted to a final concentration of 50 ng/µL. The following quantities equate to the transfection volumes required for each well. A total of 3.6 µL (180 ng) of DNA was diluted in 56.4 µL of Transfection Medium and mixed gently by repeated flicking. The transfection complex was prepared by the addition of 0.12 µL of Cytofect-2 (mixed gently by repeated flicking) and then 0.12 µL of Peptide Enhancer (mixed gently by repeated flicking). The DNA-transfection reagent mix was then incubated at 37ºC in a water bath for 25 minutes. After which, the cell culture media was aspirated from the cells, and 60 µL of transfection complex was pipetted into each well and incubated under normal cell culturing conditions for 1 hour. The transfection complex was then aspirated and replaced with 100 µL of antibiotics-free Endothelial Cell Growth Medium (with or without 40 ng/mL TNF-α and 100 ng/mL IFN-γ). The cells were then incubated for 24 hours until lysis. Transfections were conducted in triplicate for each condition on three separate occasions.

2.6.5 Dual-Luciferase Reporter Assay

The Dual-Luciferase Reporter Assay (Promega, USA) was carried out in order to maximise luminescent signal. After transfection of HUVECs as described (section 2.6.3) and subsequent incubation for 24 hours, media was aspirated from each 96-well and gently replaced with 100 µL

of PBS to removed residual media. The PBS was then entirely aspirated and 25 µL of 1x Passive Lysis Buffer was added to each well and incubated with gentle rocking at room temperature for 30 minutes. The lysate was then stored at -80ºC until analysis. After thawing to room temperature, 10 µL of homogenous lysate was transferred to a single well of a 96-well NUNC white plate. Firefly and *Renilla* luminescence was recorded using a GloMax-96 Microplate Luminometer (Promega, USA). Using the automated injector system, a total of 50 µL of reconstituted Luciferase Assay Substrate was injected into each well, with a 2 second delay between reagent addition and initiating luminescence detection and a 10 second integration time. Immediately after recording of firefly luminescence, 50 µL of Stop & Glo Reagent was injected and *Renilla* luminescence recorded using identical luminometer parameters. Each transfection was undertaken in triplicate, on three occasions. Data was then exported and background recordings (observed from wells treated as described with the exception of DNA in the transfection mix) subtracted. Firefly luciferase was then normalised to that of *Renilla* luciferase. Normalised firefly luminescence pertaining to the construct with the *GCH1* promoter (pGL4.20-GCH1-3.4kb) was expressed as a percentage of pGL4.20 relative luminescence. As the pGL4.20-GCH1-3.4kb construct subsequently formed the backbone for the addition of multiple regions of the *GCH1* intron, relative luminescence pertaining to these constructs was expressed as a percentage of pGL4.20-GCH1-3.4kb, in the presence or absence of cytokines. Data was analysed in GraphPad Prism 6.0.

2.6.6 Fluorescent microscopy

For the purpose of optimisation, transfections were undertaken as described (section 2.6.4) with the following adaptations. After resuspending of cells to a concentration of $1x10^5$ cells/mL. Two hundred and fifty microlitres of the resulting cell suspension was distributed per well of a 48-well cell culture plate and incubated for 20-24 hours. The following volumes equate to the transfection volumes required for each well. A total of 300 ng of DNA (expression vector encoding a GFP-tagged carbohydrate-responsive element-binding protein) was diluted in 100 µL of Transfection Medium and mixed gently by repeated flicking. The transfection complex was prepared by the addition of 0.25 µL of Cytofect-2 (mixed by repeated flicking) and then 0.25 µL of Peptide Enhancer (mixed by repeated flicking). The DNA-transfection reagent mix was then incubated at 37ºC in a water bath for 25 minutes. After which, the culture media was aspirated from the cells, and 100 µL of transfection complex was pipetted into the well and incubated under normal cell culturing conditions for 1 hour. The transfection complex was then aspirated and replaced with

250 µL of antibiotics-free Endothelial Cell Growth Medium. The cells were then incubated for 24 hours until visualisation.

Transfected cells were then imaged using an EVOS FL Cell Imaging System (Thermo Scientific, UK) equipped with DAPI and GFP EVOS LED Light Cubes. For images incorporating both DAPI and GFP cubes, cells were maintained for 24 hours after transfection before fixation, permeation and the application of 4,6-diamidino-2-phenylindole (DAPI) dihydrochloride. After removal of cell culture media, 500 µL of 4% formaldehyde was added to each well of the 24-well cell culture plate and incubated at room temperature for 15 minutes. The formaldehyde solution was removed and cells washed twice with 500 µL of 1x Tris-buffered saline (Thermo Scientific, UK), after which 500 µL of 1x Permeabilisation Buffer (Thermo Scientific, UK) was added and incubated at room temperature for 15 minutes. Cells were then washed once with 1x TBS followed by the addition of 200 µL of DMEM containing 5 µg/mL DAPI dihydrochloride.

2.7 EMSA

2.7.1 Cell culture and drug treatment

In order to obtain nuclear protein, HEK293 cells were maintained as described (section 2.5). Several permutations were considered based upon MatInspector analysis (section 2.8.4). Initially the effect of different concentrations of MeBio (exogenous AhR agonist) were assessed on *GCH1* and *CYP1A1* (positive control) expression, in addition to L-kynurenine (endogenous AhR agonist) and CH-223191 (AhR antagonist). This was similarly repeated in the case of cobalt chloride (a chemical mimetic of hypoxia) by the analysis of *GCH1* and *VEGFA* (positive control) expression. Cells were seeded in 500 µL of DMEM into a 24-well cell culture plate at a density of $2.5x10^5$ cells/mL. After 24 hours, media was replaced with media containing vehicle only (control) or drug containing media. Gene expression analysis was conducted after a further 24 hours by lysis of cells in 500 µL TRI Reagent (Sigma, UK) as processed as described (section 2.8.2)

In order to conduct the EMSA, cells in two T75 cell culture flasks were expanded to ~70% confluency and subject to media replacement with either complete DMEM or complete DMEM containing 250 µM of cobalt chloride and cultured for a further 24 hours prior to extraction of nuclear protein as described (section 2.7.2). This was also performed with respect to MeBio, which was used at a final concentration of 1 µM.

2.7.2 Isolation of nuclear protein

Nuclear protein from HEK293 cells (passage <30) was isolated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, UK). Cells were grown to >70% confluency and harvested as described (section 2.5.3). The resulting cell pellet was then resuspended in 1 mL of PBS and transferred to a 1.5 mL microcentrifuge tube followed by centrifugation at 500 x *g* for 3 minutes. After removal of the entire supernatant, an appropriate volume (100 µL per 10 µL of packed cell volume) of ice-cold CER1 was added to the cell pellet followed by 1 µL of Protease Inhibitor Cocktail (Sigma, UK) per 100 µL of CER1. The cell pellet was resuspended by vortexing for 15 seconds, and incubated on ice for 10 minutes. Ice-cold CERII was then added at a ratio of 5.5 µL per 10 µL of original packed cell volume, followed by vortexing for 5 seconds, incubation on ice for 1 minute and centrifugation at 4ºC for 5 minutes at 16000 x *g*. The entire supernatant (cytoplasmic isolate) was transferred to pre-chilled 1.5 mL microcentrifuge tube for storage at -80ºC. An appropriate volume (50 µL per 10 µL of original packed cell volume) of ice-cold NER was added to the pellet, followed by 1 µL of Protease Inhibitor Cocktail per 100 µL NER. The nuclei-containing pellet was then vortexed for 15 seconds and incubated on ice for 40 minutes with intermittent vortexing for 15 seconds at 10 minute intervals, followed by centriguation at 4ºC for 10 minutes at 16000 x *g*. The supernatant (nuclear extract) was then transferred to pre-chilled 1.5 mL microcentrifuge tube for immediate storage at -80ºC until use.

2.7.3 Protein quantification

Quantification of nuclear protein was undertaken using the Bradford Assay. Ten microliters of nuclear extract was diluted to 100 µL with ultrapure water to bring the concentration within the linear range of the assay. Standards were made by dissolving 1 mg of bovine serum albumin (BSA) (Sigma, UK) in 1 mL of ultrapure water. Serial dilutions of BSA ranged from 0 µg/mL to 1000 µg/mL. Ten microliters of standards and samples were dispensed, in duplicate, into a 96-well plate. Bio-Rad protein assay reagent (5x) (Bio-Rad, UK) was diluted with water to its working concentration and to each well containing a standard or unknown, 200 µL of the diluted assay reagent was added. The plate was incubated at room temperature for 5 minutes and absorbance read at 595 nm using an Infinite F50 microplate reader (Tecan, UK).

2.7.4 Annealing of oligonucleotides

Ten microliters of the forward and reverse oligonucleotides (50 µM each) were diluted with 70 µL of ultrapure water and 10 µL of 10x annealing buffer in a 0.2 mL tube. Annealing of the oligonucleotides was achieved by heating to 95ºC for 5 minutes, followed by a controlled

temperature reduction of 1°C per minute, for 70 minutes, using a Bio-Rad T100 thermal cycler. Annealed oligonucleotides were then aliquoted and stored at -20°C until use. Oligonucleotide sequences are detailed (Appendix 6).

2.7.5 Native polyacrylamide gel

Preparation of a native (6%) polyacrylamide gel was performed by mixing 16.6 mL of ultrapure water with 2.5 mL of 5x TBE. A total of 3.75 mL of 40% acrylamide (Bio-Rad, UK) and 2 mL of 2% bis-acrylamide (Bio-Rad, UK) were added, followed by 125 µL of 10% w/v ammonium persulfate (Sigma, UK) and finally, 25 µL of the polymerisation catalyst tetramethylethylenediamine (TEMED) (Bio-Rad, UK). The solution, sufficient for two gels, was immediately transferred to the gel casting apparatus and allowed to set for 1 hour at room temperature before pre-electrophoresing of the gel for 30 minutes at 70V.

2.7.6 EMSA

The electrophoretic mobility shift assay (EMSA) was performed with the Infrared EMSA Kit (LI-COR, USA). To each of three tubes per binding analysis, an appropriate volume of ultrapure water (such that the total volume reaches 20 µL) was added to a 0.2 mL tube, followed by 2 µL of 10x binding buffer. Two microliters of 25 mM DTT/2.5% Tween 20®, 1 µL of 1 µg/µL Poly (dI·dC) and 1 µL of 100 mM $MgCl_2$ were then added to all binding reactions. To the third tube, 1 µL of 10 µM excess unlabelled competitor probe was added. Nuclear protein was allowed to thaw on ice and 5 µg was added to the second and third tubes, followed by 2 µL of 50 nM Cy5 labelled probe to all tubes. For the supershift assay, a forth tube was included with the same constituents as the second tube, except for the addition of 2.5 µL of HIF-1α antibody (sc-10790; Santa Cruz Biotechnology, USA). All reactions were incubated at room temperature for 30 minutes. Two microliters of 10x Orange Loading Dye was added to each reaction before loading onto a pre electrophoresed polyacrylamide gel. Electrophoresis was performed at 70V in the dark until the dye front reached approximately two thirds distance. The polyacrylamide gel was then carefully excised from the cast and imaged using the Cy5 setting on a ChemiDoc MP Imaging System (BioRad, UK).

2.8 Other methods

2.8.1 Genomic DNA extraction from cultured cells

Extraction of gDNA from cultured cells was carried out using the GeneJet Genomic DNA Purification Kit (Thermo Scientific, UK). Adherent cells (~$5x10^6$) were tryspinised and pelleted

as described (section 2.5). After decanting of the supernatant, cells were resuspended in 5 mL of PBS and centrifuged at 500 x *g* for 5 minutes. The entire supernatant was then removed and the cell pellet resuspended in 200 µL of PBS and transferred to a 1.5 mL microcentrifuge tube, followed by the addition of 200 µL of Lysis Solution and 20 µL of proteinase K. The sample was then incubated at 56ºC for 10 minutes with occasional vortexing. Twenty microliters of RNase A solution was then added, mixed by vortexing, and incubated at room temperature for 10 minutes. Four hundred microliters of 50% ethanol was added, mixed by vortexing, and transferred to a purification column before centrifugation at 6000 x *g* for 1 minute. After discarding of the eluate, 500 µL of Wash Buffer I was added to the column and centrifuged at 8000 x *g* for 1 minute, followed by the addition of 500 µL of Wash Buffer II and centrifugation at 16160 x *g* for 3 minutes. The column was then placed in a sterile 1.5 mL microcentrifuge tube and incubated for 2 minutes at room temperature after the addition of 100 µL of Elution Buffer. The gDNA was eluted by centrifugation at 8000 x *g* for 1 minute followed by quantification using a NanoDrop ND2000 ultraviolet–visible spectrophotometer (Labtech International Ltd, UK) and stored at -20ºC.

2.8.2 RNA extraction from monolayer cells and removal of DNA

Following removal of cell culture media, cells were washed twice with DEPC treated PBS. After the addition of 1 mL of TRI Reagent per 10 cm^2 of culture plate surface area, the resulting lysate was passed subject to repeated pipetting to ensure homogeneity. The entire lysate was then transferred to a 1.5 mL microcentrifuge tube and 0.2 mL of chloroform added per 1 mL of TRI Reagent, followed by vigorous shaking for 15 seconds. After allowing phase separation for 5 minutes, the resulting mixture was centrifuged at 12000 x *g* for 15 minutes at room temperature. The resulting upper aqueous layer containing RNA was then transferred to a new 1.5 mL microcentrifuge tube containing 0.5 mL of isopropanol per 1 mL of the initial TRI Reagent volume. The sample was mixed by repeated inversion and allowed to settle for 5 minutes before centrifugation at 12000 x *g* for 10 minutes at room temperature. The resulting supernatant was then removed leaving the RNA pellet. This was washed by the addition of 1 mL of 75% ethanol, followed by vortexing and centrifugation at 7500 x *g* for 5 minutes. Excess ethanol was removed and the RNA pellet allowed to air dry for 5-10 minutes until minimal residual ethanol remained on the pellet. An appropriate volume of nuclease free/DEPC treated water was added to the pellet followed by incubation at 55ºC for 10 minutes. The RNA was then quantified using NanoDrop ND2000 ultraviolet–visible spectrophotometer (Labtech International Ltd, UK) and stored at -80ºC. Carry over DNA was then removed by DNase I treatment prior to cDNA synthesis. After

extraction, up to 1 µg of RNA was diluted to 8 µL with RNase-free/DEPC treated water and 1 µL of 10x reaction buffer added, followed by 1 µL (1 U) of DNase I. After incubation at 37ºC for 1 hour, 1 µL of 50 mM EDTA was added and incubated at 65ºC for 5 minutes. If the input RNA was less than 500 ng, the aforementioned volumes were reduced by half. Synthesis of cDNA was then carried out as described (section 2.1.2.4)

2.8.3 Western blot

Cultured HUVECs were expanded on T75 cell culture flasks until 60-70% confluent and subject to treatment with vehicle (PBS) or cytokine stimulation (40 ng/mL TNF-α and 100 ng/mL IFN-γ) for 24 hours. Media was aspirated, cells washed, trypsinised and centrifuged as described (section 2.5.3). The supernatant was discarded, and the cell pellet washed by resuspension in 5 mL of PBS. The cells were again pelleted by centrifugation at 500 x *g* for 5 minutes. After removal of the entire supernatant, 120 µL of RIPA buffer (Sigma, UK) was added to the cell pellet (containing $5x10^5$ cells), mixed briefly by pipetting, and incubated at 4ºC for 30 minutes. The lysate was centrifuged at 10000 x *g* for 10 minutes and the supernatant stored at -80ºC until analysis. Protein quantification was performed using the Bradford assay as described (section 2.7.3), to ensure equal loading. A total of 82.5 µL of sample (correlating to 82.5 µg protein) was mixed with 27.5 µL of 4x laemmli sample loading buffer, followed by incubation at 100ºC for 10 minutes. Forty microlitres (~33 µg) of the sample was loaded in a single well (in duplicate) of a 4-20% Mini-PROTEAN TGX gel (Bio-Rad, UK) followed by electrophoresis (35mA, 250V) for 45 minutes using a Mini Protean Tetra System and 1xTris-glycine-SDS (TGS) buffer (Bio-Rad, UK).

After completion of electrophoresis the polyacrylamide gel was removed and placed on an Immun-Blot PVDF membrane (Bio-Rad, UK), pre-soaked in methanol and then washed in transfer buffer. The PVDF membrane was then placed upon a piece of extra thick blot paper (Bio-Rad, UK) pre-soaked in transfer buffer. A further piece of blot paper was placed over the polyacrylamide gel and the stack placed in a Trans-Blot Turbo Transfer System (25V, 1.0A, 30 minutes). The PVDF membrane was then blocked in a 10% milk powder solution for 30 minutes with rocking followed by a rinse in TBST and a further wash in TBST for 10 minutes with rocking. Primary antibodies for GAPDH (sc-25778; Santa Cruz Biotechnology, USA) and GTPCH (sc-134574; Santa Cruz Biotechnology; USA) were diluted in 1% milk powder in TBS (to a final dilution of 1:500) and rocked overnight on ice with the membrane. The PVDF membrane was then subject to two brief rinses in TBST, washed in TBST for 5 minutes, one brief rinse and two washes with rocking for 10 minutes each. The secondary antibodies (Alexa Fluor 546 goat anti-rabbit and Alexa Fluor 647

goat anti-mouse) (Thermo Scientific, UK) were then diluted (1:10000) in 1% milk powder, applied to the PVDF membrane, and rocked for 1 hour. The membrane was then briefly rinsed in TBST twice, and washed with rocking a further 10 minutes. A final wash was done with TBS. The PVDF membrane was then visualised using a ChemiDoc MP Imaging System (Bio-Rad, UK)**.**

2.8.4 MatInspector

In-silico analysis of transcription factor binding sites pertaining to the pain protective haplotype was conducted using the MatInspector facility, which is available within the Genomatix Software Suite (v3.8). Wild-type and variant sequences of DNA, each consisting of fifty nucleotides flanking both 5' and 3' of the SNP, were individually uploaded into MatInspector using the Transcription factor binding sites (weighted matrices) library (v10.0). All matrices from the 'General Core Promoter Elements' and 'Vertebrates' were included. A default Core similarity (the degree of sequence similarity of the highly conserved matrix positions when compared to the DNA input sequence) of 0.75 was selected. The Matrix similarity was maintained at the default 'Optimised' setting to reduce false positive discovery.

2.9 Stocks, solutions and reagents

Agarose gel (1% w/v): 1 g of agarose heated in 100 mL of 1x TBE or 1xTAE until dissolved, followed by the addition of 1 µL of GreenView DNA Gel Stain (GeneCopedia, USA) or 3 µL of ethidium bromide solution.

Ammonium persulfate (10% w/v): 100 mg of ammonium persulfate (Sigma, UK) dissolved in 1 mL of ultrapure water.

Ampicillin solution (1000x): 500 mg of ampicillin sodium (Thermo Scientific, UK) dissolved in 5 mL of ultrapure water, and stored at 2-8^{o}C for up to 2 weeks.

Annealing buffer (10x): 1 mL of 100 mM tris-HCl (pH 7.5), 200 µL of 10 mM EDTA (pH 8.0) and 1 mL of 500 mM NaCl, diluted to 10 mL with ultrapure water, and filtered to remove particulates.

Calcium chloride (100 mM): 7.35 g of calcium chloride dihydrate (Sigma, UK) dissolved in 500 mL of ultrapure water, and sterilised by autoclaving.

CH-223191 (10 mM): Dissolved 1.67 mg of CH-223191 (Tocris, UK) in 500 µL of DMSO and stored at -20^{o}C.

Cobalt chloride (100 mM): 238 mg of cobalt chloride hexahydrate (Sigma, UK) dissolved in 10 mL of ultrapure water and sterile filtered.

DAPI (1 mg/mL): Dissolved 1 mg of DAPI dihydrochloride (Santa Cruz Biotechnology, USA) in 1 mL of distilled water, further diluted to a working concentration by the addition of 5 µL per millilitre of media.

Dithioerythritol (1000x): 154.25 mg of DTE (Sigma, UK) dissolved in water with heating to 55ºC to facilitate dissolution if necessary, followed by storage at -80ºC.

EDTA, pH 8.0 (0.5 M): 186.12 g of EDTA disodium salt (dihydrate) (Sigma, UK) dissolved in 800 mL of ultrapure water, pH adjusted with sodium hydroxide pellets, and autoclaved after making to a final volume of 1 L.

Ethidium bromide (10 mg/mL): 50 mg of ethidium bromide dissolved in 5 mL of ultrapure water, and stored at 2-8ºC in the dark.

Freezing media: 50 mL of freezing media was prepared by the addition of 5 mL of DMSO to 45 mL of the appropriate basal cell culture medium.

Freezing media (HUVEC): 20 mL of freezing media for HUVECs was prepared by the addition of 2 mL of DMSO to 18 mL heat inactivated FCS.

Glycerol solution (30% v/v): 30 mL of glycerol (Thermo Scientific, UK) mixed with 70 mL of ultrapure water and sterilised by autoclaving.

L-kynurenine (50 mM): Dissolved 5.21 mg of L-kynurenine (Tocris, UK) in 500 µL of ultrapure water, sterile filtered, and stored at -20ºC.

Laemmli sample loading buffer (4x): For 50 µL of laemmli sample loading buffer, added 5 µL of β-mercaptoethanol (Sigma, UK) to 45 µL of laemmli sample buffer (Bio-Rad, UK).

LB agar: 10 g of LB (Sigma, UK) and 7.5 g agar (Sigma, UK) dissolved in 500 mL of ultrapure water and sterilised by autoclaving. For selection plates, after cooling to 50ºC, 500 µL of ampicillin solution was added. The resulting mixture was then poured into petri dishes and allowed to set.

***LB broth*:** 10 g of LB broth powder (Sigma, UK) dissolved in 500 mL of ultrapure water and sterilised by autoclaving.

Loading dye: 25 mg of bromophenol blue dissolved in 7 mL of ultrapure water and added to 3 mL of glycerol (Thermo Scientific, UK).

Lysozyme (10 mg/mL): 20 mg of lysozyme (Amresco, USA) dissolved in 2 mL of 10 mM tris-HCl pH 8.0 and stored at 2-8^{o}C for up to 1 month.

MeBio (10 mM): Dissolved 1.85 mg of MeBio (Tocris, UK) in 500 µL of DMSO and stored at -20^{o}C.

Milk powder solution (1% w/v): 75 mg of dried skimmed milk (Marvel, Ireland) in 7.5 mL of TBS.

Milk powder solution (10% w/v): 1 g of dried skimmed milk (Marvel, Ireland) in 10 mL of TBST.

Mobile phase (2 L): 50 mM sodium acetate, 5 mM citric acid, 48 µM EDTA (36 mg), 160 µM DTE (49.4 mg), pH 5.22.

NaCl (5 M): Dissolved 29.22 g of sodium chloride in 100 mL of ultrapure water.

Phosphate buffered saline: 5 PBS tablets (Thermo Scientific, UK) dissolved in 500 mL of ultrapure water and sterilised by autoclaving**.**

Potassium dihydrogen phosphate, pH 7.4 (20 mM): 2.72 g of potassium dihydrogen phosphate dissolved in 900 mL of ultrapure water, pH corrected, and made to a final volume of 1 L.

Precipitation buffer: 8.17 g of trichloroacetic acid (2 M), 1.71 mL of 85% phosphoric acid (2 M) and 25 µL of 1 M DTE (1 mM final concentration), made to a final volume of 25 mL with PBS.

Reagent A: 10 mL of 1 M tris, 109.54 g of sucrose, 470 mg of magnesium chloride and 10 mL of Triton X-100 (Sigma, UK) to 800 mL of ultrapure water. Adjusted to pH 8.0 and made to 1 L with ultrapure water. Autoclaved at 10 p.s.i. for 10 minutes.

Reagent B: 400 mL of 1 M tris (pH 7.6), 120 mL of 0.5 M EDTA (pH 8.0), 8.76 g of sodium chloride, and adjustment to pH 8.0 before making to a final volume of 1 L with ultrapure water. Autoclaved for 15 minutes at 15 p.s.i. and 10 g of sodium dodecyl sulphate added.

RNase A solution: 250 mg RNase A (Thermo Scientific, UK) dissolved in 25 mL of 10 mM tris-HCl pH 7.5. The solution was then boiled for 15 minutes and cooled to room temperature before centrifugation at 12500 x *g* for 5 minutes to remove any precipitate. Aliquots were stored at -20^{o}C.

Resuspension buffer: 20 mL of 50 mM PBS, 20 µL of 1000x (1 M) DTE and 100 µL of 100 µM EDTA.

SOB media: 20 g tryptone, 5 g yeast extract, 2 mL of 5 M NaCl, 2.5 mL of 1 M potassium chloride, 10 mL of 1 M magnesium chloride, 10 mL of 1 M magnesium sulphate and made to a final volume of 1 L with ultrapure water.

Sodium chloride (5 M): 146 g of sodium chloride dissolved in 1 L of ultrapure water and sterilised by autoclaving.

Sodium perchlorate (5 M): 70 g of sodium perchlorate monohydrate dissolved in 80 mL of ultrapure water, then made to a final volume of 100 mL.

STET buffer: 40 g of sucrose, 25 mL of Triton X-100, 25 mL of 1 M tris-HCl pH 8.0 and 50 mL of 0.5 M EDTA pH 8.0, topped up to 500 mL with ultrapure water and sterilised by autoclaving.

TAE (50x): 24.2 g tris (Sigma, UK), 5.71 mL glacial acetic acid, 10 mL 0.5 M EDTA (pH 8.0) and ultrapure water to a final volume of 100 mL.

TBE (5x): 5.4 g tris (Sigma, UK), 2.75 g boric acid (Sigma, UK) and 375 mg EDTA disodium salt (Sigma, UK) were added, made to a final volume of 100 mL with ultrapure water and filtered to remove particulates.

TBE (10x): 54 g tris (Sigma, UK), 27.5 g boric acid (Sigma, UK) and 3.75 g EDTA disodium salt (Sigma, UK) were added to ultrapure water to a final volume of 500 mL.

TBS: 50 mL tris-HCl pH 7.4 (1 M) and 30 mL sodium chloride (5 M), made to volume of 1 L with ultrapure water.

TBST: 50 mL tris-HCl pH 7.4 (1 M), 30 mL sodium chloride (5 M) and 1 mL Tween-20 (Thermo Scientific, UK), to a final volume of 1 L with ultrapure water.

TE buffer, pH 7.6: 10 mL of 1 M tris-HCl pH 7.6, 2 mL of 0.5 M EDTA, ultrapure water added to 900 mL, pH adjustment to 7.6 and made to a final volume of 1 L with ultrapure water. Autoclaved for 15 minutes at 15 p.s.i.

Trichloroacetic acid (5% w/v): 500 mg of trichloroacetic acid was dissolved in 10 mL of ultrapure water.

Tris-HCl, pH 4, 7.5, 7.6 and 8.0 (1 M): Dissolved 121.1 g of tris (Sigma, UK) in 800 mL of ultrapure water, adjusted pH as necessary, and made to a final volume of 1L with ultrapure water. Autoclaved to sterilise.

Tris-HCl pH 8.0 (10 mM): Dilution of 10 mL of 1 M tris-HCl pH 8.0 with 990 mL of ultrapure water.

Chapter 3 The Role of Tetrahydrobiopterin Synthesis in Chronic Neuropathic Pain

3.1 Introduction

Sensitivity to pain, the propensity to develop chronic pain and the subsequent potential for variable response to pharmacotherapy highlights great challenges for the clinical management of patients with chronic pain, with potential implications for accurate diagnosis, prognosis and treatment efficacy (Lacroix-Fralish *et al.* 2009). It is common for individuals to regard themselves as particularly sensitive or insensitive to pain, though beyond quantitative sensory testing, it is difficult to ascertain the mechanisms underpinning such claims (Coghill *et al.* 2003). Indeed, the somewhat subjective nature of reporting using pain intensity scales has been ameliorated by the identification of correlations between the extremes of pain sensitivity and observations from functional magnetic resonance imaging of the brain (Coghill *et al.* 2003).

One such apparent, and somewhat unexplained variation, is the likelihood for an individual to develop chronic pain. After trauma, or the onset of a disease or infection associated with chronic pain, such as stroke, herpes zoster or diabetes mellitus, studies have consistently highlighted that only a proportion of these patients will ultimately develop chronic pain (Andersen *et al.* 1995, Yawn *et al.* 2007) (Davies *et al.* 2006). This suggests a prominent role for genetic predisposition, with chronic pain more likely to result from an insult to the nervous system in susceptible patients (Lacroix-Fralish *et al.* 2009). Interestingly, heightened sensitivity to experimentally induced pain, or a diminished pain-inhibitory system, has been associated with greater propensity to develop severe chronic pain (Edwards 2005). It has been considered that patients with chronic pain who disclose chronic pain as a common feature in their family history may have impaired function of the endogenous opioid pathway, thereby suggesting an underlying genetic basis for variation in pain sensitivity and susceptibility (Bruehl *et al.* 2006).

Studies analysing experimental pain sensitivity have highlighted that the majority of the measured response to painful stimuli is underpinned by the individual subject, rather than the stimulus parameter or intensity (Nielsen *et al.* 2005). Multiple factors, including gender, ethnicity, cultural beliefs and behaviours, and temperament have been shown to influence pain sensitivity (Edwards *et al.* 2001, Rahim-Williams *et al.* 2007), potentially through interactions with genotype (Kim *et al.* 2004, Lacroix-Fralish *et al.* 2009). Studies incorporating monozygotic and dizygotic twins have

generally demonstrated considerable genetic contribution towards experimental pain sensitivity (Norbury *et al.* 2007, Nielsen *et al.* 2008), yet others have attributed experimental pain sensitivity to familial influences on behavioural patterns, rather than a predominantly genetic contribution (MacGregor *et al.* 1997). Estimates of heritability associated with nociceptive sensitivity and analgesic efficacy in mice has varied widely between 28% and 76% (Mogil 1999).

3.1.1 Tetrahydrobiopterin

3.1.1.1 Tetrahydrobiopterin synthesis

The *de novo* biosynthesis of BH_4 requires three enzymatic steps, initiated by GTPCH (Figure 3.1). Active GTPCH is a homodecamieric tunnel-fold protein with a quaternary structure formed through the face-to-face dimerisation of two pentamers (Nar *et al.* 1995). The GTPCH-mediated biosynthesis of 7,8-dihydroneopterin triphosphate (DHNTP) from guanosine triphosphate (GTP) represents the committing and rate-limiting step of BH_4 synthesis. The fate of DHNTP is bidirectional and is influenced by the cell type and the relative expression of 6-pyruvoyl-tetrahydropterin synthase (PTPS), a homohexamer consisting of six actives sites (Nar *et al.* 1994). Oxidation and dephosphorylation of DHNTP leads to the production of neopterin, which functions as a marker of GTPCH activity. This occurs at the expense of BH_4 synthesis, which requires the PTPS-mediated conversion of DHNTP to 6-pyruvoyl-tetrahydropterin. Sepiapterin reductase (SPR), a homodimer (Auerbach *et al.* 1997), subsequently undertakes the final enzymatic process in the *de novo* pathway, synthesising BH_4 from 6-pyruvoyl-tetrahydropterin. However, there is also a secondary route, the 'salvage pathway', which enables BH_4 synthesis in the event of SPR deficiency. This involves both aldose reductase (AR) and carbonyl reductase (CR) resulting in the synthesis of 7,8-dihydrobiopterin (BH_2) synthesis, which is then converted to BH_4 by NADPH-dependent dihydrofolate reductase (DHFR) (Latremoliere *et al.* 2011).

Feedback regulation of GTPCH activity and related BH_4 synthesis was initially attributed to BH_4 (Bellahsene *et al.* 1984), based on the ability of BH_4 to reduce urinary neopterin in patients with atypical phenylketonuria (PKU) (Niederwieser *et al.* 1982). However, it was later determined that a protein, termed p35 (GTP cyclohydrolase 1 feedback regulatory protein; GFRP), was required alongside BH_4 to facilitate complex formation with GTPCH (Harada *et al.* 1993). In the presence of BH_4, one GFRP pentamer complexes with a single GTPCH pentamer resulting in the formation of a complex which inhibits GTPCH activity (Harada *et al.* 1993, Yoneyama *et al.* 1997). Moreover, it was also found that inhibition of GTPCH by complex formation with GFRP and BH_4

was inhibited by phenylalanine, thereby promoting BH_4 synthesis and subsequent conversion of phenylalanine to tyrosine in situations of elevated phenylalanine (Harada *et al.* 1993).

Regeneration or recycling of BH_4 is a critical process allowing maintenance of basal cofactor levels. After oxidation of BH_4, which occurs during its use as a cofactor, pterin 4-α-carbinolamine is formed. This is subsequently converted to quinonoid dihydrobiopterin (qBH_2) by pterin 4-α-carbinolamine dehydratase (PCBD1). Quinonoid dihydrobiopterin is then converted to BH_2 by dihydropteridine reductase (DHPR), or undergoes spontaneous non-enzymatic oxidation to biopterin. The final step of BH_4 regeneration is mediated by DHFR (Nichol *et al.* 1985).

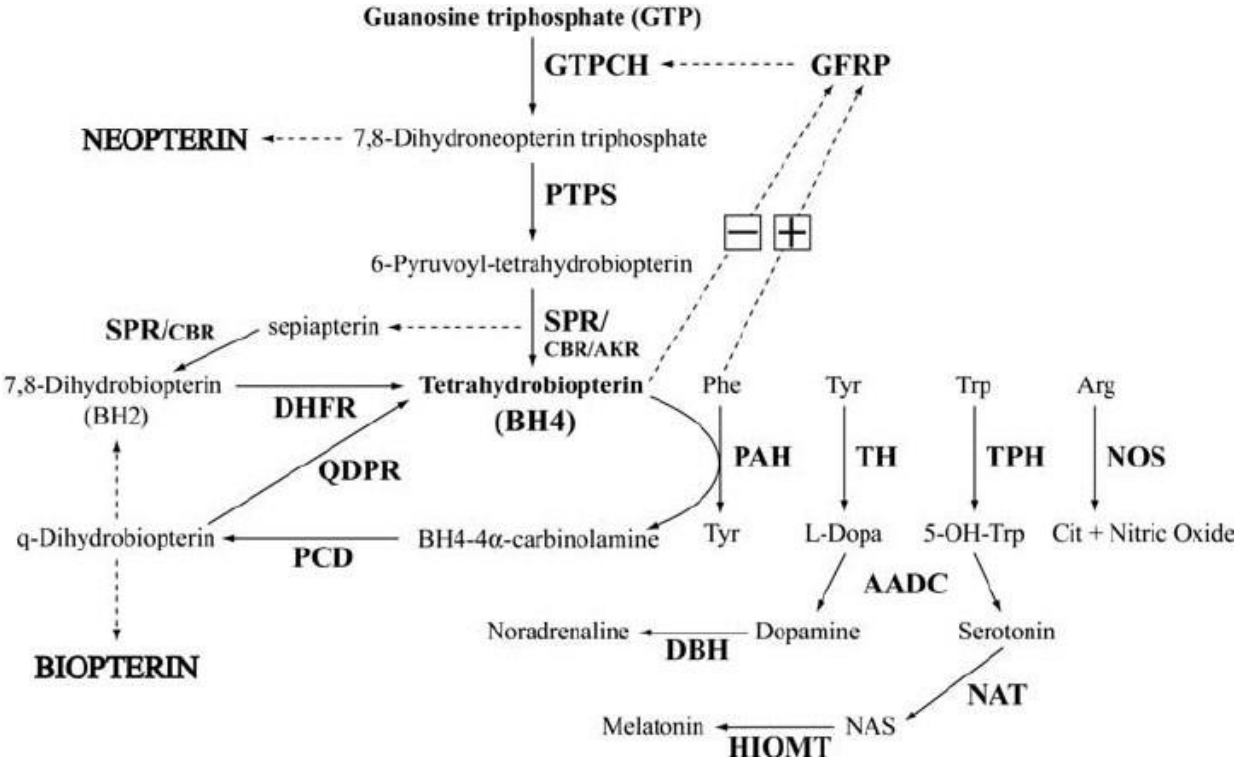

Figure 3.1: Mechanisms involved in tetrahydrobiopterin synthesis

The *de novo* synthesis of BH_4 involves sequential enzymatic reactions involving GTP cyclohydrolase I (GTPCH), 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase (SPR). Instead of conversion via the final step mediated by SPR, 6-pyruvyl-tetrahydrobiopterin can be converted to BH_4 by aldose reductase (AR/AKR) or carbonyl reductase (CB/CBR), followed by dihydrofolate reductase (DHFR). Regeneration of BH_4 also occurs via pterin-4acarbinolamine dehydratase (PCBD1/PCD) and dihydropteridine reductase (QDPR). GTPCH activity is regulated by GTP cyclohydrolase feedback regulator (GFRP) in conjuction with the effector molecules, BH_4 and phenylalanine. Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; Arg, arginine; Cit, citrulline; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; NOS, nitric oxide synthase; 5-OH-Trp, 5-hydroxytryptophan; AADC, aromatic amino-acid decarboxylase; DBH, dopamine b-hydroxylase; NAT, N-acetyltransferase; NAS, N-acetylserotonin; HIOMT, hydroxyindole-O-methyltransferase. Permissions obtained (McHugh *et al.* 2011).

3.1.1.2 Role of tetrahydrobiopterin

Tetrahydrobioptin, a reduced and biologically active form of biopterin, was initially identified as the necessary cofactor for the production of nitric oxide (Kwon *et al.* 1989, Tayeh *et al.* 1989). It is also an essential cofactor for several other enzymes, including phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase and is therefore integral to the synthesis of the monoamine neurotransmitters dopamine, serotonin and noradrenaline, in addition to phenylalanine hydroxylation (Figure 3.1).

In addition to its central role as a cofactor, BH_4 has been associated with multiple and variable cellular roles. It has been suggested that BH_4 mediates the release of neurotransmitters including dopamine, serotonin and glutamate, independent of its cofactor activity, thereby regulating neuronal activity (Mataga *et al.* 1991, Wolf *et al.* 1991, Koshimura *et al.* 1992).

3.1.1.3 Inherited deficiencies of tetrahydrobiopterin

Phenylketonuria (PKU) is an autosomal recessive disorder, most frequently associated with missense mutations in the phenylalanine hydroxylase gene, which catalyses the hydroxylation of phenylalanine to tyrosine (Williams *et al.* 2008). PKU is therefore a consequence of reduced enzymatic activity leading to an accumulation of phenylalanine. Phenotypically, PKU is of variable severity, but typically presents as stunted growth, developmental retardation and neurological signs such as seizures. Patients tend also to be of fair skin, due to restricted melanin synthesis (Farishian *et al.* 1980). However, early diagnosis and dietary modification can provide symptomatic amelioration.

Given that BH_4 functions as a necessary cofactor for phenylalanine hydroxylase, deficiencies in the function of the *de novo* synthesis or regeneration of BH_4 have variable phenotypic presentations. Mutations in the genes responsible for the *de novo* biosynthesis and regeneration of BH_4 are autosomal recessive and have been extensively described (Thony *et al.* 2006). Of 104 *GCH1* mutations previously described, only 5 have been linked with autosomal recessive hyperphenylalaninemia, alongside deficiencies in monoamine neurotransmitters, whereas the majority of the remaining mutations are autosomal dominant and result in dopa-responsive dystonia (DRD) with comparatively reduced penetrance (Thony *et al.* 2006). DRD typically emerges after one year of age and gradually worsens, with a dystonia and parkinsonism like

symptoms, featuring diurnal variation, increasing in severity towards the evening and alleviating after sleep (Blau *et al.* 2001).

3.1.1.4 Role of tetrahydrobiopterin in neuropathic pain

The degree of interest pertaining to BH_4 synthesis in neuropathic pain was greatly accelerated by the determination that two genes within the *de novo* BH_4 synthesis pathway, *Gch1* and *Spr*, were differentially regulated in the DH after sciatic nerve injury (Costigan *et al.* 2002). Further investigation highlighted significant upregulation of *Gch1* in the L4-5 dorsal root ganglion (DRG) after spared nerve injury (SNI), alongside more modest increases in *Spr* and *Qdpr* (Tegeder *et al.* 2006). Similar outcomes have been observed in multiple studies, including the determination that *Gch1* is upregulated in macrophages proximal to peripheral nerve injury, and in injured neurons, which remained consistent from 7 to 21 days after injury (Latremoliere *et al.* 2015b). This highlights the potential for immunological contributions to localised BH_4 production, which may subsequently facilitate the development of CNP. Indeed, mice with a *Gch1* knockout specific to sensory neurons showed similar signs of thermal hyperalgesia after CFA injection, suggesting that infiltrating immune cells may be a dominant contributor to BH_4 production (Latremoliere *et al.* 2015b). In addition to *Gch1* upregulation, elevations in neopterin and biopterin were also observed, indicating increased GTPCH activity (Tegeder *et al.* 2006). Moreover, *Gchfr* is also known to be differentially regulated after nerve injury, thus potentially altering the stoichiometric balance leading to BH_4 accumulation and increases in nitric oxide and monoamine neurotransmitter synthesis (Tegeder *et al.* 2006).

The mechanism underpinning the role of BH_4 in pain sensitivity is therefore often attributed to its role as a cofactor. After intrathecal administration of BH_4, increases in response to noxious radiant heat in naïve rats and increased sensitivity to painful stimuli have been observed in neuropathic (SNI) and inflammatory (complete Freund's adjuvant; CFA) animal pain models (Tegeder *et al.* 2006). After SNI, both tryptophan hydroxylase and neuronal nitric oxide synthase (*Nos1*) were upregulated, although phenylalanine hydroxylase and inducible nitric oxide synthase (*Nos2*) remained unchanged, and tyrosine hydroxylase was downregulated. Although serotonin in the DRG remained undetectable after injury, increased nitric oxide was observed. Elevations in nitric oxide were strongly attenuated by the prototypical GTPCH inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP), whilst the NOS inhibitor, Nω-Nitro-L-arginine methyl ester (L-NAME), also ameliorated mechanical and cold allodynia after SNI (Tegeder *et al.* 2006). Administration of DAHP failed to significantly decrease serotonin concentrations in the spinal

cord and brain stem, suggesting that changes to descending inhibitory pathways were not responsible for the efficacy of DAHP. It was therefore concluded that as BH_4-induced pain is of rapid onset and is underpinned by a mechanism that does not encompass transcriptional changes, neuronal cell death or microglia activation (Tegeder *et al.* 2006). Moreover, the efficacy of DAHP in the formalin test, a model of peripheral inflammation, and in multiple models of neuropathic pain, points to a common BH_4-dependent mechanism in a diverse range of pain aetiologies (Tegeder *et al.* 2006). There are multiple suggested mechanisms for increased pain perception resulting from heightened nitric oxide synthesis (Tegeder *et al.* 2006), including protein nitrosylation (Hara *et al.* 2005), interaction with NMDA receptor activity (Lipton *et al.* 1993) and increasing glutaminergic neurotransmission (Lewin *et al.* 1999, Tegeder *et al.* 2004).

Aside from the reputed role of BH_4 in nitric oxide synthesis, it has also been demonstrated that BH_4 induces calcium influx, which is influenced in-part by nitric oxide synthesis (Tegeder *et al.* 2006). The BH_4-induced calcium influx, in conjunction with TRPV1/TRPA1 results in PI3K pathway activation which is associated with hypersensitivity in patients with neuropathic pain (Zhuang *et al.* 2004, Latremoliere *et al.* 2011).

3.1.1.5 Inhibition of tetrahydrobiopterin synthesis

Pharmacological inhibition of BH_4 synthesis has been shown to alleviate pain in various animal models, including those pertaining to pain of neuropathic and inflammatory origin (Latremoliere *et al.* 2015b). Thus far research has focussed on inhibition of BH_4 synthesis by targeting of the *de novo* synthesis pathway. Whilst knockdown of GTPCH and DHFR both resulted reductions in BH_4, only DHFR knockdown resulted in notable increases in BH_2, thereby diminishing the BH_4/BH_2 ratio, which has been discussed (section 3.4.3). The BH_4/BH_2 ratio is a key determinant of nitric oxide production and reductions in the ratio facilitate uncoupling of NOS and subsequent superoxide production, promoting endothelial cell dysfunction (Crabtree *et al.* 2009a).

Administration of DAHP has been shown to successfully reverse mechanical and cold hypersensitivity after SNI (Tegeder *et al.* 2006). It has been demonstrated that DAHP inhibition occurs indirectly, requires GFRP, and is reversed by L-phenylalanine. It is therefore considered that the degree of DAHP-mediated inhibition of GTPCH will be greatest in cells with comparatively high expression of GFRP and relatively low levels of L-phenylalanine (Kolinsky *et al.* 2004). Treatment with DAHP also ablated injury-induced elevations in neopterin, whilst attenuating increases in biopterin levels which were maintained, to a degree, through continued

BH_4 regeneration. There was also no distinguishable change in mechanical or heat pain sensitivity in uninjured animals, with no apparent negative effects of DAHP administration. Administration of DAHP was also efficacious in reducing heat hyperalgesia after intraplantar injection of CFA, suggesting that BH_4 may be implicated in pathways that converge between pain of predominantly neuropathic or inflammatory origin (Tegeder *et al.* 2006).

Of the *de novo* BH_4 synthesis pathway enzymes, scrutiny of active sites led to the conclusion that both GTPCH and SPR exhibit promising druggability (Naylor *et al.* 2010). However, the risk of pronounced side effects and the relative inaccessibility of GTPCH active sites, due to their inward facing position, has made the prospect of analgesia by GTPCH inhibition less enticing (Latremoliere *et al.* 2015b). In contrast, inhibition of SPR will continue to permit limited BH_4 synthesis by aldose reductase and carbonyl reductase, although this pathway will of limited function in the CNS due to a relative deficiency of DHFR (Blau *et al.* 2001, Costigan *et al.* 2012). Similarly, administration of N-acetylserotonin (NAS), an SPR inhibitor and metabolite formed in the melatonin synthesis pathway (Katoh *et al.* 1982, Haruki *et al.* 2015), reduced mechanical and cold allodynia after SNI and thermal hyperalgesia after intraplantar CFA injection (Tegeder *et al.* 2006). However, the systemic use of NAS is restricted due to conversion to melatonin by acetylserotonin methyltransferases (Latremoliere *et al.* 2015b). A more potent SPR inhibitor, SPRi3, has been shown to reduce allodynia in models of neuropathic pain and attenuate thermal hyperalgesia after CFA injection, without implicating nociceptive pain (Latremoliere *et al.* 2015b).

Repurposing of drugs with current regulatory approval for alternative indications poses a multitude of benefits over the conventional drug discovery process (Oprea *et al.* 2012). The opportunity for the targeting of BH_4 synthesis with such compounds is prominent. It has been determined that sulfasalazine and its metabolites, sulfapyridine and mesalamine, inhibit SPR, which has been considered as a potential mechanism contributing to the clinical efficacy of these compounds (Chidley *et al.* 2011). It is also thought that SPR inhibition by these compounds, and other similar sulfa-compounds, may be responsible for several documented side effects caused by these drugs (Yang *et al.* 2015). It has been noted that the CNS side effects of sulfamethoxazole, a potent SPR inhibitor with notable CNS penetration, overlap with those of SPR deficiency, which may be exacerbated by a relatively low expression enzymes involved in BH_4 regeneration, such as DHFR, in the CNS (Blau *et al.* 2001, Chidley *et al.* 2011). Further scrutiny has also elucidated a range of other sulfa-based drugs, including the antidiabetic sulfonylureas, as inhibitors of SPR (Haruki *et al.* 2013) Interestingly, sulfamethoxazole is used in combination with trimethoprim, an inhibitor

of DHFR, in the treatment of bacterial infection. Considerable CNS side effects are observed at higher doses, which may result from dual inhibition of BH_4 synthesis (Haruki *et al.* 2013). Latterly, methotrexate, another inhibitor of DHFR, has been scrutinised in various animal models of neuropathic pain, although DHFR inhibition was overlooked as a rationale for the observed reduction in neuropathic pain behaviour (Scholz *et al.* 2008).

3.1.2 The pain protective haplotype

The *GCH1* gene (Figure 3.17) consists of a single haploblock spanning 72 kb (Tegeder *et al.* 2006)with four transcript variants, of which variant 1 and 2 encode for the functional GTPCH protein (isoform 1). Transcript variants 3 (encoding isoform 2) and 4 (encoding isoform 3) result in non-functional proteins. It was suggested that, given the potential role of GTPCH in pain sensitivity in animal models, polymorphisms within, or flanking *GCH1*, may confer a distinguishable pain phenotype. *In vitro* analysis of cultured white blood cells (WBCs) from patients with varying copies of the pain protective haplotype demonstrated that *GCH1* expression did not differ between carriers and non-carriers of the pain protective haplotype. However, after forskolin treatment, *GCH1* expression was significantly elevated in cultures from non-carriers, which became incrementally reduced with increasing copies of the pain protective haplotype (Tegeder *et al.* 2006). As such, surgical discectomy patients with persistent lumbar root pain due to intervertebral disc herniation were originally genotyped for 15 single nucleotide polymorphisms spanning *GCH1*. It was determined that 5 SNPs were significantly associated with leg pain severity scores one year after surgery and 2 SNPs (rs8007267 and rs3783641) were associated with low pain scores. A specific haplotype, with an allelic frequency of 15.4%, was strongly predictive of low leg pain scores (Tegeder *et al.* 2006). It has been suggested that the pain protective haplotype results in changes to the transcriptional regulation of *GCH1*, and this regulatory modification was likely to be either in the *GCH1* 5' flanking region or within the large first intronic region, when taking into consideration the loci of the two SNPs (rs8007267; rs3783641) exclusively present in the pain protective haplotype (Tegeder *et al.* 2006). Further scrutiny of the pain protective haplotype illustrated that, after accounting for linkage disequilibrium, three (rs8007267 G>A, rs3783641 A>T and rs10483639 C>G) or fewer SNPs (Figure 3.17) were sufficient to identify the pain protective haplotype. Indeed, the use of any one of these SNPs alone conferred >95% sensitivity and specificity for identification of the pain protective haplotype (Lötsch *et al.* 2007).

A vast array of studies (Table 3.1) have been undertaken to find associations between the pain protective haplotype and pain susceptibility associated with disease (e.g. cancer), acute painful

events (e.g. labour) and sensitivity in experimental pain tests. Notable variation exists between studies, including the target population, type of pain (which may be underpinned by variable pathophysiological mechanisms) and inherent differences in the methodology used for population/patient screening and pain assessment. Such contrast between studies complicates the feasibility of meta-analysis, leaving the evidence for the role of the pain protective haplotype in pain sensitivity/susceptibility somewhat suggestive, rather than established.

Considering the *GCH1* pain protective haplotype appears to reduce the susceptibility and sensitivity to pain in animal models, and carriers of the haplotype do not have a clearly distinguishable phenotype, it is proposed that pharmacologically replicating the effect of the pain protective haplotype by attenuating increases in *de novo* BH_4 synthesis may ameliorate neuropathic pain with few adverse effects, whilst leaving acute nociceptive pain sensitivity unaltered (Tegeder *et al.* 2006, Latremoliere *et al.* 2015b).

Table 3.1: Comprehensive summary of research seeking to determine associations between various pain aetiologies and *GCH1* genotype

Origin of Pain	Cohort Size	Ethnicity/ Population	*GCH1* SNPs	Pain Measures	Outcomes	Reference
Provoked vestibulodynia	98 cases, 102 controls	Swedish females	rs8007267 rs3783641 rs10483639	Coital pain (measured by visual analogue scale) and pressure pain thresholds.	No association between the pain protective haplotype and sensitivity to pressure-induced pain or coital pain. Significance was observed between a subset of patients taking hormonal contraceptives and *GCH1* genotype.	(Heddini *et al.* 2012)
Labour pain	676 cases	Caucasian	rs8007267 rs3783641 rs10483639	Labour related parameters (e.g. analgesia, duration, cervical dilation).	Homozygous carriers of the pain protective haplotype were more likely to require second-line analgesia.	(Dabo *et al.* 2010)
Experimental pain models	39 subjects	Any ethnicity	rs752688 rs4411417 rs8007201 rs3783641 rs8007267	Capsaicin-induced pain (measured by visual analogue scale).	Three SNPs (rs3783641, rs4411417 and rs752688) were significantly associated with lower pain ratings.	(Campbell *et al.* 2009)
Pancreatitis	131 (recurrent acute) and 265 (chronic) cases, 236 controls	Caucasian	rs8007267 rs3783641	Questionnaire for assessment of pancreatitis, including the duration and severity of pain.	No association between the pain protective haplotype and recurrent acute pancreatitis, chronic pancreatitis or pain severity.	(Lazarev *et al.* 2008)

Chronic widespread pain	197 cases, 197 controls	Primarily Caucasian population	rs8007267 rs3783641 rs10483639	Pain questionnaire and body manikins (using American College of Rheumatology Criteria).	No association between the pain protective haplotype and pain sensitivity or susceptibility to chronic widespread pain.	(Holliday *et al.* 2009)
Experimental pain models	10 (homozygous pain protective haplotype), 22 non-carriers	Caucasian	15 SNPs	Cutaneous inflammation, capsaicin-induced thermal pain, mechanical pain, pressure pain and tolerance to electrically induced pain.	Carriers of the pain protective haplotype were less sensitive to mechanical pain and to capsaicin-induced pain following sensitisation. No association was found for heat, pressure and electrically induced pain without sensitisation.	(Tegeder *et al.* 2008)
Cancer pain	251 cases	Caucasian	rs8007267 rs3783641 rs10483639	Analgesia related parameters, including steady state morphine concentration, pain severity assessment (Brief Pain Inventory questionnaire) and time since diagnosis.	The interval between cancer diagnosis and opioid therapy initiation was sequentially longer in homozygous carriers of the pain protective haplotype than in heterozygous and non-carriers.	(Lotsch *et al.* 2010)
Advanced cancer pain patients with inadequate analgesia	240 cases	Not stated (study based in The Netherlands)	rs8007267 rs3783641 rs10483639	Pain intensity and opioid requirement were assessed after interventions by a palliative care team	No associations were observed with *GCH1* genotype.	(Matic *et al.* 2017)

Pain therapy requirement	424 cases	Caucasian	rs8007267 rs3783641 rs10483639	Analgesia medication (including opioid doses), pain treatment duration and 24 hour pain intensity score (0-10 rating scale).	Patients with the pain protective haplotype required comparatively shorter durations of specialised pain therapy, and tended to require lower opioid doses and had lower 24 hour pain scores.	(Doehring *et al.* 2009)
HIV-associated sensory neuropathy	159 cases	Black African	rs10483639 rs752688 rs4411471 rs8007201 rs3783641 rs8007267	Peripheral neuropathy screening tool and sensory testing.	Patients with the pain protective haplotype or a 6-SNP *GCH1* haplotype had a reduced pain risk. Associations did not persist after correction for age, gender and CD4 T-cell count.	(Wadley *et al.* 2012)
Experimental pain models/molar extraction	735 subjects (221 molar extractions)	Varied	38 SNPs	Thermal and cold stimuli, extraction of impacted third molar. Pain ratings obtained with visual analogue scale.	No associations were found between *GCH1* genotype and measures of pain sensitivity.	(Kim *et al.* 2007)
Surgical discectomy for chronic lumbar root pain and experimental pain models	147 cases 547 subjects (both cohorts assessed individually)	Caucasian (cases), not stated (subjects)	15 SNPs	Severity of pain after discectomy. Healthy volunteers were subject to heat, mechanical and ischaemic pain.	The pain protective haplotype was associated with significantly lower pain scores post-discectomy. Healthy controls homozygous for the pain protective haplotype exhibited greater tolerance to experimentally induced pain.	(Tegeder *et al.* 2006)

Fibromyalgia syndrome	409 cases 422 controls	Korean population	rs3783641 rs841 rs752688 rs4411417	Eighteen tender points assessed by survey.	The investigated polymorphisms did not influence the clinical features of fibromyalgia or prescribing of medication. A specific *GCH1* haplotype was associated with reduced pain sensitivity.	(Kim *et al.* 2013)
Persistent pain after breast surgery	51 cases (20 with persistent pain)	Caucasian	rs8007267 rs3783641 rs10483639	McGill Pain Questionnaire, pain severity (measured by visual analogue scale), analgesia requirement and hyperalgesia near surgery site.	No associations were found between *GCH1* genotype and persistent pain after breast surgery.	(Lee *et al.* 2103)
Lumbar degenerative disc disease	69 cases	White only	15 SNPs	Back pain severity (measured by numerical rating scale).	An association was observed between the minor allele of a *GCH1* polymorphism (rs998259) and reduced disability and pain scores.	(Kim *et al.* 2010)
Pain crises (sickle-cell anaemia)	228 cases (discovery), 513 cases (replication)	African	DNA sequencing	Emergency department presentation or hospitalisation due to acute sickle cell pain.	Two *GCH1* polymorphisms (rs8007267 and rs7147286) were associated with the case cohort. Both a *GCH1* 6-SNP haplotype and the rs8007267 major allele were associated with severe painful events.	(Belfer *et al.* 2014)

Persistent pain following lumbar discectomy	53 cases	Not stated	rs8007267 rs3783641 rs10483639	McGill Pain Questionnaire, pain severity (measured by visual analogue scale), Roland-Morris Questionnaire and thresholds to electrical stimulation.	No association was observed between the studied SNPs or the pain protective haplotype and limited pain persistence.	(Hegarty *et al.* 2012)
Post-arthroscropic shoulder surgery pain	150 cases	Any ethnicity	rs3783641	Brief Pain Inventory questionnaire.	An interaction between patients homozygous for the rs3783641 minor allele and anxiety was observed, predicting pain ratings at 12 months post-surgery.	(George *et al.* 2016)
Labour pain	97 cases	Swedish population	rs8007267 rs3783641 rs10483639	Labour pain rating (visual analogue scale) and analgesia requirement.	An association was observed between the pain protective haplotype and an increased requirement for second-line analgesia.	(Pettersson *et al.* 2016)
Postoperative pain (molar extraction)	100 cases	White (Irish ancestry)	rs8007267 rs3783641 rs10483639	McGill Pain Questionnaire, pain severity (measured by visual analogue scale) and analgesia requirement.	The presence of the major allele for each SNP correlated to a shorter duration of analgesia use after surgery. Patients lacking the pain protective haplotype also exhibited a reduced period of analgesia use after surgery.	(Lee *et al.* 2011)

Opioid use in cancer pain	2201 cases	Caucasian	rs3783641 rs4411417 rs752688	Opioid requirement, pain intensity (Brief Pain Inventory) and pain mechanism (Edmonton Staging System).	No associations were found between the studied *GCH1* polymorphisms and opioid requirement in cancer pain.	(Klepstad *et al.* 2011)
Mastectomy related persistent pain	42 cases (10 controls for QST analysis)	Not stated (based in Ireland)	rs8007267 rs3783641 rs10483639	McGill Pain Questionnaire, pain severity (measured by visual analogue scale) and thresholds to electrical stimulation.	No associations were found between the pain protective haplotype and pain measures.	(Hickey *et al.* 2011)
HIV-associated sensory neuropathy	158 cases	Black African	31 SNPs	Pain severity (measured by numerical pain rating scale).	No associations were found between *GCH1* SNPs or haplotypes and pain intensity.	(Hendry *et al.* 2013)
Exercise-induced shoulder pain	190 cases	Any ethnicity	rs3783641	Questionnaires pertaining to the fear of pain and pain catastrophizing. Exercise-induced muscle injury and Brief Pain Inventory questionnaire.	No associations found between rs3783641 genotype and exercise-induced shoulder pain.	(George *et al.* 2014)
Temporomandibular disorder and experimental pain model	200 cases, 198 controls	White only	10 SNPs	Tender points assessed by examination, thermal pain threshold, central sensitisation (measured by numerical rating scale) and pressure pain.	An interaction was observed between the *COMT met* allele and the *GCH1* rs10483639 minor allele (homozygotes only). Increase mechanical pain thresholds were observed with this genotype.	(Smith *et al.* 2014)

3.1.3 Neopterin

3.1.3.1 Role of neopterin

Neopterin, pyrazino-pyrimidine derivative, was first isolated from urine in 1967 (Sakurai *et al.* 1967) and was identified as the fluorescent constituent of urine previously detected in mice with Ehrlich ascites tumour, and in humans with malignant disease (Hamerlinck 1999). Neopterin is synthesised from GTP by GTPCH which cleaves the purin leading to the production of DHNTP, followed by dephosphorylation to 7,8-dihydroneopterin and subsequent oxidation to neopterin (Murr *et al.* 2002). The biological roles of neopterin have been summarised in significant detail (Hamerlinck 1999). Neopterin production is increased following upregulation of GTPCH, a consequence of stimulation though multiple pathways, commonly via IFN- γ. Neoptein is therefore considered an indicator of endogenous IFN-γ release (Huber *et al.* 1983, Huber *et al.* 1984, Widner *et al.* 2000, Feldman 2004) and consequently of T-cell activation. Both neopterin and 7,8-dihydroneopterin have been shown to activate redox sensitive transcription factors, AP-1 and NF-κB, resulting in changes gene expression, including *NOS2* upregulation (Hoffmann *et al.* 1996, Baier-Bitterlich *et al.* 1997).

3.1.3.2 Neopterin as a clinical utility

Research seeking to determine the value of neopterin, as an indicator of immune activation in various infections and diseases, flourished in the late 20th century. As a marker of IFN-γ activity, neopterin possesses significant advantages in that the measurement of circulating IFN-γ, which can be complicated by complexation with soluble and cell surface receptors, leading to variations in measurable IFN-γ and subsequent misleading data. In renal allograft patients, both serum neopterin and IFN-γ correlated with rejection episodes, though the suitability of neopterin for this purpose remained when IFN-γ levels fell below the limit of detection (Woloszczuk *et al.* 1986). This illustrates the potential advantages for circulating neopterin levels to reflect pathophysiological and immune related changes in isolated tissues.

Subsequent research has led to a wealth of studies considering a diverse range of conditions. These include changes in neopterin levels due to chronic infections such as tuberculosis (Fuchs *et al.* 1984), HIV (Fuchs *et al.* 1987), as a marker of disease progression and as both a predictor and indictor of treatment efficacy in hepatitis C (Feldman 2004). Similarly, changes in neopterin were also detected in non-infectious conditions. Neopterin has been investigated as a marker of coronary disease and its severity (Lyu *et al.* 2015), a differentiator of acute coronary syndrome and chronic

stable angina pectoris (Kaski *et al.* 2005) and as a predictive marker of major coronary events in chronic stable angina patients (Avanzas *et al.* 2005). More recently, circulating and urinary neopterin levels have been shown to be elevated in relation to CNS disorders and disease, including major depressive disorder (Taymur *et al.* 2015), Parkinson's disease (Widner *et al.* 2002) and multiple sclerosis (Bagnato *et al.* 2003), which highlights the potential for neopterin levels to reflect CNS disease.

In addition, neopterin levels are also known to reflect pharmacological modulation of the immune system, which both allows for the monitoring of treatment efficacy, but also complicates the use of neopterin as a biomarker of disease. Unsurprisingly, patients receiving cytokine therapy may exhibit elevated neopterin levels (Datta *et al.* 1987, Durastanti *et al.* 2011), whilst the opposite effect is observed in patients receiving an immunosuppressant, such as ciclosporin (Wehrmann *et al.* 1987, Hamerlinck 1999, Feldman 2004). It is perhaps more pertinent to consider that neopterin may vary with more common conditions, such as psoriasis (Sanchez-Regana *et al.* 2000), and may even increase after strenuous exercise (Sprenger *et al.* 1992). It is also noteworthy that peaks in neopterin levels are generally observed in subjects under 18 and above 75 years of age (Maloney *et al.* 1997, Hamerlinck 1999), with increased body mass index (Spencer *et al.* 2010), smoking status (Djordjevic *et al.* 2008) and exhibit a degree of diurnal variation (Garcia-Gonzalez *et al.* 2006).

3.1.4 Nitric oxide

3.1.4.1 Synthesis and key functions of nitric oxide

Nitric oxide is a key effector molecule in various physiological process and changes in nitric oxide regulation have been associated with several disease states. The production of nitric oxide is mediated by the nitric oxide synthases (NOSs), a family of enzymes which catalyse the conversion of L-arginine to citrulline, leading to the release of nitric oxide. Of the three NOSs, endothelial (eNOS) and neuronal (nNOS) are generally considered to be constitutively expressed and their activity is dependent on intracellular Ca^{2+}-calmodulin, whereas inducible NOS (iNOS) functions independent of Ca^{2+}-calmodulin, is induced by inflammatory processes, and can be upregulated in immune cells, such as macrophages and glial cells (Bredt *et al.* 1994, Petho *et al.* 2012). Nitric oxide facilitates a range of physiological effects, including vasodilation (Gruetter *et al.* 1979), plasma extravasation and cytotoxicity, in addition to implications on neurotransmission (Garthwaite *et al.* 1995).

3.1.4.2 Role of nitric oxide in disease

The role of nitric oxide in the CNS is diverse and has been shown to implicate a range of physiological processes. Nitric oxide is potentially neurotoxic and neuroprotective in stroke (Garry *et al.* 2015) and both proconvulsive and anticonvulsive in epilepsy (Banach *et al.* 2011). Inducible NOS and the subsequent increase in nitric oxide production has been shown to implicate wound healing and regeneration of tissue (Yamasaki *et al.* 1998) and after experimentally induced traumatic brain injury in mice, the administration of iNOS inhibitors resulted in reduced cognitive function when compared non-treated mice (Sinz *et al.* 1999). Both plasma neopterin and the citrulline-arginine ratio, an indicator of nitric oxide synthesis, were decreased in bipolar affective patients, indicating reduced BH_4 activity (Hoekstra *et al.* 2006), although somewhat contradictory observations have been made (Yanik *et al.* 2004). Moreover, serum nitrite/nitrate was able to distinguish active and inactive inflammatory bowel disease with reasonable sensitivity and specificity (Avdagic *et al.* 2013), whilst significantly higher plasma nitrite/nitrate levels were detected in untreated coeliac disease (Murray *et al.* 2003).

3.1.5 Aims and objectives

The overall aims and objectives of chapter 3 are as follows:

- To determine whether molecules pertaining to the tetrahydrobiopterin synthesis pathway may be differentially abundant in the plasma of patients with CNP, when compared to health controls
- To determine whether the *GCH1* pain protective haplotype may influence circulating nitric oxide and pterin levels in patients with CNP, when compared to healthy controls
- Use reporter gene assays to predict whether specific polymorphisms within the pain protective haplotype may influence *GCH1* expression
- Formulate potential hypotheses relating to the role of the pain protective haplotype by considering reporter gene assay data, transcription factor binding prediction tools and ESMAs

3.2 Methods

Specific methods relating to this chapter are detailed within chapter 2.

3.3 Results

3.3.1 Nitric oxide analysis

3.3.1.1 Nitric oxide method development

Nitiric oxide is a highly unstable free radical with poor aqueous solubility. The physiological half-life is typically less than a second, with nanomolar plasma concentrations, which means accurate routine quantification lacks feasibility. Autoxidation of nitric oxide in aqueous media occurs at a rate which is inversely proportional to its concentration (Ford *et al.* 1993), leading to the formation of nitrite (NO^{2-}), which itself exhibits a relatively short half-life of 110 seconds in blood and a typical plasma concentration of 100-500 nmol/L. Further oxidation leads to the formation of nitrate (NO^{3-}) which is comparatively stable in circulating blood with a half-life of up to 5-8 hours and a plasma concentration of 30-60 µmol/L (Kelm 1999).

The Griess test was first developed in the 19th Century by Peter Griess. It uses a diazotization reaction which allows for the colourmetric determination of nitrite in aqueous solution (Griess 1858, Griess 1879). Initial assessment of the Griess assay therefore sought to clarify the feasibility of nitrite quantification in plasma, without nitrate reduction to nitrite. Using the Griess Reagent System (Promega, USA), plasma was thawed on ice, followed by the addition of 50 µL of 1% sulphanilamide (in 5% phosphoric acid) to 50 µL of plasma in a 96-well assay plate. The plate was incubated for 10 minutes at room temperature in the dark. Fifty microliters of 0.1% N-1-napthylethylenediamine was added followed by spectrophotometric analysis at 570 nm using a Tecan Infinite F50 microplate reader (Tecan, UK). There was no discernible colour change or increase in baseline absorbance. A slight elevation of baseline absorbance was observed with non-deproteinised samples. Herein plasma was invariably deproteinised to reduce the effect of sample turbidity and the interference of precipitated proteins on the assay (Moshage *et al.* 1995, Guevara *et al.* 1998), although this process did not facilitate the Griess reaction using the aforementioned methodology. There are several methods used to deproteinise plasma, but acid precipitation must be avoided to prevent acid-induced conversion of nitrite to dinitrogen trioxide, which leads to the release of nitrogen dioxide (Miranda *et al.* 2001). Therefore, a total of 1 mL of plasma was centrifuged at 4^{o}C for 20 minutes at 3800 x *g* using an Amicon Ultra-4 (10 kDa) Centrifugal Filter Unit (Sigma, UK). The clear filtrate was analysed, using the protocol described for the Griess Reagent System (Promega, USA). There was no discernible colour change. It was therefore confirmed that the Griess assay method must be modified for the reduction of nitrate to nitrite.

Reduction of nitrate is typically undertaken chemically with reducing metals or with a nitrate reductase system. The use of chemical methods with biological samples usually centres on cadmium (Casey *et al.* 2000), though as with many chemical methods, there are significant drawbacks. Cadmium, aside from the associated chemical hazards, is capable of further reducing nitrite to nitric oxide leading to erroneous quantification and is susceptible to experimental variation depending on multiple factors, such as pH and the cadmium surface area to sample volume ratio (Sun *et al.* 2003). Cadmium reduction has also been shown to correlate poorly with other methods at low nitrate concentrations (Marzinzig *et al.* 1997). Another chemical method involves the use of vanadium (III) chloride, which exhibits some advantages over cadmium. Vanadium (III) chloride is able to readily reduce nitrate to nitric oxide at high temperatures (Braman *et al.* 1989) but at lower temperatures, the reaction leads to the steady accumulation of nitrite (Miranda *et al.* 2001). In contrast to cadmium, vanadium (III) chloride is also less toxic and does not require removal prior to analysis. Using a previously described method for serum analysis with slight modifications (Kalugalage *et al.* 2013), plasma was thawed on ice and followed by the addition of 10 µL of zinc sulphate heptahydrate (1.5 g/mL) for deproteinisation. The sample was vortexed for 1 minute, and centrifuged at room temperature for 10 minutes at 10000 x *g*. The supernatant was separated from the precipitate, followed by further centrifugation using identical conditions. To 50 µL of the supernatant, 50 µL of a vanadium (III) chloride (8 mg/mL) solution in 1 M hydrochloric acid was added, alongside 50 µL of sulphanilamide and 50 µL of N-1-napthylethylenediamine (Promega Griess Reagent System). The reaction was allowed to proceed at room temperature for 30 minutes, but colour change was not discernible. The rationale for a lack of reactivity using this method is unclear, but this may be explained by the susceptibility of vanadium to oxidation (Miranda *et al.* 2001), by rapid reduction of nitrate to nitric oxide (Yang *et al.* 1997) or due to the documented low reaction efficiency of this method (García-Robledo *et al.* 2014).

Another method consisting of nitrate reductase-mediated reduction of nitrate to nitrite was considered an as alternative to the aforementioned methods. This has been shown to provide adequate sensitivity for the quantification of nitrite and nitrate in biological fluids (Grisham *et al.* 1996). One potential disadvantage of the nitrate reductase method is the necessity for NADPH which can interfere with the Griess reaction, although this can be circumvented by limiting the amount of NADPH alongside the use of a catalytic system for recycling of $NADP^+$ to NADPH (Verdon *et al.* 1995, Miranda *et al.* 2001). The use of zinc sulphate for deproteinisation has been

associated with a decline in assay reproducibility when used in combination with nitrate reductase and NADPH, so this was avoided (Guevara *et al.* 1998). Using the Nitrite/Nitrate Colourmetric Assay Kit (Cayman Chemical, UK) according to manufacturer's instructions after column filtration of plasma, a definitive colour change was observed, equating to a plasma total nitrate concentration of 48 µM. There was no difference in the absorbance readings with filtered and non-filtered ultrapure water, which demonstrated that contact with the filtration membrane does not contribute to sample nitrite and nitrate quantitation. The complete method for nitrite/nitrate quantification is described (section 2.2.7).

3.3.1.2 Nitric oxide

Analysis of plasma nitrite/nitrate as a surrogate marker of nitric oxide production in the discovery cohort showed a mean (±SD) in healthy controls (n = 8) of 17.10 (±13.45) µM (range: 1.190-40.09 µM). The mean (±SD) in patients with CNP (n = 7) was 34.31 (±26.62) µM (range: 12.28-88.32 µM). Statistical analysis (unpaired t-test) highlighted a trend towards significance (p = 0.129) (Figure 3.2). An insufficient quantity of plasma prevented deproteinisation and analysis of both healthy controls (n = 2) and patients with CNP (n = 3).

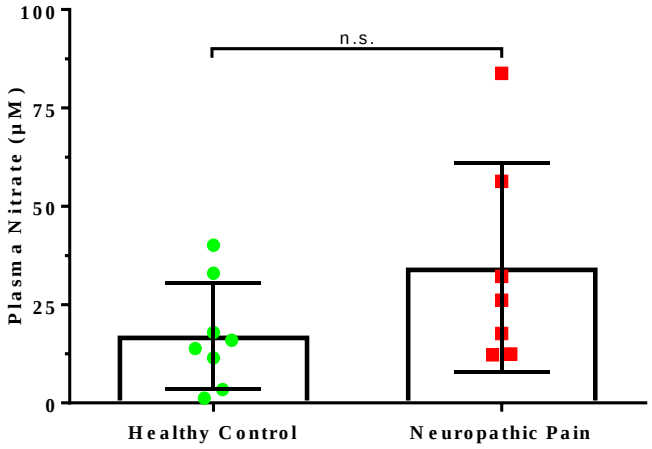

Figure 3.2: Analysis of plasma nitrate levels in healthy control and neuropathic pain patients in the discovery cohort

After isolation of plasma and removal of proteins using a 10 kDa cut-off filter, analysis of the nitrite/nitrate concentration within the filtrate was conducted by enzymatic reduction of nitrate to nitrite followed by the Griess reaction (section 2.2.7). Absorbance data was analysed by unpaired t-test (±SD). n.s: not statistically significant. Data is available in the electronic supplementary material within the folder entitled 'Nitric Oxide' and has a file name of 'Discovery'.

The discovery/validation cohort was sufficiently sized to perform additional analysis according the result of the S-LANSS test. The mean (±SD) nitrate level in healthy controls (n = 23) was 23.53 (±2.69) µM (range: 9.10-61.76 µM). In patients with chronic neuropathic pain (n = 23), the mean (±SD) level was 22.81 (±7.52) µM (range: 13.45-35.45 µM). For those with CNP, patients with an S-LANSS result of <12 (n = 8) had a mean (±SD) nitrate level of 22.23 (±8.70) µM (range: 13.45-35.45 µM) whilst those with an S-LANSS result of ≥12 (n = 15) had a mean (±SD) level of 23.53 (±12.90) µM (range: 14.28-37.24 µM). Statistical analysis (unpaired t-test) highlighted no significance between healthy control and CNP (p = 0.820) (Figure 3.3). There was also no difference when comparing healthy control and CNP patients with an S-LANSS result of <12 (p = 0.952) or between healthy control and those with an S-LANSS score of ≥12 (p = 0.862) (Figure 3.3). Similarly, no difference was observed between patients with an S-LANSS result of <12 and ≥12 (p = 0.789).

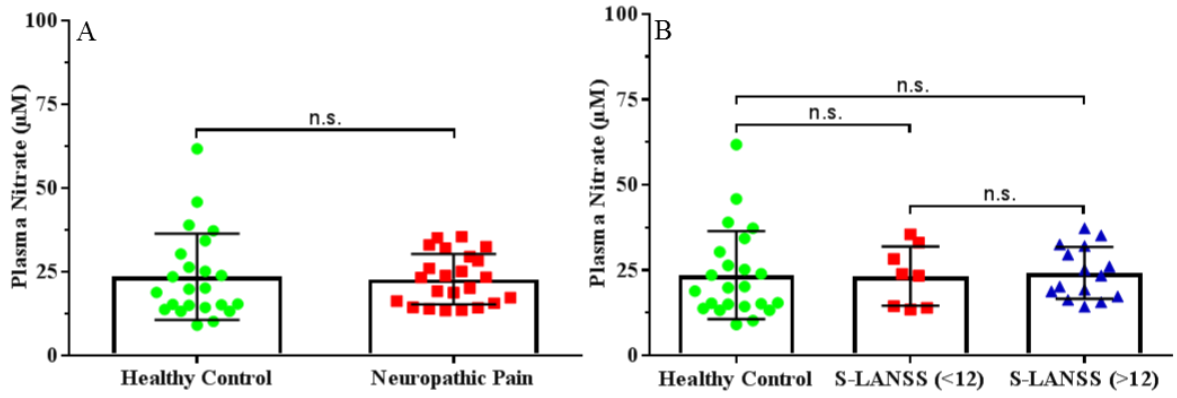

Figure 3.3: Analysis of plasma nitrate levels in healthy control and neuropathic pain patients in the discovery/validation cohort

After isolation of plasma and removal of proteins using a 10 kDa filter, analysis of the nitrite/nitrate concentration within the filtrate was conducted by enzymatic reduction of nitrate to nitrite followed by the Griess reaction (section 2.2.7). (A) Healthy controls and patients with CNP were analysed followed by (B) separation of the neuropathic pain cohort by S-LANSS score. Absorbance data was analysed by unpaired t-test (±SD). n.s: not statistically significant. Data is available in the electronic supplementary material within the folder entitled 'Nitric Oxide' and has a file name of 'Discovery validation'.

3.3.2 Neopterin analysis

3.3.2.1 Neopterin method development

In order to quantify plasma neopterin levels using high performance liquid chromatography (HPLC), a number of additional factors must be therefore considered before the processing of samples in preparation for analysis. Blood plasma is an abundant source of circulating metabolites and as a clinical utility, provides a wealth of information pertaining to underlying disease. However, plasma is also highly complex, consisting of a wide range of organic and inorganic molecules, with a 200 fold lower concentration of neopterin than urine, which complicates analysis (Hamerlinck 1999, Daykin *et al.* 2002). Proteins are a highly abundant constituent of plasma and of which, albumin comprises of approximately 55% of the total (Nicholson *et al.* 2000). In order to allow for analysis using HPLC, the primary consideration must be given to plasma deproteinisation. Early deproteinisation methods involved the use of ion exchange solid phase extraction allowing isolation and concentration of neopterin (Werner *et al.* 1987b), but such methodology is now considered unreliable (Flavall *et al.* 2008). More recently, deproteinisation of plasma for HPLC purposes has been achieved using a variety of chemicals and solvents, including ethanol (Lee *et al.* 1992), methanol (Contin *et al.* 2008), acetone (Kwadijk *et al.* 2002), acetonitrile (ACN) and trichloroacetic acid (TCA) (Flavall *et al.* 2008). The use of such solvents and acids facilitate the precipitation of plasma proteins by causing alterations in solubility. It is a noteworthy consideration that proteins will vary significantly in their solubility under a given set of conditions, and thus the analytes solubility and potential for ligand-protein co-precipitation should also be considered (Daykin *et al.* 2002).

Initial method development involved the replication of a previously documented method using ACN as the deproteinising agent (Flavall *et al.* 2008). Comparative studies seeking to determine optimal HPLC conditions using ACN and TCA deduced that the former improved the signal-to-noise ratio and produced larger neopterin peak areas (Flavall *et al.* 2008), although this has not been universally observed (Agilli *et al.* 2012). Therefore, plasma (or standard) was combined with acetonitrile at a 1:1 ratio, vortexed for 5 seconds and centrifuged for 10 minutes at 10300 x *g.* An isocratic mobile phase consisting of 5% methanol in 20 mM ammonium phosphate (pH 6.0), with a flow rate of 1 mL/min, was used. A single peak was initially observed with neopterin standards, and adequate separation was achieved with deproteinised plasma. However, after serial dilution of neopterin standard, a linear association between peak area and neopterin concentration was not observed. This was particularly apparent at physiologically relevant concentrations, of ~10 nM

(Feldman 2004), and was attributed to the presence of an overlapping peak which remained present when injecting a sample consisting of 1:1 mobile phase and acetonitrile. The presence of the peak persisted despite the use of alternative solvent and buffer sources, and the peak area declined disproportionately when the 1:1 dilution of acetonitrile was combined with an equal volume of mobile phase, given a total final concentration of 25% acetonitrile. This suggested interference by 'system peaks' may have occurred due to equilibrium disruption caused by the contrasting solvent composition of the sample and mobile phase.

Further analysis was carried out using a method previously described (Carru *et al.* 2004), which involved the deproteinisation of plasma with an equal volume of 5% TCA, followed by vortexing for 10 seconds, centrifugation at 3000 x *g* for 10 minutes and dilution of 50 µL of supernatant with 200 µL of ultrapure water. Due to inadequate chromatographic separation the proportion of ACN in the mobile phase was increased incrementally to 3% but as the proportion of ACN increased, negative peak formation became problematic and interfered with neopterin quantification.

Due to apparent interactions between the mobile phase solvent and the injected sample, a fully aqueous mobile phase was considered. Previously, urine (Werner *et al.* 1987a, Groetsch *et al.* 1991, Zis *et al.* 2017) and serum (Groetsch *et al.* 1991) neopterin analysis has been achieved using a mobile phase comprising of potassium phosphate and in the case of serum, acid precipitation with TCA was used. Indeed, urinary neopterin analysis using similar methodology was achieved in a separate study prior to quantification of plasma neopterin for CNP biomarker identification (Zis *et al.* 2017). Therefore, a method comprising of deproteinisation with an equal volume of 5% TCA, and a 15 mM potassium phosphate mobile phase (pH 6.4) was used. This provided clear peak separation for plasma samples with no overlapping peaks previously observed with acetonitrile. Peak shape was subsequently enhanced by shifting of the pH to 7.4, and increasing the concentration of potassium phosphate in the mobile phase to 20 mM. The complete method for neopterin quantification is described (section 2.2.8).

In order to quantify plasma neopterin levels using high performance liquid chromatography (HPLC), a number of additional factors must be therefore considered before the processing of samples in preparation for analysis. Blood plasma is an abundant source of circulating metabolites and as a clinical utility, provides a wealth of information pertaining to underlying disease. However, plasma is also highly complex, consisting of a wide range of organic and inorganic molecules, with a 200 fold lower concentration of neopterin than urine, which complicates analysis

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Initial method development therefore involved the replication of a previously documented method using ACN as the deproteinising agent (Flavall *et al.* 2008). Comparative studies seeking to determine optimal HPLC conditions using ACN and TCA deduced that the former improved the signal-to-noise ratio and produced larger neopterin peak areas (Flavall *et al.* 2008), although this has not been universally observed (Agilli *et al.* 2012). Therefore, plasma (or standard) was combined with acetonitrile at a 1:1 ratio, vortexed for 5 seconds and centrifuged for 10 minutes at 10300 x *g.* An isocratic mobile phase consisting of 5% methanol in 20 mM ammonium phosphate (pH 6.0), with a flow rate of 1 mL/min, was used. A single peak was initially observed with neopterin standards, and adequate separation was achieved with deproteinised plasma. However, after serial dilution of neopterin standard, a linear association between peak area and neopterin concentration was not observed. This was particularly apparent at physiologically relevant concentrations, of ~10 nM (Feldman 2004), and was attributed to the presence of an overlapping peak which remained present when injecting a sample consisting of 1:1 mobile phase and acetonitrile. The presence of the peak persisted despite the use of alternative solvent and buffer sources, and the peak area declined disproportionately when the 1:1 dilution of acetonitrile was combined with an equal volume of mobile phase, given a total final concentration of 25% acetonitrile. This suggested interference by ‘system peaks’ may have occurred due to equilibrium disruption caused by the contrasting solvent composition of the sample and mobile phase.

Further analysis was carried out using a method previously described (Carru *et al.* 2004), which involved the deproteinisation of plasma with an equal volume of 5% TCA, followed by vortexing for 10 seconds, centrifugation at 3000 x *g* for 10 minutes and dilution of 50 µL of supernatant with 200 µL of ultrapure water. Due to inadequate chromatographic separation the proportion of ACN in the mobile phase was increased incrementally to 3% but as the proportion of ACN increased, negative peak formation became problematic and interfered with neopterin quantification.

Due to apparent interactions between the mobile phase solvent and the injected sample, a fully aqueous mobile phase was considered. Previously, urine (Werner *et al.* 1987a, Groetsch *et al.* 1991, Zis *et al.* 2017) and serum (Groetsch *et al.* 1991) neopterin analysis has been achieved using a mobile phase comprising of potassium phosphate and in the case of serum, acid precipitation with TCA was used. Indeed, urinary neopterin analysis using similar methodology was achieved in a separate study prior to quantification of plasma neopterin for CNP biomarker identification (Zis *et al.* 2017). Therefore, a method comprising of deproteinisation with an equal volume of 5% TCA, and a 15 mM potassium phosphate mobile phase (pH 6.4) was used. This provided clear peak separation for plasma samples with no overlapping peaks previously observed with acetonitrile. Peak shape was subsequently enhanced by shifting of the pH to 7.4, and increasing the concentration of potassium phosphate in the mobile phase to 20 mM. The complete method for neopterin quantification is described (section 2.2.8).

3.3.2.2 Neopterin quantification

Analysis of plasma neopterin as an indicator GTPCH activity in the discovery cohort showed a mean (±SD) level in healthy controls (n = 8) of 22.86 (±22.15) nM (range: 9.75-75.25 nM). The mean (±SD) in patients with CNP (n = 10) was 29.97 (±22.82) nM (range: 10.41-85.88 nM). Statistical analysis (unpaired t-test) highlighted a lack of statistical significance (p = 0.515) (Figure 3.4). An insufficient quantity of plasma prevented neopterin quantification in a proportion of healthy controls (n = 2).

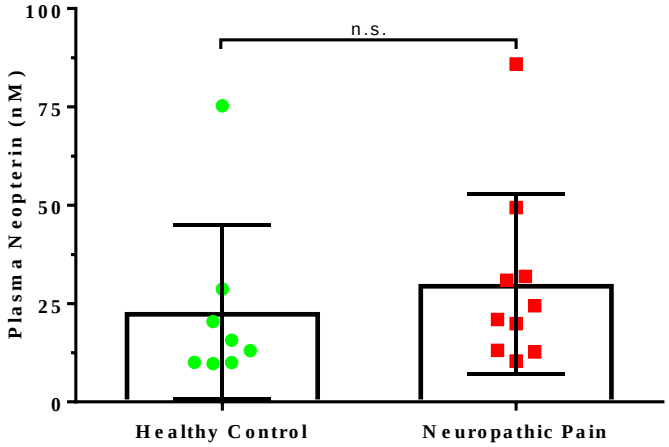

Figure 3.4: Analysis of plasma neopterin levels in healthy control and neuropathic pain patients in the discovery cohort

After isolation of plasma and removal of proteins by acid precipitation, HPLC was performed (section 2.2.8) using a 20 mM potassium dihydrogen phosphate pH 7.4 mobile phase (1 mL/min), SphereClone™ 5µM ODS(2) 250x4.6mm column and an Agilent Technologies 1100 HPLC system. Neopterin was quantified by fluorescence detection with an excitation and emission of 353nm and 438nm, respectively. Data was analysed by unpaired t-test (±SD). n.s: not statistically significant. Data is available in the electronic supplementary material within the folder entitled 'Neopterin' and has a file name of 'Discovery'.

In the discovery/validation cohort, the mean (±SD) neopterin level in healthy controls (n = 24) was 13.02 (±7.27) nM (range: 5.18-27.70 nM). In patients with CNP (n = 23), the mean (±SD) level was 9.78 (±6.59) nM (range: 1.58-25.45 nM). For those with CNP, patients with an S-LANSS result of <12 (n = 8) had a mean (±SD) nitrate level of 6.97 (±3.71) nM (range: 1.58-12.38 nM) whilst those with an S-LANSS result of ≥12 (n = 15) had a mean (±SD) level of 10.73 (±7.15) nM (range: 4.05-25.45 nM). Statistical analysis (unpaired t-test) highlighted a trend towards significance between healthy control and CNP (p = 0.117). There was a significant difference when comparing healthy control and CNP patients with an S-LANSS result of <12 (p = 0.033) (Figure 3.5). In contrast, there was no statistical significance between healthy controls and those with an S-LANSS score of ≥12 (p = 0.352) or between patients with an S-LANSS result of <12 and ≥12 (p = 0.200).

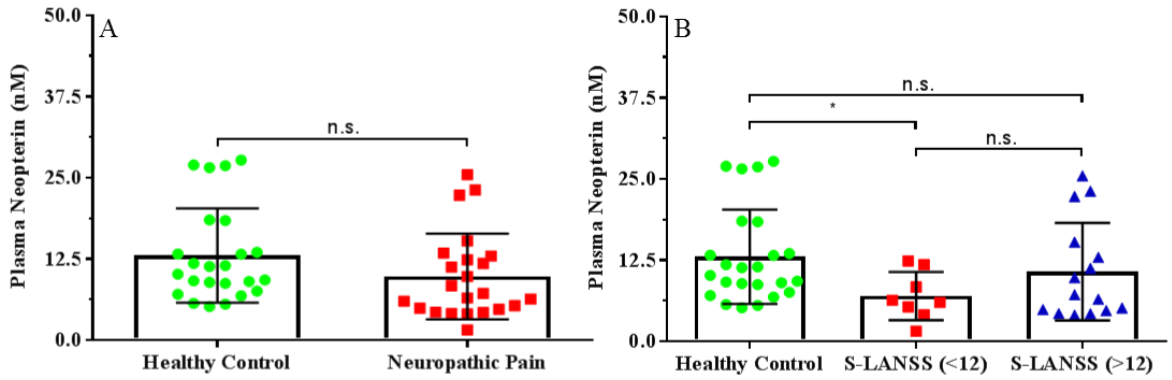

Figure 3.5: Analysis of plasma neopterin levels in healthy control and neuropathic pain patients in the discovery/validation cohort

After isolation of plasma and removal of proteins by acid precipitation, HPLC was performed (section 2.2.8) using a 20 mM potassium dihydrogen phosphate pH 7.4 mobile phase (1 mL/min), SphereClone™ 5µM ODS(2) 250x4.6mm column and an Agilent Technologies 1100 HPLC system. Neopterin was quantified by fluorescence detection with an excitation and emission of 353nm and 438nm, respectively. Healthy controls and patients with CNP were analysed (A) followed by separation of the neuropathic pain cohort by S-LANSS score (B). Data was analysed by unpaired t-test (±SD). n.s: not statistically significant, * denotes $p = \leq 0.05$. Data is available in the electronic supplementary material within the folder entitled 'Neopterin' and has a file name of 'Discovery validation'.

3.3.3 Biopterin

Quantification of reduced and oxidised biopterins highlighted significant variations between groups within the discovery cohort. The mean (±SD) plasma biopterin concentration (n = 10) was 3.00 (±0.81) nM (range: 1.86-4.41 nM) in healthy controls and 1.78 (±0.78) nM (range: 0.75-3.11 nM) in CNP patients (n = 10). When considering the same subjects, BH_2 levels were similar, with a mean level of 10.52 (±2.90) nM (range: 6.94-16.77 nM) in the control groups, compared to a mean of 12.02 (±2.95) nM (range: 7.78-18.11) for CNP patients. Moreover, the mean plasma BH_4 concentration in the control cohort was 41.45 (±25.68) nM (range: 18.47-86.33 nM) whereas the mean level in the CNP group was 21.65 (±11.18) nM (range: 8.77-42.98). Similarly, the mean total biopterin levels in healthy controls was 54.97 (±26.49) nM (range: 29.30-102.30) compared to CNP patients wherein a mean of 35.44 (±13.86) nM (range: 20.02-63.64) was observed. Both biopterin (p = 0.002) and BH_4 (p = 0.038) showed significant downregulation in CNP patients. There was no difference in BH_2 levels (p = 0.268), although total biopterin strongly trended (p = 0.054) towards downregulation in CNP patients (Figure 3.6). The mean ratio of neopterin to biopterin (N/B) in the healthy controls was 0.475. In contrast, the mean ratio for CNP patients was

0.922, although this was not statistically significant (p = 0.180). Moreover, the mean BH_4/BH_2 ratio in healthy controls was 4.046, which was significantly greater (p = 0.010) than the mean ratio in CNP patients of 1.766 (Figure 3.6).

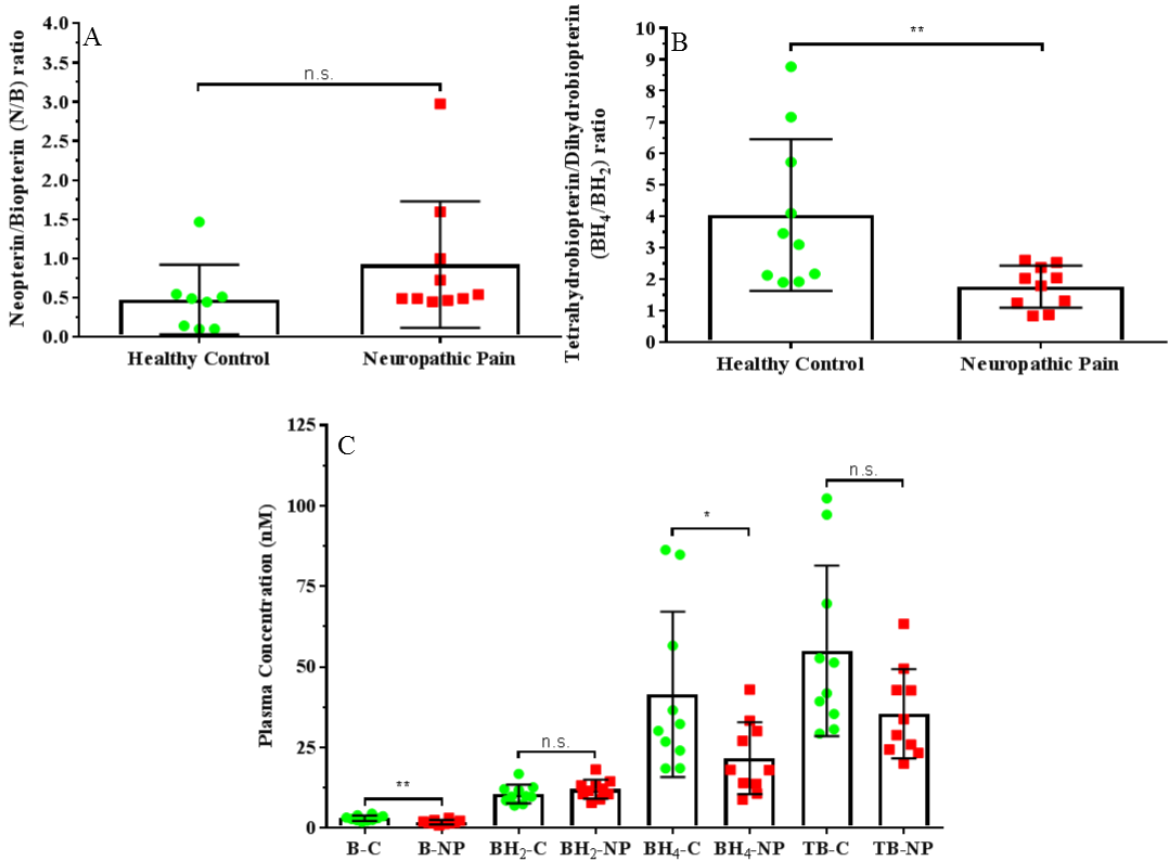

Figure 3.6: Analysis of pterins in healthy control and neuropathic pain patients in the discovery cohort

After isolation of plasma and removal of proteins, HPLC was conducted (section 2.1.1.5). Quantification was enabled by sequential electrochemical and fluorescence detection. The mobile phase consisted of 50 mM sodium acetate, 5 mM citric acid, 48 µM EDTA, and 160 µM DTE (pH 5.2) at a flow rate of 1.3 mL/min. Quantification of BH_4 was enabled by electrochemical detection (background currents of +500 nA and -50 nA). Biopterin and BH_2 were measured as using a Jasco FP2020 fluorescence detector. Analysis was undertaken with consideration for (A) the neopterin/biopterin ratio, (B) the tetrahydrobiopterin/dihydrobiopterin ratio and (C) between the plasma levels of biopterin (B), dihydrobiopterin (BH_2) and tetrahydrobiopterin (BH_4) in healthy controls and CNP patients. Data was analysed by unpaired t-test (±SD). TB: total biopterins. n.s: not statistically significant, * denotes p = ≤0.05, **denotes p = ≤0.01. Data is available in the electronic supplementary material within the folder entitled 'Biopterins' and has a file name of 'Discovery'.

3.3.4 Pain protective haplotype

Genotyping of healthly controls and CNP patients for three SNPs (rs8007267, rs3783641, rs10483639) permitted screening for the pain protective haplotype in both discovery and discovery/validation cohorts. In the discovery cohort, four subjects were carriers of the pain protective haplotype (20.00%), of which one was homozygous (5.00%). In the discovery/validation cohort, nine healthy controls (37.50%) were carriers of the pain protective haplotype, of which one was homozygous (4.17%). Seven (30.43%) CNP patients were homozygous for the pain protective haplotype. Allele and genotype frequencies were determined for the three SNPs (see electronic supplementary material entitled 'genotype and allele frequencies'). Genotyping data pertaining to the discovery/validation cohort was subsequently used for categorisation of healthy controls and patients for analysis of plasma nitrate, neopterin and *GCH1* expression. However, there were insufficient data points to permit further analysis of CNP patients by categorised of the S-LANSS score. One patient with CNP in the discovery/validation cohort could not be genotyped due to difficulties obtaining venous blood.

3.3.4.1 Pain protective haplotype – nitric oxide

After grouping all study participants in the discovery/validation cohort according to genotype, carriers of the pain protective haplotype (n = 15) had a mean (±SD) nitrate level of 25.92 (±13.53) µM (range: 10.17-61.76 µM). Similarly, non-carriers (n = 31) had a mean (±SD) nitrate level of 21.84 (±8.52) µM (range: 9.10-38.98 µM). The mean (±SD) nitrate level in healthy controls assigned as carriers (n = 8) was 27.65 (±17.90) µM (range: 10.17-61.76 µM) whereas the mean (±SD) nitrate level for non-carriers (n = 15) was 21.33 (±9.29) µM (range: 9.10-38.98 µM). Chronic neuropathic pain patients with the pain protective haplotype (n = 7) had a mean (±SD) nitrate level of 23.94 (±6.70) µM (range: 13.94-33.01 µM). Similarly, non-carriers with CNP (n = 16) had a mean (±SD) level of 22.32 (±8.01) µM (range: 13.45-35.45 µM). There was no significant difference between carriers and non-carriers for all study participants (p = 0.218), controls alone (p = 0.273) or CNP alone (p = 0.647) (Figure 3.7).

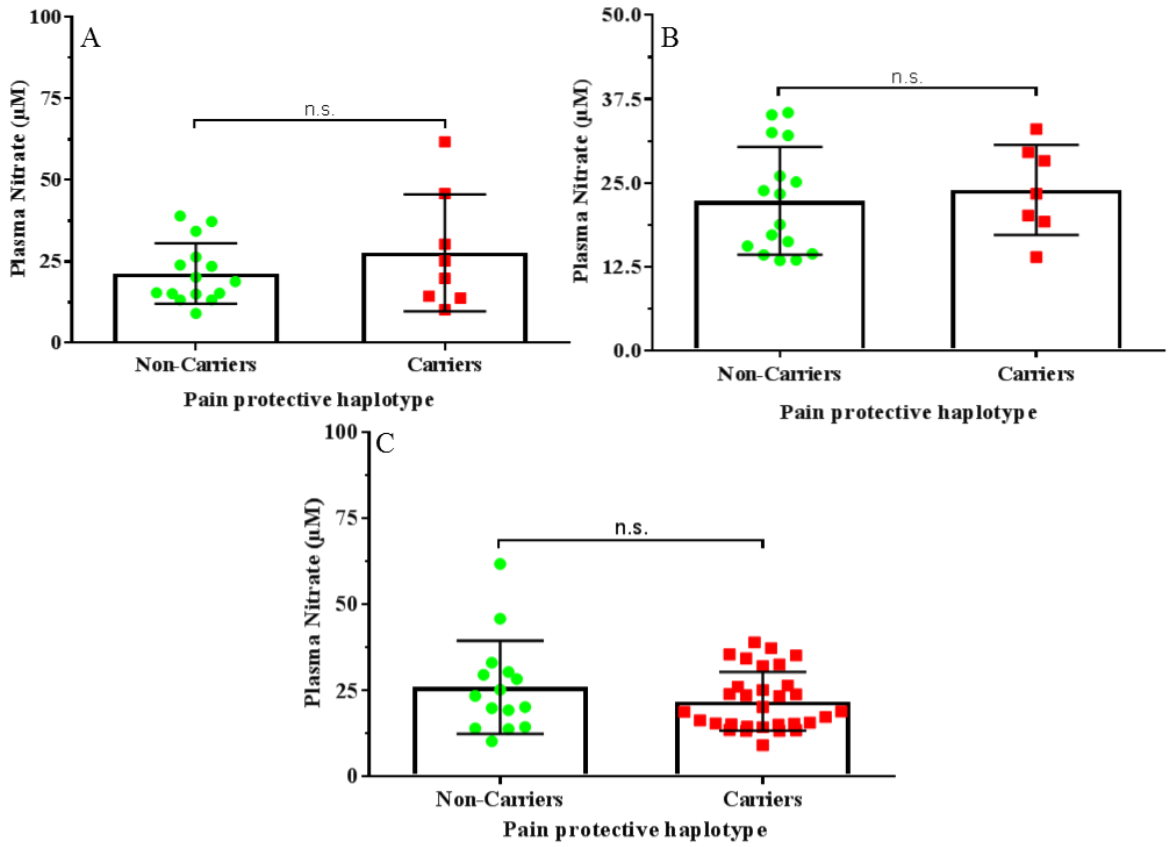

Figure 3.7: Analysis of plasma nitrate in the discovery/validation cohort according to *GCH1* genotype

After isolation of plasma and removal of proteins using a 10 kDa filter, analysis of the nitrite/nitrate concentration within the filtrate was conducted by enzymatic reduction of nitrate to nitrite followed by the Griess reaction (section 2.2.7). Genotypes pertaining to the pain protective haplotype were determined (section 2.2.5) followed by assessment of plasma nitrate according to genotype in (A) controls subjects only, (B) CNP patients only and (C) with all study participants grouped together. Absorbance data was analysed by unpaired t-test (±SD). n.s: not statistically significant.

3.3.4.2 Pain protective haplotype – neopterin

All healthy controls and patients were initially grouped together and segregated into those who were carriers of the pain protective haplotype (n = 16) and those without a single copy (n = 31). The mean (±SD) neopterin level in carriers was 12.48 (±6.93) nM (range: 4.72-27.70 nM). In non-carriers, the mean (±SD) level was 10.89 (±7.18) nM (range: 1.56-26.93 nM). When considering only healthy controls for categorisation by genotype, carriers (n = 9) had a mean (±SD) neopterin level of 12.48 (±6.84) nM (range: 5.67-27.70 nM) whilst non-carriers (n = 15) had a mean (±SD) neopterin level of 13.35 (±7.72) nM (range: 5.18-26.93 nM). Patients with CNP designated as

carries (n = 7) had a mean (±SD) neopterin level of 12.51 (±7.57) nM (range: 4.72-23.11 nM) whilst non-carriers (n = 16) had a mean (±SD) neopterin level of 8.59 (±5.98) nM (range: 1.56-25.45 nM). There was no significance observed between carriers and non-carriers when considering all participants (p = 0.468), controls alone (p = 0.784) or CNP alone (p = 0.196) (Figure 3.8).

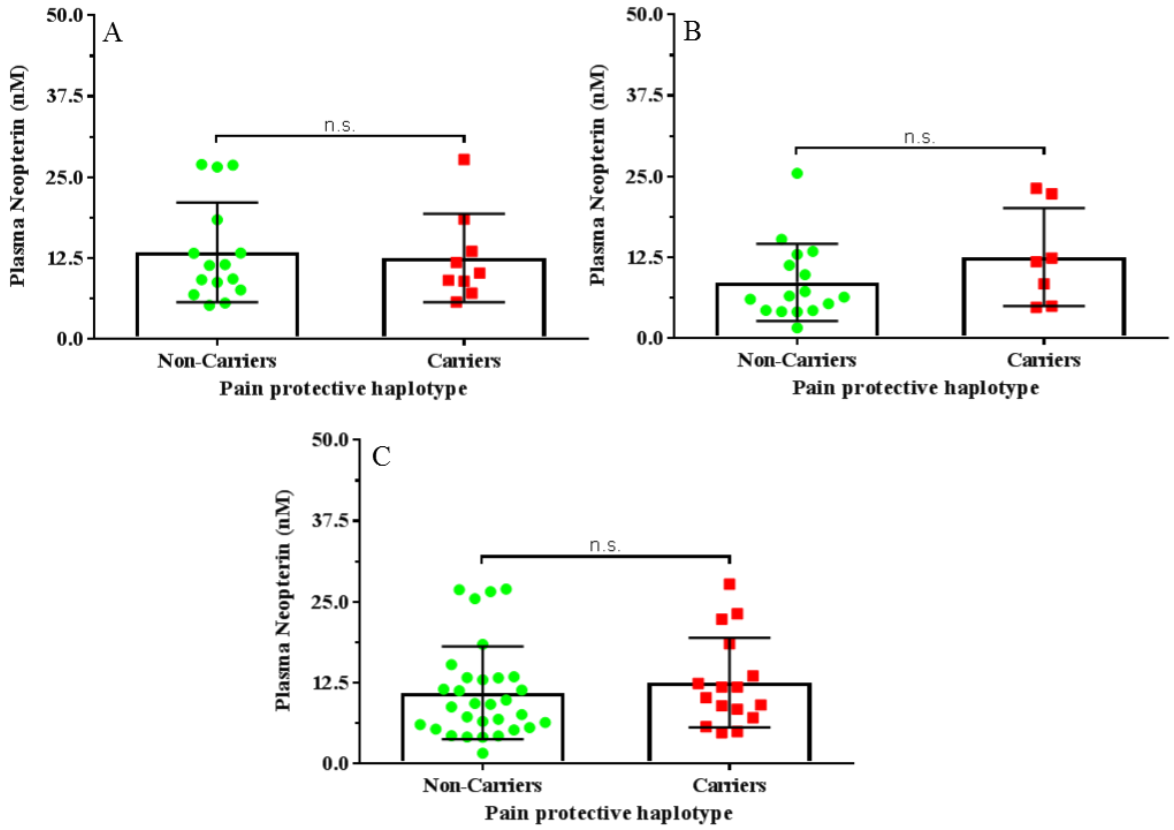

Figure 3.8: Analysis of plasma neopterin levels in healthy control and neuropathic pain patients in the discovery/validation cohort

After isolation of plasma and removal of proteins by acid precipitation, HPLC was performed (section 2.2.8) using a 20 mM potassium dihydrogen phosphate pH 7.4 mobile phase (1 mL/min), SphereClone™ 5µM ODS(2) 250x4.6mm column and an Agilent Technologies 1100 HPLC system. Neopterin was quantified by fluorescence detection with an excitation and emission of 353nm and 438nm, respectively. Genotypes pertaining to the pain protective haplotype were determined (section 2.2.5) followed by assessment of plasma neopterin according to genotype in (A) controls subjects only, (B) CNP patients only and (C) with all study participants grouped together. Data was analysed by unpaired t-test (±SD). n.s: not statistically significant.

3.3.4.3 Pain protective haplotype – *GCH1* expression

Analysis of *GCH1* expression and the influence of the pain protective haplotype was conducted in qbase+ (section 4.1.2.2). There was no significant difference (p = 0.416) between *GCH1* expression in carriers (n = 16) than non-carriers (n = 32) of the pain protective haplotype. The fold change in carriers indicated a marginal upregulation of *GCH1* (fold change: 1.05). In contrast, analysis of healthy control participants showed a significant (p = 0.018) upregulation in carriers (n = 9) when compared to non-carriers (n = 15), though a similar small fold change was observed (fold change: 1.13). Participants with CNP who were carriers (n = 7) of the pain protective haplotype did not have significantly different (p = 0.649) *GCH1* expression than healthy controls (n = 17). There was a marginal downregulation in CNP patients separated by genotype (fold change: 1.05) (Figure 3.9).

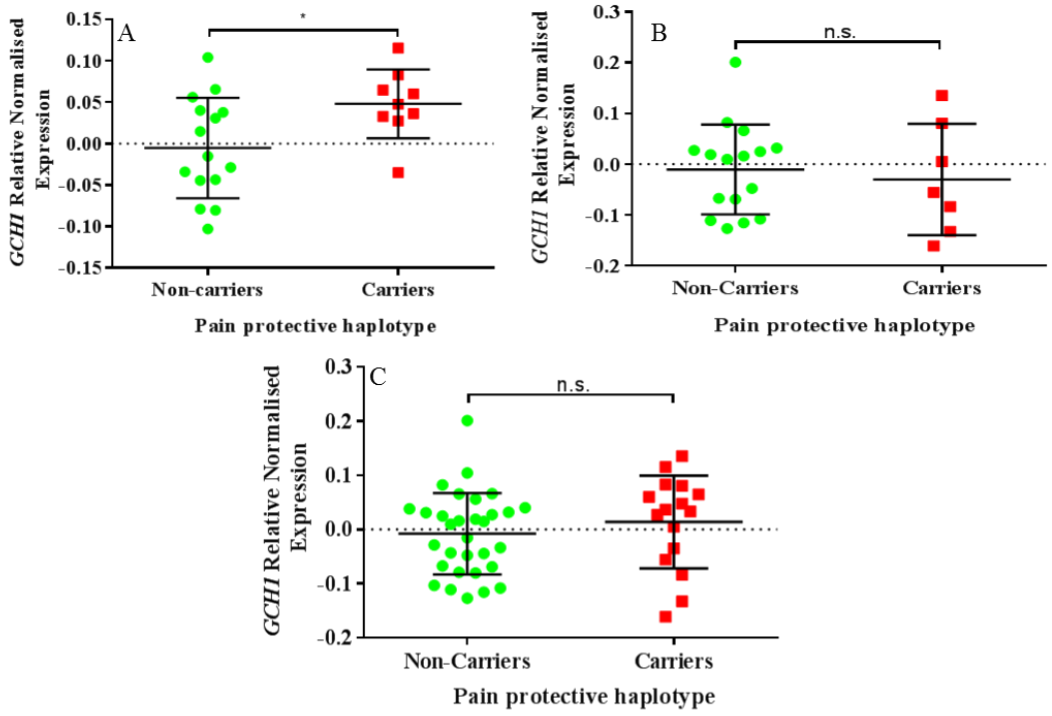

Figure 3.9: Analysis of *GCH1* expression in healthy control and neuropathic pain patients in the discovery/validation cohort according to genotype

After extraction of RNA (section 2.1.2.2) and subsequent qRT-PCR (section 2.1.2.5), the expression of *GCH1* was determined and normalised to the geometric mean of *CYC1* and *YWHAZ*. Genotypes pertaining to the pain protective haplotype were determined (section 2.2.5) followed by assessment of plasma nitrate according to genotype in (A) controls subjects only, (B) CNP patients only and (C) with all study participants grouped together. Absorbance data was analysed by unpaired t-test (±SD). n.s: not statistically significant. * denotes $p = \leq 0.05$. Individual genotypes are available in the electronic supplementary material within the folder entitled 'Genotyping' and has a file name of 'Genotypes'.

3.3.5 Correlation analysis

Correlation analysis of multiple parameters, including plasma nitrite/nitrate and neopterin, were undertaken to determine if variations in Pearson's correlation may be indicative of CNP. Multiple correlations incorporating either all participants, healthy controls alone or patients with CNP were performed using data from the discovery cohort, which included parameters pertaining to plasma biopterins (Table 3.2). A relatively strong positive correlation was present between plasma nitrate and neopterin levels when grouping healthy controls and CNP patients (r = 0.701, p = 0.008). However, when analysing both groups independently, the positive correlation between nitrate and nitrate was considerably stronger in CNP patients (r = 0.811, p = 0.027) than healthy controls (r = 0.547, p = 0.259). Correlation analysis incorporating *GCH1* expression could not be performed due to insufficient data points.

Data correlation in the discovery/validation cohort was performed as described in the discovery cohort, although the CNP group was also further categorised depending on the S-LANSS score (Table 3.3). In contrast to the discovery cohort, there was no strong correlation between nitrate and neopterin with all permutations, though a weak positive correlation was observed in CNP patients with an S-LANSS score of ≥12 (r = 0.219, p = 0.432). In contrast, clear correlations were observed when analysing nitrate and *GCH1* and between neopterin and *GCH1*. The degree of positive correlation between nitrate and *GCH1* was notably greater in CNP patients (r = 0.396, p = 0.055) than health controls (r = 0.041, p = 0.855) with a further clear distinction between those with an S-LANSS score of <12 (r = 0.284, p = 0.495) and ≥12 (r = 0.585, p = 0.022). Similar results were observed between neopterin and *GCH1*, wherein the positive correlation was greater in CNP patients (r = 0.623, p = 0.001) than in healthy controls (r = 0.156, p = 0.468) whilst analysis of patients with CNP alone showed that those with an S-LANSS score of ≥12 had a notably stronger correlation (r = 0.773, p = 0.001) than those scoring <12 (r = 0.297, p = 0.497).

Table 3.2: Pearson correlation analysis of pterins and nitric oxide in the discovery cohort

		Nitrate *vs.* Neopterin	BH_4 *vs.* Neopterin	BH_4 *vs.* Nitrate	Total Biopterin *vs.* Neopterin	$BH_4/(BH_2+B)$ *vs.* Nitrate	BH_4/BH_2 *vs.* Nitrate
Pain and healthy control							
	N	13	18	15	18	15	15
	Correlation (*r*)	0.701	-0.182	-0.145	-0.164	-0.194	-0.229
	p	0.008	0.469	0.607	0.516	0.489	0.411
Healthy control							
	N	6	8	8	8	8	8
	Correlation (*r*)	0.547	-0.283	0.005	-0.250	-0.174	-0.203
	p	0.259	0.498	0.991	0.551	0.680	0.629
Pain							
	N	7	10	7	10	7	7
	Correlation (*r*)	0.811	0.165	0.047	0.128	0.353	0.389
	p	0.027	0.649	0.921	0.726	0.438	0.388

Table 3.3: Pearson correlation analysis of neopterin, nitric oxide and *GCH1* in the discovery/validation cohort

		Nitrate *vs.* Neopterin	Nitrate vs. *GCH1*	Neopterin *vs. GCH1*
Pain and health control				
	n	47	47	48
	Correlation (*r*)	0.080	0.193	0.439
	p	0.593	0.194	0.002
Healthy control				
	n	23	23	24
	Correlation (*r*)	0.058	0.041	0.156
	p	0.794	0.855	0.468
Pain only				
	n	24	24	24
	Correlation (*r*)	0.123	0.396	0.623
	p	0.577	0.055	0.001
Pain with S-LANSS <12				
	n	8	8	8
	Correlation (*r*)	-0.037	0.284	0.297
	p	0.930	0.495	0.497
Pain with S-LANSS ≥12				
	n	15	15	15
	Correlation (*r*)	0.219	0.585	0.773
	p	0.432	0.022	0.001

3.3.6 Transcriptional regulation in the synthesis of tetrahydrobiopterin

Differential regulation of genes encoding for enzymes involved in *de novo* BH_4 synthesis and regeneration of BH_4 were analysed. In the discovery cohort, no single gene was found to be significantly differentially regulated, though a trend towards *QDPR* downregulation was observed in CNP (Table 3.4). Analysis in the discovery/validation cohort showed significant down regulation of *GCHFR* in CNP patients (Table 3.5). Several other genes trended towards differential regulation, in particular *PCBD1* and *PTS*, both of which were marginally upregulated in CNP patients.

Table 3.4: Expression of genes involved in BH_4 systhesis in the discovery cohort

Accession Number	Gene Name	Gene Symbol	*p* value (qRT-PR)	FC in CNP (qRT-PCR)
NM_000791	Dihydrofolate reductase	*DHFR*	0.413	↓1.55
NM_000161	GTP cyclohydrolase I	*GCH1*	0.905	↑1.42
NM_005258	GTP cyclohydrolase I feedback regulator	*GCHFR*	0.905	↓1.01
NM_000281	Pterin-4 alpha-carbinolamine dehydratase 1	*PCBD1*	1.000	↓1.18
NM_000317	6-pyruvoyltetrahydropterin synthase	*PTS*	0.111	↓1.75
NM_000320	Quinoid dihydropteridine reductase	*QDPR*	0.063	↓1.53
NM_003124	Sepiapterin reductase	*SPR*	0.286	↓1.79

Gene expression analysis was conducted by qRT-PCR. Data was normalised to the geometric mean of *ATP5B*, *SHDA* and *YWHAZ* using qbase+ after geNorm analysis. Linear fold changes and *p* values are shown (Mann-Whitney). $p = \leq 0.05$ considered statistically significant. Data files, including geNorm analysis, are available in the electronic supplementary material under the file path; qRT-PCR > Clinical Samples > Discovery.

Table 3.5: Expression of genes involved in BH_4 systhesis in the discovery/validation cohort

Accession Number	Gene Name	Gene Symbol	*p* value (array)	FC in CNP (array)	*p* value (qRT-PCR)	FC in CNP (qRT-PCR)
NM_000791	Dihydrofolate reductase	*DHFR*	0.555	↑1.02	0.607	↑1.03
NM_000161	GTP cyclohydrolase I	*GCH1*	0.204	↓1.12	0.192	↓1.07
NM_005258	GTP cyclohydrolase I feedback regulator	*GCHFR*	0.801	1.00	0.038	↓1.08
NM_000281	Pterin-4 alpha-carbinolamine dehydratase 1	*PCBD1*	0.975	↑1.01	0.066	↑1.17
NM_000317	6-pyruvoyltetrahydropterin synthase	*PTS*	0.027	↑1.06	0.087	↑1.11
NM_000320	Quinoid dihydropteridine reductase	*QDPR*	0.042	↓1.03	0.184	↓1.10
NM_003124	Sepiapterin reductase	*SPR*	0.493	↑1.02	0.254	↓1.09

Gene expression analysis was conducted by microarray and qRT-PCR. Data obtained by qT-PCR was normalised to the geometric mean of *CYC1* and *YWHAZ* using qbase+ after geNorm analysis. Fold changes and *p* value for microarray data (ANOVA) and qRT-PCR (unpaired t-test) are shown ($p = \leq 0.05$ considered statistically significant). Data files are available in the electronic supplementary material under the file path; qRT-PCR > Clinical Samples > Discovery validation > Group 3 (BH_4 pathway).

3.3.7 Luciferase reporter assays

3.3.7.1 Stimulation and transfection development

In order to confirm a suitable method to analyse the potential impact of stimulation upon the luciferase reporter assay constructs, stimulation of HUVEC and RAW264.7 cells was conducted with reference to a previous study seeking to elucidate the molecular mechanisms of *GCH1* upregulation upon stimulation (Liang *et al.* 2013).

Initially, stimulation of RAW264.7 cells was conducted with lipopolysaccharide (LPS) (Sigma, UK). Cells were seeded at a density of $2x10^5$ cells/mL in 1 mL of complete DMEM on a 24-well plate for 24 hours before complete replacement with media containing a range of LPS concentrations, varying between 0 and 1000 ng/mL. Similarly, to determine the degree of *GCH1* upregulation in response to TNF-α and IFN-γ, HUVECs were seeded on a 24-well plate with 1 mL of cell suspension at a density of $5x10^4$ cells/mL. After 24 hours, the media was changed and the

cells subject to 1 mL of equivalent media containing varying concentrations of TNF-α, IFN-γ, or a combination of both cytokines. Data analysis revealed an upregulation of *Gch1* in the presence of LPS (Figure 3.10**Error! Reference source not found.**), ranging from a 2.88 fold with 100 ng/mL LPS to a 3.71 fold upregulation with 1000 ng/mL. After incubation for 24 hours, RNA was extracted as described (section 2.8.2) using 500 µL Tri Reagent (Sigma, UK) followed by DNAse treatment (section 2.8.2), cDNA synthesis (section 2.1.2.4) and qRT-PCR (section 2.1.2.5). Data was analysed using Bio-Rad CFX Manager 3.1 and the data extracted for graphical representation in GraphPad Prism 6.0.

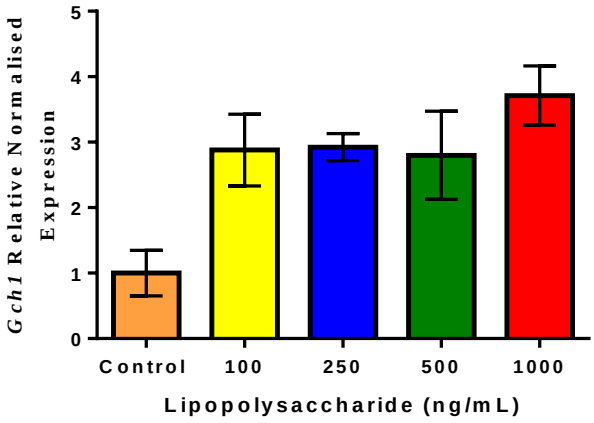

Figure 3.10: The effect of LPS on *Gch1* expression in cultured RAW264.7 cells

Cultured RAW264.7 cells were subject to different concentrations of LPS for 24 hours. qRT-PCR was then used to determined *Gch1* expression relative to that of *Gapdh*, and expressed relative to control. Data was exported from Bio-Rad CFX Manager for analysis in GraphPad Prism 6.0 (± SEM).

Universal upregulation of *GCH1* was observed in all cells exposed to cytokines (Figure 3.11**Error! Reference source not found.**). The application of TNF-α resulted in the lowest degree of upregulation, ranging from 2.57 fold with 20 ng/mL to 5.46 fold at 100 ng/mL. Similar response was also observed when using IFN-γ alone, with a maximal observed response, a 22.00 fold upregulation, at 100 ng/mL. However, as previously described (Huang *et al.* 2005), a combination of IFN-γ and TNF-α resulted in an upregulation on a scale significantly greater than when using IFN-γ and TNF-α alone. A combination of TNF-α (20 ng/mL) and IFN-γ (50 ng/mL) resulted in a 181.37 fold *GCH1* upregulation. This was increased to 269.77 fold and further to 313.90 fold as both IFN-γ and TNF-α concentrations increased.

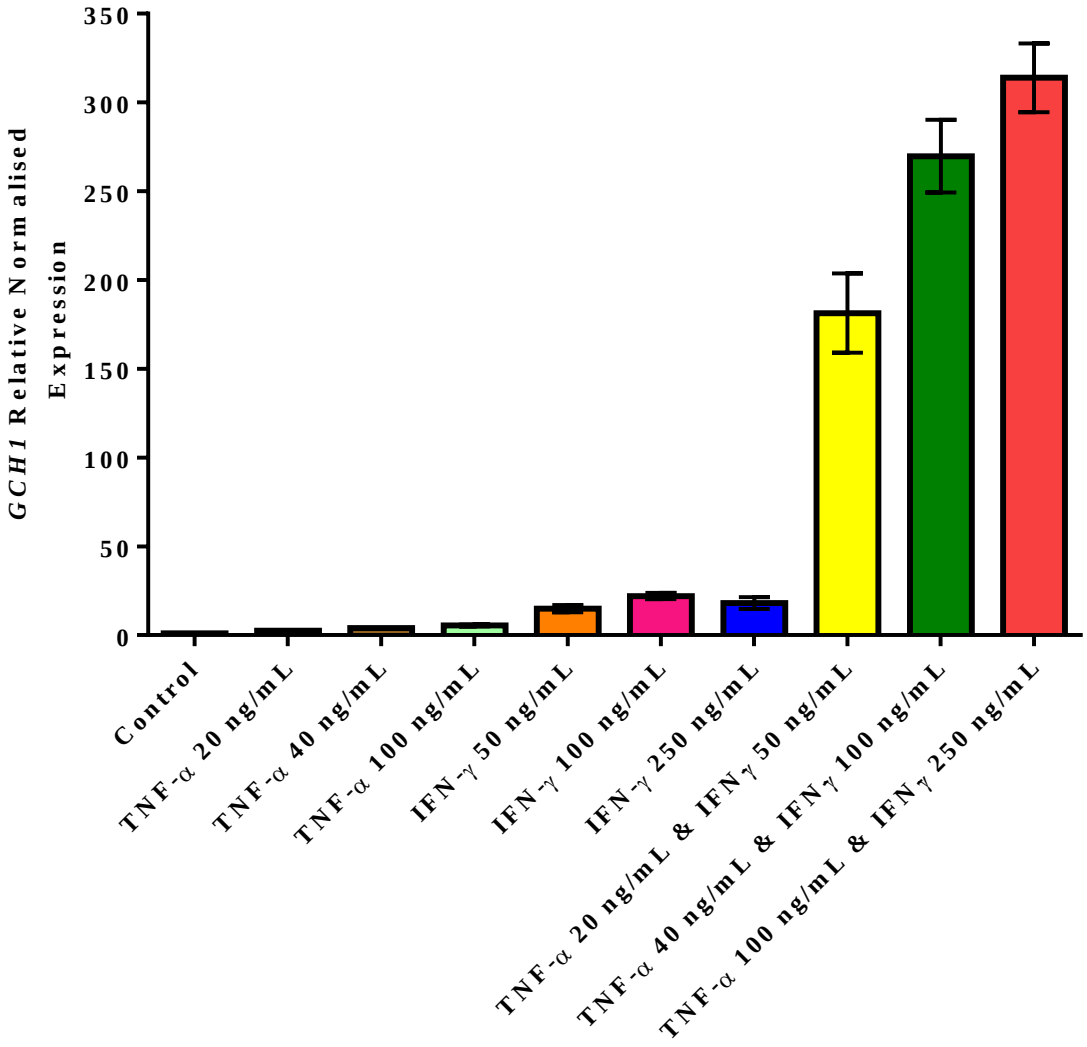

Figure 3.11: Relative normalised expression of *GCH1* illustrating upregulation in HUVECs exposed to cytokines
Cultured HUVECs were subject to different concentrations of IFN-γ and/or TNF-α for 24 hours. qRT-PCR was then used to determined *GCH1* expression relative to that of *GAPDH* and expressed relative to control. Data was exported from Bio-Rad CFX Manager for analysis in GraphPad Prism 6.0 (± SEM).

Cytokine-induced upregulation of GTPCH was then confirmed using western blot, as detailed section 2.8.3). Two polyacrylamide gel lanes consisting of protein from non-stimulated cells and cytokine stimulated cells were used. Two distinct bands corresponding to GTPCH were present in the stimulated cells alone, which corresponded to GTPCH (Figure 3.12**Error! Reference source not found.**).

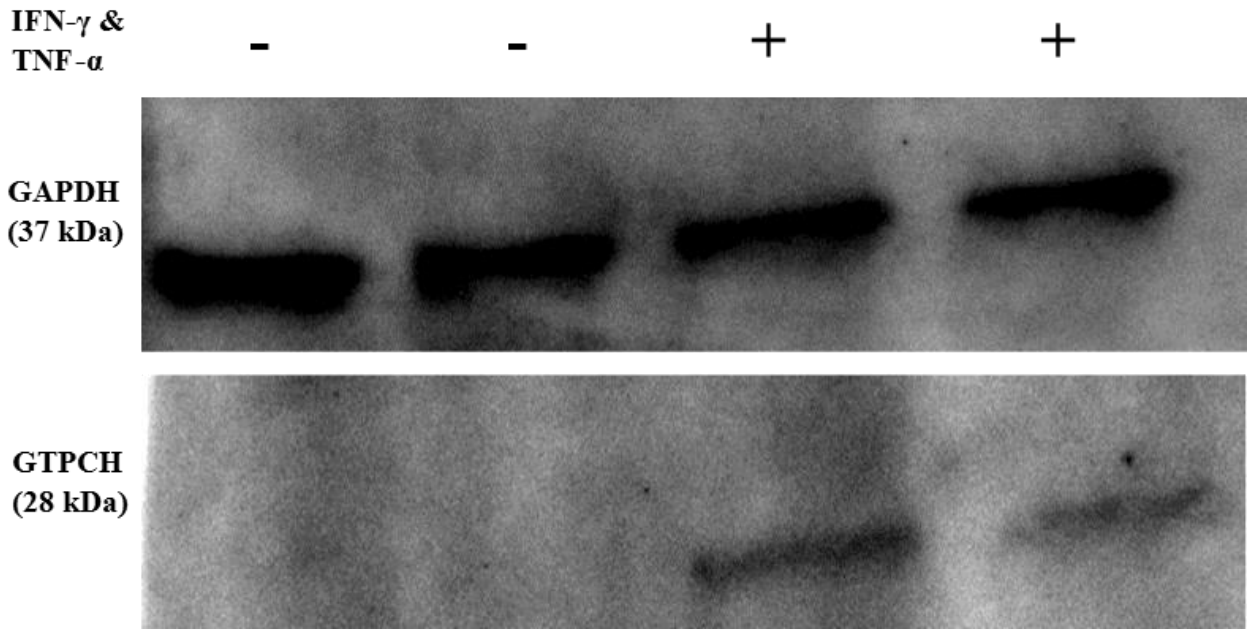

Figure 3.12: Western blot of HUVEC lysate after cytokine stimulation

HUVECs were cultured until near confluent on two T75 cell culture flasks, with (+) and without (-) cytokine treatment (40 ng/mL of TNF-α and 100 ng/mL of IFN-γ), for 24 hours. Protein was then isolated and a westen blot performed (in duplicate) as described (section 2.8.3). Primary antibodies for GTPCH and GAPDH (loading control) were used. Images and densitometry analysis were obtained using a ChemiDoc MP Imaging System (Bio-Rad, UK). Densitometry analysis of GAPDH bands showed an average volume intensity of 34,598,120 for non-stimulated cell extract and 27,628,196 for protein extract from stimulated cells, thereby illustrating that any differences in protein loading cannot be attributable for the absence of bands corresponding to GTPCH in non-stimulated cells.

3.3.7.2 Transfection

3.3.7.2.1 Chemical transfection

Both RAW264.7 and HUVECs are generally considered difficult to efficiently transfect. Initial attempts to assess reporter assay signal included the transfection of RAW264.7 cells using luciferase reporter constructs established for assessment of *GCH1* stimulation. These vector constructs were extracted using the GeneJet Plasmid Miniprep kit (Thermo Scientific, UK) as described (section 2.6.1). Multiple transfection parameters were considered for each reagent, including those suggested in the manufacturers protocol in addition to further adaptations for optimisation. The Dual-Luciferase Reporter Assay System was used rather than the Dual-Glo Luciferase Assay System due to increased sensitivity, and a greater luminescent output, associated with the 'flash' kinetics of this assay. Transfection of RAW264.7 cells was undertaken with multiple transfection reagents, including X-tremeGENE HP (Roche, UK), polyethylenimine (Polyscience Inc, USA), Fugene 6 (Promega, USA) and Fugene HD (Promega, USA). However,

all luminescence recordings were consistently indistinguishable from background level. Similarly, transfection of HUVECs was carried out as described for RAW264.7 cells, aside from variations in cell culture produces (Section 2.5.2). There was a distinct lack of luminescent signal which was relatively inconsistent between replicates and often indistinguishable from non-transfected cells. Transfections were subsequently undertaken with TransIT-Jurkat (RAW264.7 only) (Mirus, USA) and TransIT-2020 (Mirus, USA). Despite a comparative increase in luminescent signal with TransIT-2020, this was insufficient and did not improve by varying DNA concentration, changing transfection reagent-DNA ratios, alongside media changes at either 4 or 8 hours post-transfection to minimise toxicity. Only when transfecting 250 ng (per 48-well) of pRL-SV40 or pRL-CMV alone was luminescent signal reliably observed, thereby illustrating low transfection efficiency coupled with comparatively low transcriptional activity of the *GCH1* firefly vector constructs. As such, RAW264.7 and HUVEC transfections were also performed using an expression vector encoding GFP-tagged carbohydrate-responsive element-binding protein (ChREBP) to allow visualisation of transfection efficiency using fluorescent microscopy. Moreover, the aforementioned transfections procedures were also repeated with DNA extracted using the PureLink HiPure Plasmid Midiprep Kit, followed by solvent precipitation (section 2.6.3). Such methodology has been described in detail for the successful transfection of RAW264.7 cells (Cheung *et al.* 2015), but failed to distinguishably improve transfection efficiency when using previous methodology.

3.3.7.2.2 Electroporation

Transfection of RAW264.7 and HUVECs by means of electroporation was considered. Initially, HUVECs were resuspended in Opti-MEM reduced serum media (Thermo Scientific, UK) to a concentration of $2x10^6$ cells/mL. Two hundred microliters of cell suspension was transferred to an electroporation cuvette containing 5 µg of plasmid DNA encoding either firefly/Renilla luciferase or ChREBP-GFP. Electroporation was then carried out using a Gene Pulser XCell Electroporation System (Bio-Rad, UK). Multiple parameters were varied in order to optimise transfection efficiency whilst maintaining sufficient cell viability. Two decay patterns were used; square wave and exponential wave. Multiple parameters were initially considered, including varying the square wave pulse duration, ranging between 15-25 milliseconds (ms) and between 200-300 microfarads (µF), and varying the exponential wave pulse from 200-250 V and 250, 500 or 950 µF. The extremes of these parameters tended to either fail to transfect HUVECs (e.g. 15 ms, 200 µF) or left few viable cells after electroporation (e.g. 25 ms, 300 µF), as observed by (fluorescent)

microscopy. The amount of DNA was also varied, but no clear advantage was observed. The optimal electroporation parameters, which are similar to those previously described for RAW264.7 cells (e.g. 20 ms/250 µF or 225 V/950 µF), resulted in a distinct agglomeration of dead cells and whilst many remained seemingly viable. The transfection efficiency remained inadequate.

3.3.7.2.3 Cytofect HUVEC

Transfection of HUVECs with the Cytofect HUVEC Transfection Kit (Cell Applications, USA) was performed using several luciferase reporter constructs for *GCH1* analysis, extracted using the GeneJet Plasmid Miniprep kit as described (section 2.6.1). Multiple parameters were considered for optimisation, including the incubation time after transfection (12, 18, 24, 36 or 48 hours), the duration of incubation with the transfection complex (1 hour or 2 hours), the volume of transfection complex (as per protocol, or double volume) and the ratio of firefly to *Renilla* (pRL-SV40). Fluorescent microscopy of cells transfected with GFP encoding vector showed few positive cells, indicating low transfection efficiency. This was reaffirmed by low luminescent signals, though *Renilla* luminescence was observed. However, the use of the PureLink HiPure Plasmid Midiprep Kit (section 2.6.3) dramatically improved transfection efficiency, as demonstrated by fluorescent microscopy (Figure 3.13**Error! Reference source not found.**). This was also reflected by a dramatic increase in firefly and *Renilla* luminescence. The transfection optimisation process described above was subsequently repeated before commencement of reporter assays to assess *GCH1* polymorphisms and intronic regions under cytokine stimulation. Transfection of HUVECs using this methodology is detailed within the methodology section (section 2.6.4).

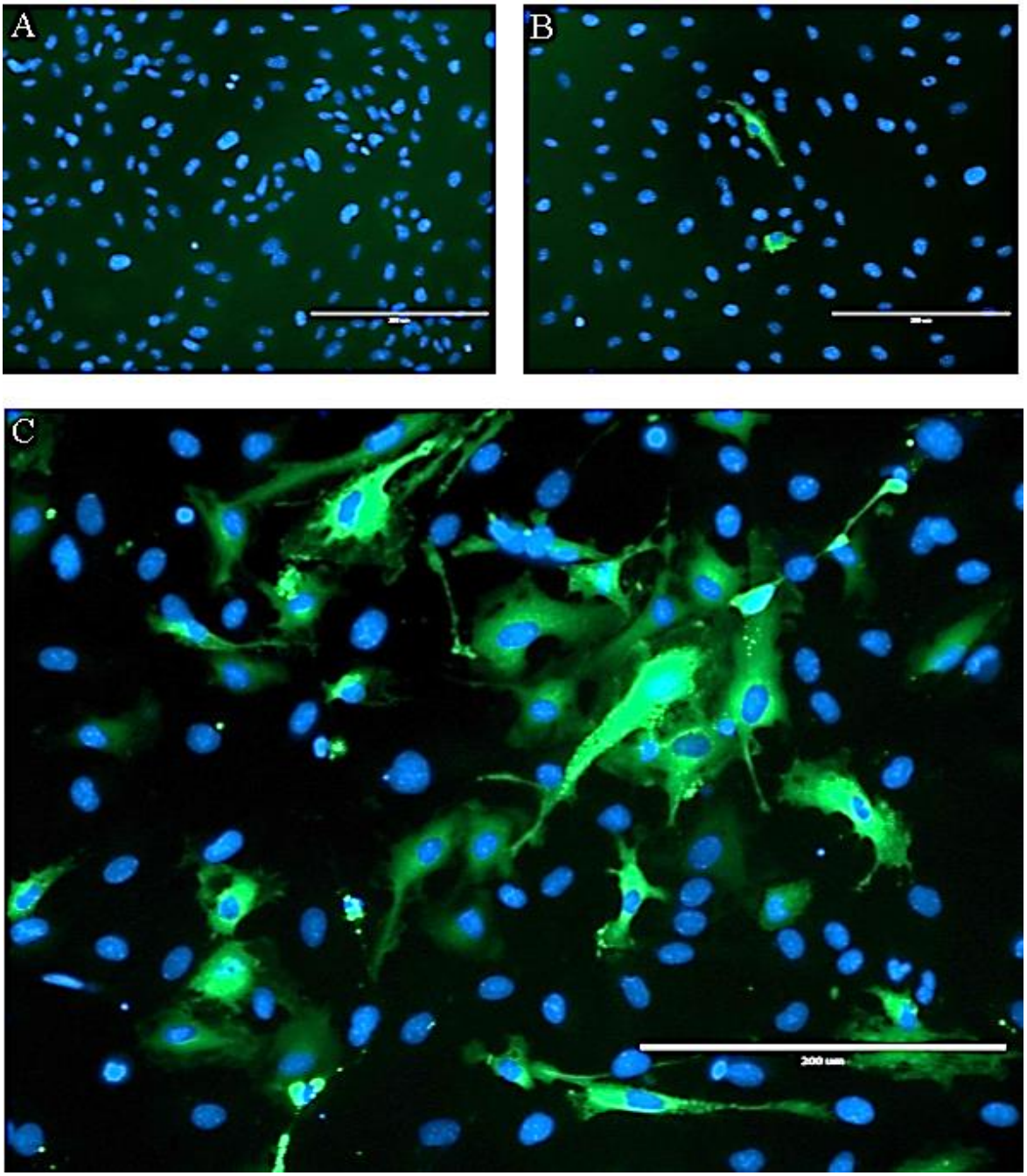

Figure 3.13 Fluorescent microscopy of transfected HUVECs after DAPI staining

Visulisation of transfection efficiency was carried out with an expression vector encoding GFP-tagged ChREBP, followed by DAPI staining. HUVECs were transfected as described (section 2.6.4 and 2.6.6) with DNA obtained using different extraction methods. Cells were either (A) subject to the transfection reagents in the absence of DNA (negative control), (B) transfected with 300 ng of DNA extracted using the GeneJET Gel Extraction Kit (section 2.6.1) or (C) with the PureLink HiPure Plasmid Midiprep Kit followed by an addition solvent purification step (section 2.6.3). Images were obtained with an EVOS FL Cell Imaging System.

3.3.7.3 Luciferase assays and the pain protective haplotype

Initial luciferase reporter assays using the constructs representing the three SNPs constituting the pain protective haplotype (rs8007267, rs3783641 and rs10483639) were performed using HEK293 cells (Figure 3.14). After normalisation of firefly to *Renilla* (pRL-TK), data analysis highlighted a significant reduction in relative luminescence for the variant construct representing the *GCH1* 5' SNP (rs8007267). There was no significant difference between constructs relating to the intronic (rs3783641) and 3' (rs10483639) SNPs. This was subsequently repeated using SH-SY5Y cells, yielding similar results with a significant reduction in relative luminescence pertaining to the (rs8007267) variant construct (Figure 3.14). Both the wild-type and variant pGL4.20-GCH1-10kb constructs (consisting of 10.1 kb of the *GCH1* 5' region) were then analysed using HEK293 cells (Figure 3.14). Again, relative luminescence was significantly reduced in cells transfected with the (rs8007267) variant construct.

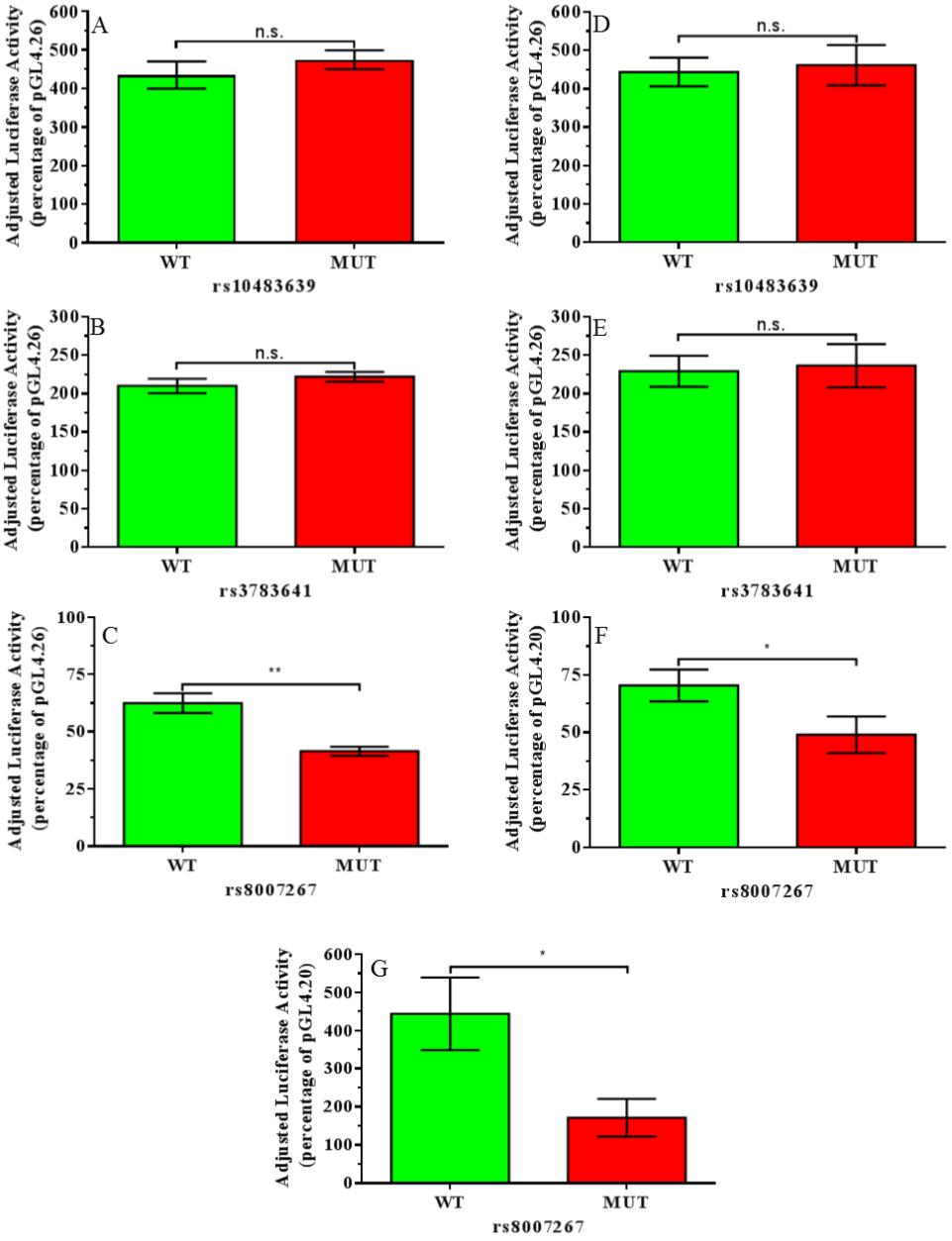

Figure 3.14: Luciferase assays assessing the potential regulatory role of the pain protective haplotype on *GCH1* expression

Transfections were undertaken using both (A-C) HEK293 cells and (D-F) SH-SY5Y cells followed by luminescence assays (section 2.6.2) to determine the regulatory potential of the constructs representing the pain protective haplotype (rs10483639, rs3783641 and rs8007267). This was also repeated (G) using HEK293 cells with the constructs pertaining to the *GCH1* 5' SNP (rs8007267) cloned within ~10 kb of the *GCH1* 5' region. Firefly luciferase was normalised to that of *Renilla*, either encoded by (A-F) pRL-TK or (G) pRL-SV40, followed by representation of data as a percentage of control (pGL4.20 or pGL4.26). Assays were conducted in triplicate on three separate occasions. Statistical analysis was undertaken (unpaired t-test). n.s: not statistically significant. * denotes $p = \leq 0.05$. ** denotes $p = \leq 0.01$. Raw data is available in the electronic supplementary material within the folder entitled 'Luciferase' and has a file name of 'Luciferase data'.

Further analysis was then carried out with HUVECs, in the absence and presence of INF-γ and TNF-α (Figure 3.15). The pGL4.26-GCH1 constructs pertaining to the 3' SNP (rs10483639) showed significantly lower relative luminescence with the variant construct regardless of INF-γ and TNF-α. A general increase in relative luminescence was observed in both wild-type and variant constructs in the presence INF-γ and TNF-α. In contrast, relative luminescence was reduced with the wild-type construct representing the intronic SNP (rs3783641), though statistical significance was only observed in the presence of INF-γ and TNF-α. The variant constructs pertaining to the 5' SNP (rs8008267) with the pGL4.26 backbone showed consistently reduced relative luminescence regardless of INF-γ and TNF-α, although the presence of cytokines notably reduced overall relative luminescence. Latterly, the wild-type and variant pGL4.20-GCH1-10kb constructs showed no difference under normal conditions, yet a significant reduction in relative luminescence was observed with the variant construct in the presence of INF-γ and TNF-α (Figure 3.15).

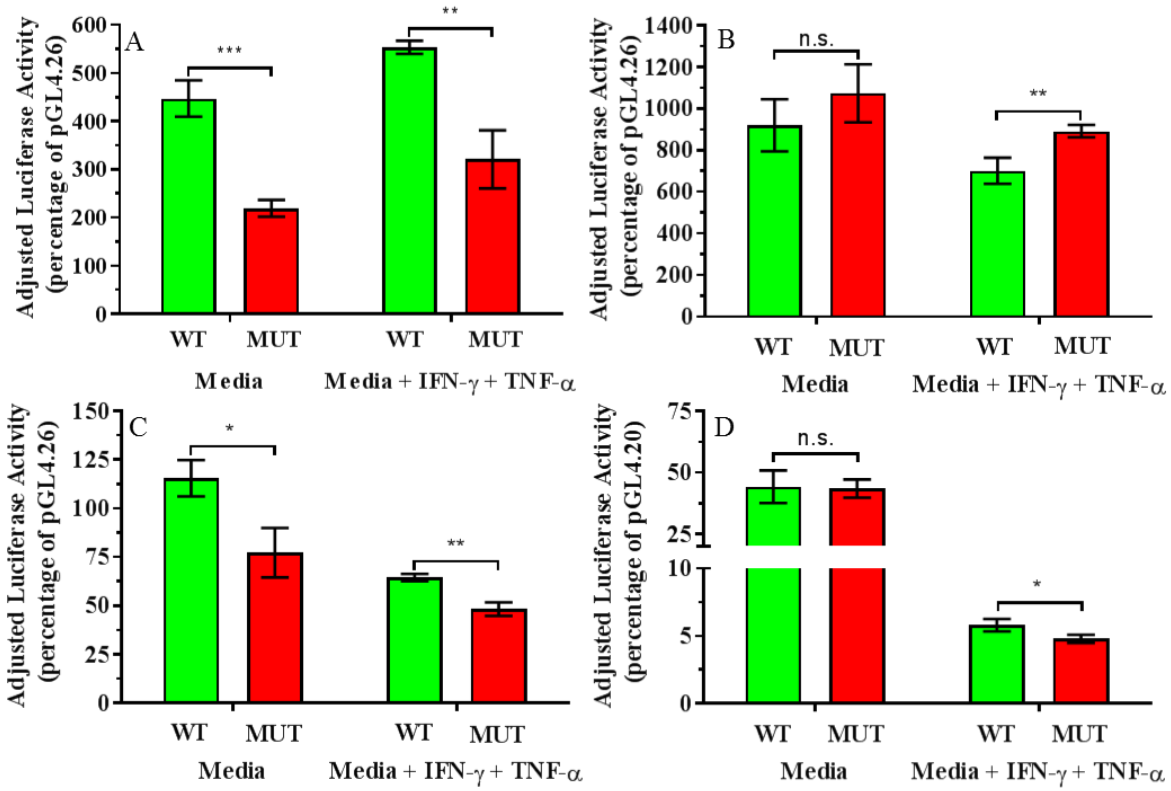

Figure 3.15: Luciferase assays assessing the potential regulatory role of the pain protective haplotype on *GCH1* expression

Transfections were undertaken using HUVECs (section 2.6.4) under cytokine stimulation (40 ng/mL TNF-α and 100 ng/mL IFN-γ) followed by luciferase assays (section 2.6.5) to determine the regulatory potential of the constructs representing the pain protective haplotype; (A) rs10483639, (B) rs3783641 and (C) rs8007267. This was also repeated (D) with the constructs pertaining to the *GCH1* 5' SNP (rs8007267) cloned within ~10 kb of the *GCH1* 5' region. Firefly luciferase was normalised to that of Renilla (pRL-SV40), followed by representation of data as a percentage of control (pGL4.20 or pGL4.26). Assays were conducted in triplicate on three separate occasions. Statistical analysis was undertaken (unpaired t-test). n.s: not statistically significant. * denotes p = ≤0.05. ** denotes p = ≤0.01. *** denotes p = ≤0.001. Raw data is available in the electronic supplementary material within the folder entitled 'Luciferase' and has a file name of 'Luciferase data'.

3.3.7.4 Luciferase reporter assays and *GCH1* regulation

Initially the pGL4.20-GCH1-3.4kb promoter construct was transfected into HUVECs in the absence and presence of INF-γ and TNF-α. A highly significant reduction in relative luminescence was observed in the presence of INF-γ and TNF-α. This construct then formed the vector backbone for multiple segments of *GCH1* intron 1 (Figure 3.17). A total of nine vector constructs were then analysed (Figure 3.16). There was a clear trend towards reduced relative luminescence in the presence of INF-γ and TNF-α in all permutations, apart from pGL4.20-GCH1-3.4kb-Int.1A wherein a slight non-significant trend towards increased relative luminescence was observed in the presence of INF-γ and TNF-α. All other constructs, apart from those labelled Int.1B, Int.1C and Int.1D, showed a statistically significant reduction in relative luminescence in the presence of INF-γ and TNF-α.

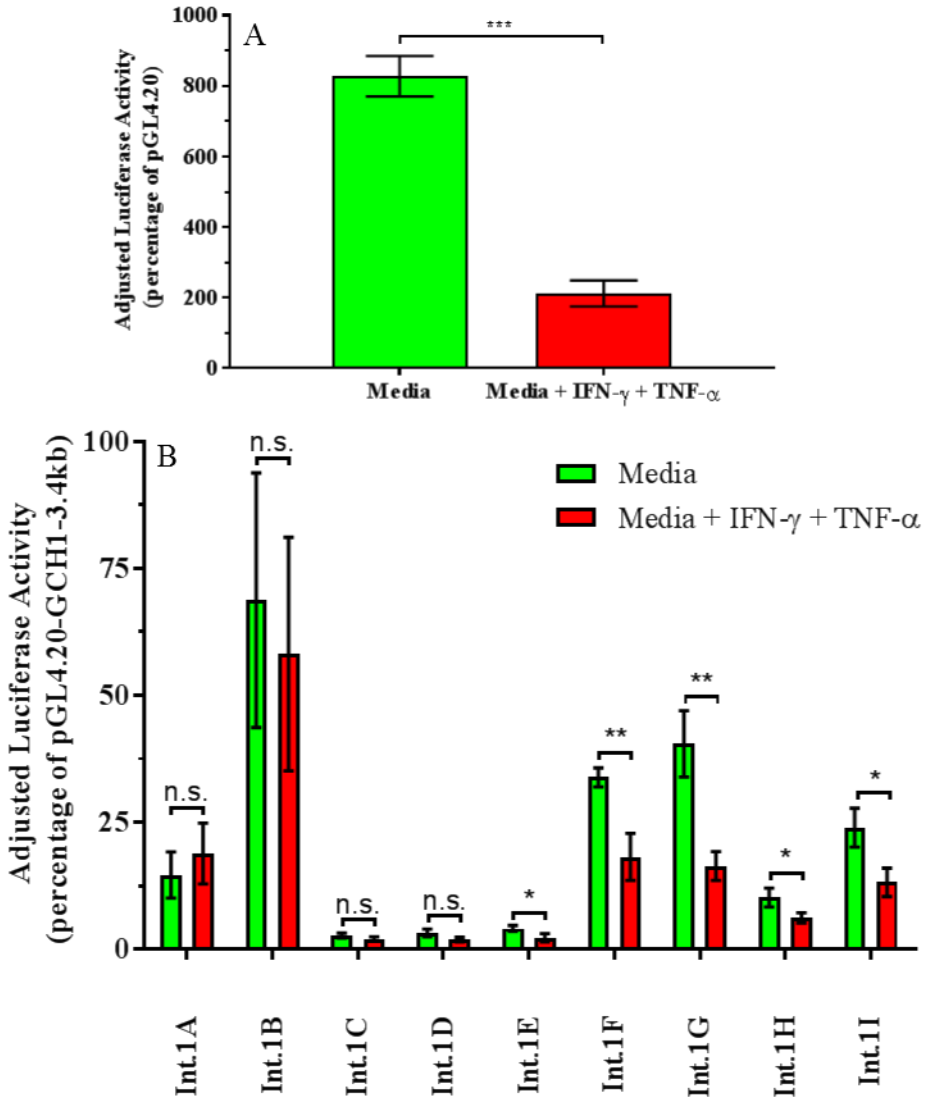

Figure 3.16: Luciferase assays for the assessment of immune regulation of *GCH1* via regulatory elements in the promoter and intronic regions

Transfections were undertaken using HUVECs (section 2.6.4) followed by luciferase assays (section 2.6.5) to determine the influence of cytokines (40 ng/mL TNF-α and 100 ng/mL IFN-γ) on the firefly luciferase expression with cloned regions of the (A) *GCH1* promoter and (B) *GCH1* first intron. Firefly luciferase was normalised to that of *Renilla* (pRL-SV40), followed by representation of data as a percentage of control (pGL4.20 or pGL4.20-GCH1-3.4kb). Assays were conducted in triplicate on three separate occasions. Statistical analysis was undertaken (unpaired t-test). n.s: not statistically significant. * denotes $p = \leq 0.05$. ** denotes $p = \leq 0.01$. *** denotes $p = \leq 0.001$. Raw data is available in the electronic supplementary material within the folder entitled 'Luciferase' and has a file name of 'Luciferase data'.

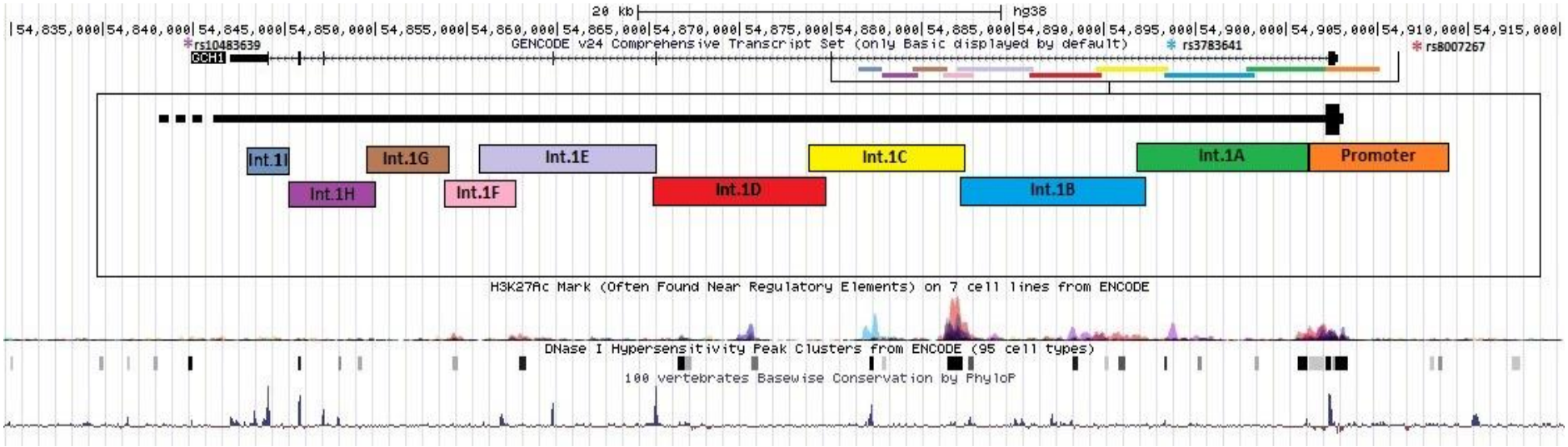

Figure 3.17: Diagrammatic representation of the *GCH1* intronic cloning process used in preparation for luciferase reporter assays

A modified caption from the UCSC genome browser (https://genome.ucsc.edu/). *GCH1* is represented in a 3'-5' orientation. The polymorphisms of the pain protective haplotype are represented by asterisks located directly above the *GCH1* transcript, as represented within the genome browser. The narrow coloured lines directly beneath the *GCH1* transcript are representative of the PCRs undertaken to derive the different luciferase constructs and are located in order to encompass a large proportion of the first intron with consideration for regions of prominent DNase I hypersensitivity and histone acetylation. These coloured lines are proportional to the PCR amplicon size and are also annotated and represented on a larger scale within the centre of the figure.

3.3.8 Transcription factor binding site analysis

Analysis of predicted changes in transcription factor binding based upon wild-type or variant alleles associated with the pain protective haplotype highlighted multiple potential changes within MatInspector (Table 3.6). A change was observed in relation to the *GCH1* 3' SNP (rs10483639) variant allele wherein a PAX-3 binding site is created which meets the required core and matrix similarity with results of 1.0 and 0.77, respectively. An SPI-1 proto-oncogene binding site is also predicted with the wild-type allele only, with relatively strong core and matrix similarities of 1.00 and 0.992, respectively. Similarly, multiple potential changes were observed with regards to the *GCH1* intronic SNP (rs3783641). A Tax/CREB complex binding site is present only with the wild-type allele, scoring a high core similarity (1.00), yet modest matrix similarity (0.71). Moreover, a nuclear factor 1 binding site showed high core similarity (1.00) and matrix similarity (0.97) with the wild-type allele, but did not reach thresholds with the variant allele. A slight decrease in matrix similarity, but not core similarity, was predicted for a PTF1 binding site with the variant allele. A near identical variation was also observed with a slight reduction in SRY box 9 matrix similarity, but not core similarity, when compared to wild-type. A similar reduction in matrix similarity, but not core similarity, was observed for Zinc finger protein Gfi-1, with reduced binding affinity predicted with the wild-type allele. Latterly, changes relating to the *GCH1* 5' SNP (rs8007267) were also observed, including a small reduction in RB/E2F-1/DP-1 heterotrimeric complex matrix similarity with the variant allele, though no difference in core similarity was predicted. Binding of aryl hydrocarbon receptor/ARNT heterodimers was strongly predicted with the wild-type sequence with high core similarity (1.00) and matrix similarity (0.93), whilst the variant allele failed to meet predetermined thresholds.

Table 3.6: MatInspector analysis highlighting differences in transcription factor binding predictions for the three SNPs in the pain protective haplotype

Matrix Information	Core sim.	Matrix sim.	Sequence
rs10483639 major allele (C)			
Pax-3 paired domain protein, expressed in embryogenesis, mutations correlate to Waardenburg Syndrome	1.00	0.77	t**TCGT**ctcaggctattgat
rs3783641 major allele (A)			
Tax/CREB complex	1.00	0.71	cccacc**TGAC**tcatttgccag
PTF1 binding sites are bipartite with an E-box and a TC-box (RBP-J/L) spaced one helical turn apart	1.00	0.86	ccca**CCTG**actcatttgccag
Non-palindromic nuclear factor I binding sites	1.00	0.97	acctgactcattt**GCCA**gtga
SRY (sex-determining region Y) box 9, dimeric binding sites	0.75	0.75	a**CTCA**tttgcctgtgatttctat
rs3783641 minor allele (T)			
PTF1 binding sites are bipartite with an E-box and a TC-box (RBP-J/L) spaced one helical turn apart	1.00	0.82	ccca**CCTG**actcatttgccag
SRY (sex-determining region Y) box 9, dimeric binding sites	0.75	0.69	a**CTCA**tttgcctgtgatttctat
rs8007267 major allele (G)			
Aryl hydrocarbon receptor / Arnt heterodimers	1.00	0.93	ctgaagtttgg**CGTG**tactgttcaa
RB/E2F-1/DP-1 heterotrimeric complex	0.77	0.75	gtttg**GCGT**gtactgtt
rs8007267 minor allele (A)			
RB/E2F-1/DP-1 heterotrimeric complex	0.77	0.72	gtttg**GCGT**gtactgtt

The information presented within this table represents the predicted differences in transcription factor binding between the major and minor allelles of the SNPs within the *GCH1* pain protective haplotype. In cases where a transcription factor is only presented once for a specific SNP, this indicates that the minimum threshold for transcription factor binding was not met for the other allele. For instance, AhR/ARNT binding is strongly predicted for the major allele of rs8007267 but fails to meet the pre-determined cut-off (Core sim: 0.75) and is therefore not displayed. The locus of the SNP in question is denoted by highlighted text. The Core similarity (Core sim) is an indicator of similarity between the input DNA sequence and the bases within the ideal binding motif with the highest degree of conservation (indicated in bold). The Matrix similarity (Matrix sim) is an indicator of similarity between the input DNA sequence and the entire predicted binding motif (sequence).

3.3.9 Electrophoretic mobility shift assays and related qRT-PCR

3.3.9.1 Effect of AhR modulation and hypoxia on *GCH1* expression

Prior to completion of electrophoretic mobility shift assays (EMSAs), qRT-PCR analysis was undertaken in order to clarify whether modulation of AhR or the use of a hypoxia mimetic resulted in changes in *GCH1* expression (Figure 3.18). Matinspector analysis (section 2.8.4) highlighted that the aryl hydrocarbon receptor (AhR) may interact with the locus pertaining the *GCH1* 5' SNP (rs8007267). The effect of hypoxia was considered as both AhR and HIF-1α share the same binding partner (ARNT). It was demonstrated that MeBio had no distinguishable effect on *GCH1* expression, with apparent decreases in MeBio (exogenous AhR agonist) at higher concentrations, a probable consequence of cell death due to toxicity and/or visable precipitation of MeBio. A more pertinent observation was made with the AhR antagonist (CH-223191), wherein a significant increase in *GCH1* expression was observed in contrast to a significant decrease in *CYP1A1* expression. Meanwhile all concentrations of cobalt chloride resulted in upregulation of both *GCH1* and *VEGFA*.

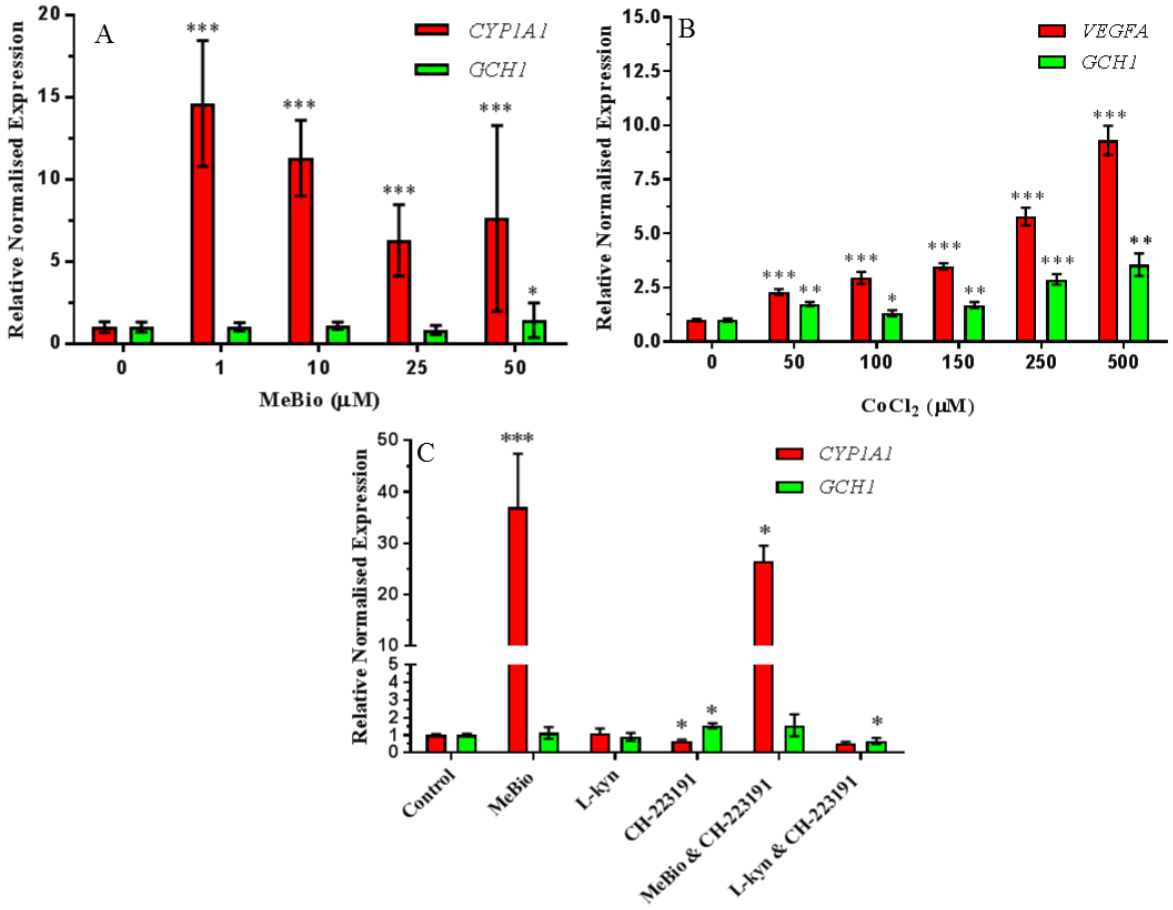

Figure 3.18: Effect of cobalt chloride and AhR modulation on *GCH1* expression

Analysis was undertaken to determine whether a hypoxia mimetic (cobalt chloride), exogenous AhR agonist (MeBio), endogenous AhR agonist (L-kynurenine) and AhR antagonist (CH-223191) influenced transcriptional regulation of *GCH1*. HEK293 cells were subject to different concentrations of (A) MeBio and (B) cobalt chloride for 24 hours. Multiple permutations were then considered (C) including MeBio (1 µM), L-kynurenine (50 µM) and CH-223191 (10 µM). Gene expression data was normalised to that of *CYC1* and is represented as relative to control (vehicle only). Positive controls were included to verify the effect of the compound on an established transcriptional target (*CYP1A1* and *VEGFA*). * denotes $p = \leq 0.05$. ** denotes $p = \leq 0.01$. *** denotes $p = \leq 0.001$ (±SEM).

3.3.9.2 Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) consistently showed specific protein-DNA binding of greater intensity with the probe representing the major allele of the *GCH1* 5' SNP (rs8007267) (Figure 3.19). Multiple permutations were considered, including the use of nuclear protein derived from HEK293 cells exposed to cobalt chloride, with or without HIF-1α antibody (Figure 3.19), and the use of the AhR agonist MeBio (Figure 3.20).

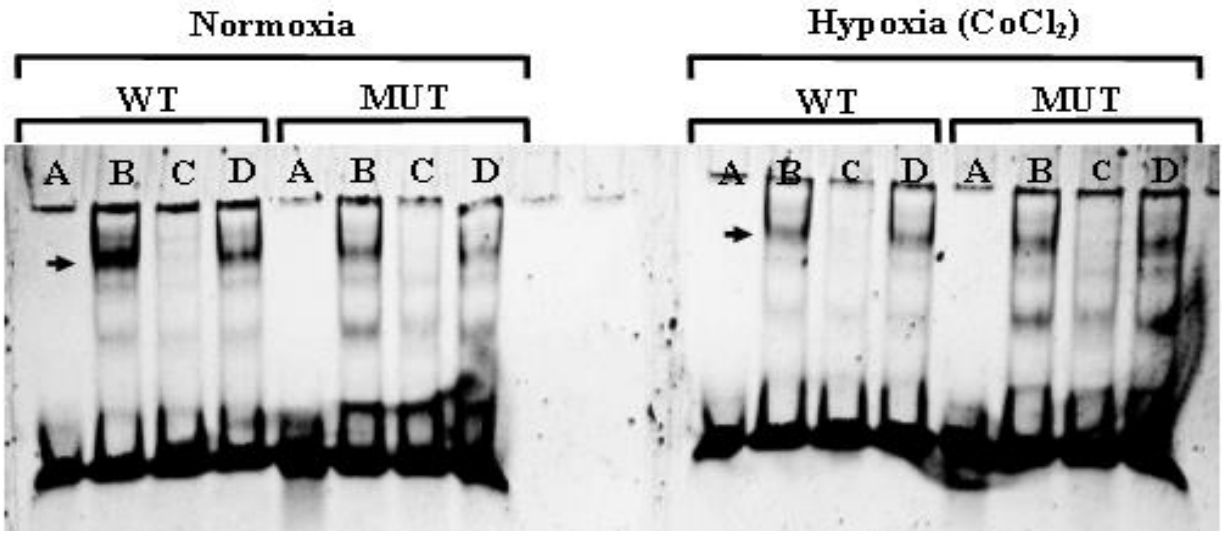

Figure 3.19: Effect of a hypoxia mimetic on protein-DNA binding by EMSA

The EMSA was performed as described (section 2.7). Four binding reactions were used for each parameter (A-D). In addition to the basic reaction components (section 2.7.6), the following was added; (A-D) 2 µL of 50 nM Cy5 labelled probe, (B-D) nuclear protein extract from HEK293 cells (subject to media with or without 250 µM cobalt chloride), (C) 1 µL of 10 µM excess unlabelled competitor probe and (D) 2.5 µL (500 ng) of HIF-1α antibody. Electrophoresis and detection was performed as described (section 2.7.6). Arrows highlight potential areas of specific protein-DNA binding which appear to contrast between the presented permutations.

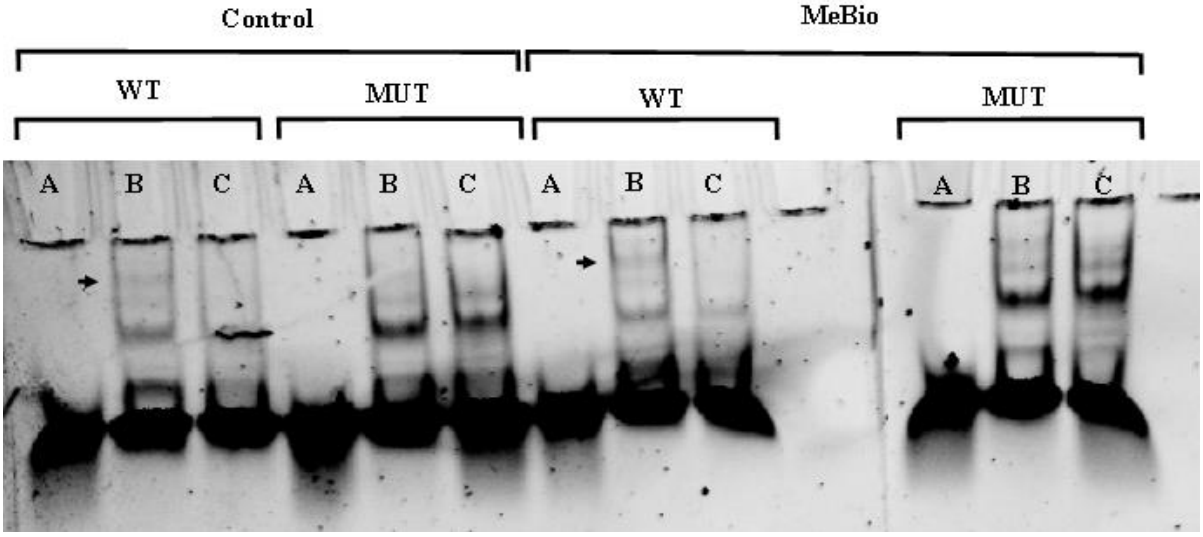

Figure 3.20: Effect of an AhR agonist on protein-DNA binding by EMSA

The EMSA was performed as described (section 2.7). Three binding reactions were used for each parameter (A-C). In addition to the basic reaction components (section 2.7.6), the following was added; (A-C) 2 µL of 50 nM Cy5 labelled probe, (B-C) nuclear protein extract from HEK293 cells (subject to media with or without 1 µM MeBio) and (C) 1 µL of 10 µM excess unlabelled competitor probe. Electrophoresis and detection was performed as described (section 2.7.6). Arrows highlight potential areas of specific protein-DNA binding which appear to contrast between the presented permutations.

3.4 Discussion

3.4.1 Nitric oxide

The role of nitric oxide in pain is diverse, with multiple reputed implications of elevated nitric oxide at various sites within the nervous system. Evidence exists supporting a role for nitric oxide both contributing towards and prevention inflammation and related inflammatory pain (Durate *et al.* 1990, Duarte *et al.* 1992, Paul-Clark *et al.* 2001), through a wide range of suggested mechanisms on primary afferent neurons (Levy *et al.* 2004). Animal models have demonstrated that nerve injury results in localised elevations in nerve blood flow which were subject to reduction by broad spectrum NOS inhibition, but not selective inhibition of nNOS and iNOS, thereby implicating eNOS, which has been identified as expressed proximal to the site of CCI, although evidence suggests this is short-lived (Ialenti *et al.* 1992, Levy *et al.* 2004). However, during Wallarian-like degeneration, both Schwann cells and migrating macrophages contribute significantly to nitric oxide production through iNOS upregulation which is likely to contribute to a degree of persistence in elevated nitric oxide (Levy *et al.* 2004). Upregulation of nNOS has also been observed in the L4-6 DRG after peripheral nerve injury (and to a lesser degree with peripheral inflammation) and has been shown to localise to interneurons within the DH (Verge *et al.* 1992, Terenghi *et al.* 1993, Vizzard *et al.* 1995, Lam *et al.* 1996, Levy *et al.* 2004). Indeed, reduced spinal inhibition evoked through the nitric oxide/cGMP pathway, which leads to PKC-mediation phosphorylation of membrane proteins, has been suggested as a contributor towards central sensitisation (Lin *et al.* 1999, Levy *et al.* 2004).

Analysis of plasma nitrate, after reduction of nitrite, highlighted a trend towards statistical significance in the discovery cohort. However, this was not replicated in the discovery/validation group, nor were significant differences in plasma nitrate observed when categorising participants by the S-LANSS score. The potential of plasma nitrite/nitrate as a marker of CNP is somewhat underpinned by disruption of the blood-nerve-barrier (BNB), and subsequent macrophage infiltration, which, as described, is associated with nerve injury during Wallerian degeneration (Fregnan *et al.* 2012, Lim *et al.* 2014). Upregulation of iNOS in macrophages and Schwann cells has been observed after CCI leading to localised elevations of nitric oxide, increasing nerve blood flow and contributing to the localised inflammatory response (Levy *et al.* 1999, Naik *et al.* 2006). Therefore, should the increased BNB permeability permit dissemination of activated macrophages

or inflammatory mediators, it is perhaps feasible that an increase in plasma nitrate may reflect ongoing neuropathic pain. Indeed, it has been shown that neuroimmune stimulation and nitric oxide can alter BBB permeability (Thiel *et al.* 2001) with transmembrane secretion and crossing of cytokines in both blood-brain and brain-blood directions (Banks 2009). Moreover, brain microvascular endothelial cells may produce and secrete prostaglandins, nitric oxide and cytokines (Banks 2009). For instance, it has been shown that exposure of the adluminal surface membrane of BBB endothelial cells to LPS resulted in a drastic increase in IL-6 release from the luminal surface membrane (Verma *et al.* 2006), which lends support to the hypothesis that localised neuroimmune interactions may lead to systemic dissemination through diffusion and interactions via microvascular endothelial barriers. Indeed, a reduction in plasma nitrate has previous been used as an indicator of clinical improvement in distinct localised inflammatory changes (Rocha *et al.* 2015).

A plethora of studies utilising animal models have highlighted that nerve injury induces a localised increase in nitric oxide through upregulation of NOS isoform(s). Upregulation of *Nos2*, elevated NOS catalytic activity and increased nitrite/nitrate have all been observed in the DRG or sciatic nerve tissue after nerve injury (Cizkova *et al.* 2002, Naik *et al.* 2006, Tegeder *et al.* 2006), though none of the models used in these studies surpassed 21 days post-surgery. Therefore, observations of elevated nitrite/nitrate may reflect acute changes associated inflammation and the development of neuropathic pain, rather than reflecting the chronicity of neuropathic pain. Interestingly, the study by Naik *et al* also sought to determine changes in serum nitrite/nitrate after CCI, but found no variation when comparing sham and CCI rats (Naik *et al.* 2006).

Whilst BH_4 is a necessary cofactor for oxidation of L-arginine to L-citrulline (Tayeh *et al.* 1989), it is noteworthy to consider whether elevated nitrite/nitrate is a consequence of GTPCH and/or NOS upregulation, particularly as GTPCH has been regarded as the rate limiting component in nitric oxide synthesis (Golderer *et al.* 2001). Indeed, both GTPCH and iNOS may be co-induced (Galley *et al.* 2001). The utilisation of hph-1 mice, which exhibit a relative BH_4 deficiency, have shown reduced cerebellar nitric oxide/cGMP pathway function resulting from decreased nitric oxide production (Brand *et al.* 1996), thereby illustrating the consequence of reduced BH_4 levels on NOS activity. However, contrasting conclusions have been drawn with regards to the influence of excess BH_4 concentration on NOS catalytic activity. Whilst the addition of BH_4 has been shown to augment IL-1β-induced nitric oxide production in rat glomerular mesangial cells (Muhl *et al.* 1994), Shimizu *et al* used rat aorta and demonstrated that LPS-induced the expression of *Gch1*

(and subsequently BH_4) and *Nos2*, resulting in L-arginine-induced tissue relaxation. Although a high concentration DAHP diminished BH_4 and prevented relaxation, normalisation of elevated BH_4 by DAHP did not attenuate relaxation of rat aorta, indicating that additional BH_4 was not required for L-arginine-induced relaxation (Shimizu *et al.* 1999). However, considering clear physiological variation, it is unclear whether this system is of relevance to neuropathic pain as it does not account for both elevated NOS expression and nitric oxide production (Steel *et al.* 1994, Cizkova *et al.* 2002) and the efficacy of DAHP and NOS inhibitors after nerve injury (Tegeder *et al.* 2006, Annedi *et al.* 2011, Annedi *et al.* 2012). Clearly changes in nitrate within both cohorts were not pronounced, though correlations analysis (section 3.3.5) suggests that particularly in CNP patients, increased nitric oxide production may be BH_4-dependent.

3.4.2 Neopterin

Neopterin, a marker of immune activation, is produced as a consequence of increased GTPCH activity. Plasma neopterin levels have been shown to be indicative of a plethora of medical conditions. It is unclear whether neuropathic pain may lead to significant increases in circulating neopterin levels, and whether such increases would be consequence of indirect systemic processes or the dissemination of high levels of localised neopterin. This is reaffirmed by the lack of evidence from animal models which are of insufficient duration to demonstrate persistent upregulation of *Gch1* after nerve injury (Tegeder *et al.* 2006).

It is pertinent to consider that unlike HUVECs (Linscheid *et al.* 1998) and certain other non-neuronal cells (Werner *et al.* 1990, Latremoliere *et al.* 2011), cytokine stimulation does not trigger upregulation of PTPS activity in the DRG, resulting in rapid neopterin accumulation. This can lead to potentially toxic neopterin accumulations (Weiss *et al.* 1993) which may contribute to the onset of neuropathic pain (Latremoliere *et al.* 2011). Although there was no clear difference between plasma neopterin when comparing healthy controls and CNP patients in both cohorts, there was a slight trend for elevated neopterin in patients with an S-LANSS of ≥12 in comparison to those scoring <12. Taken alone, this result may suggest that neopterin holds discriminatory value to potentially differentiate CNP from pain which is predominantly non-neuropathic. However, this is perhaps of limited clinical value as plasma neopterin was indistinguishable between healthy controls and those with an S-LANSS of >12. This, in addition to the significant difference observed between plasma neopterin in healthy controls and subjects with an S-LANSS score of <12, suggests that a pathological or clinical commonality may exist between patients in the group scoring <12. Consideration may be given to the potential for pharmacotherapeutic influences on

neopterin levels. Indeed, non-steroidal anti-inflammatory drugs have been shown to inhibit upregulation of monocytic genes, such as *TNF*, in response to LPS (Housby *et al.* 1999) whilst tramadol, which is taken by 37.5% of patients scoring <12 when compared to 25% scoring ≥12, has been shown to reduced TNF-α levels in patients with CNP (Kraychete *et al.* 2009).

3.4.3 Biopterin

Analysis of biopterins yielded variable results. There was significantly higher plasma biopterin and BH_4 in healthy controls, than in CNP patients. No significant difference was observed with BH_2 levels. As such, total biopterin strongly trended towards higher overall levels in healthy controls. Considering the relatively small sample size, the observed variations in BH_4 may be influenced by underlying diseases or confounding environmental factors. For instance, certain diseases, such as diabetes and atherosclerosis, are associated with increased oxidative stress (Channon 2004), thereby promoting oxidation of BH_4 to BH_2. Indeed, both BH_2 and BH_4 have similar affinities for eNOS, although interaction with BH_2 leads to peroxide release rather than NO, thereby exacerbating oxidative stress (Latremoliere *et al.* 2011). As such, it is considered that BH_4/BH_2 ratio, rather than BH_4 alone, is crucial for eNOS function (Crabtree *et al.* 2009a). The BH_4/BH_2 ratio was notably reduced in the CNP group, though whether this is attributable to neuropathic pain is unclear. It is noteworthy that superoxide and peroxynitrite, rather than just nitric oxide, are capable of inducing hyperalgesia after SNL and contribute to neuropathic pain (Kim *et al.* 2009c, Janes *et al.* 2012).

In addition, an apparent trend towards downregulation of *QDPR* in CNP patients may further lead to elevations in oxidative stress by permitting BH_2 accumulation, as occurs in hypertensive patients (Lee *et al.* 2009), although animal models have demonstrated contrasting data, illustrating transient upregulation of *Qdpr* after nerve injury, albeit in the DRG (Tegeder *et al.* 2006).

3.4.4 Correlaton analysis

Taken alone, relatively little variation was observed between plasma neopterin, nitrite/nitrate and *GCH1* expression. However, a range of positive correlations were observed throughout both cohorts. In the discovery cohort, nitrate and neopterin were positively correlated, which was notably stronger in CNP patients than healthy controls. Similar outcomes were observed between nitrite/nitrate and *GCH1*, and neopterin and *GCH1*, in the discovery/validation cohort. In both instances, CNP patients displayed stronger positive correlations than healthy controls and within the CNP group, correlations were notably stronger in those with an S-LANSS score indicative of neuropathic pain. The rationale for such observations may be underpinned by tight regulation of

GCH1 expression in the absence of stimulatory factors, in order to maintain basal BH_4 levels (Latremoliere *et al.* 2011). Such regulation of *GCH1* coupled with a wide normal variation in nitrite/nitrate due to genotype, diet or comorbidities (Wang *et al.* 1997, Ersoy *et al.* 2002, Shiekh *et al.* 2011), may lead to a lack of discernible correlation. However, changes in *GCH1* transcription coupled with subsequent changes in neopterin or nitrite/nitrate may ameliorate the overall contributions of basal variation, leading to comparatively remarkable correlations.

3.4.5 *In silico* analysis

In order to assess the potential functional role of the SNPs within the pain protective haplotype, the luciferase reporter assay was used to determine changes in relative luminescence. Analysis of relative luminescence obtained after transfection into the HEK293 cells, a relatively simple-to-transfect embryological kidney cell line, and SH-SY5Y, a neuroblastoma cell line, showed a significant difference in relative luminescence for the vector constructs relating to the *GCH1* 5' SNP (rs8007267). In both instances there was a significant decrease in relative luminescence relating to the mutated construct, reflecting the minor allele. There was no significant difference pertaining to the intronic SNP (rs3783641) or the 3' SNP (rs10483639). Cloning and mutagenesis was subsequently repeated for the *GCH1* 5' region, spanning 10.1 kb. Transfection of vector constructs using HEK293 cells resulting in a near-identical outcome. Further analysis in cytokine stimulated HUVECs demonstrated a clear reduction in relative luminescence pertaining to the construct representing the 3' SNP (rs10483639), although this occurred at a similar magnitude regardless of cytokine exposure. Further analysis with the constructs representing the *GCH1* 5' SNP indicated similar observations to those made in HEK293 and SH-SY5Y cells, although a significant difference was only observed with cytokines in the mutated construct harbouring 10.1 kb of the GCH1 5' region. It was therefore deduced that the SNP may function to reduce the transcription of *GCH1*. Changes in transcription factor binding were subsequently assessed using the MatInspector tool.

3.4.5.1 rs10483639

A PAX3 binding site was predicted with the minor allele of the *GCH1* 3' SNP. Studies have highlighted the role of *Pax3* in early neurogenesis (Goulding *et al.* 1991) and differentiation of peripheral neurons (Koblar *et al.* 1999). Differential regulation of *Pax3* has been observed in Schwann cells at the distal nerve stump during regeneration, though *Pax3* was not induced in the DRG after nerve crush injury (Vogelaar *et al.* 2004). The precise mechanism for changes in relative luminescence when transfecting HUVECs, rather than HEK293 or SH-SY5Y cells, and the role of

PAX3, remains unclear, particularly as cytokine stimulation had little impact upon the pattern of relative luminescence between constructs. Further analysis would be necessary to elucidate whether PAX3 binding occurs, and the potential impact of this SNP on *GCH1* regulation.

3.4.5.2 rs3783641

Nuclear factor 1 (NF1) is ubiquitously expressed and consists of four subtypes of transcription factors which share the same DNA binding motif (Gaussin *et al.* 2012). NF1 promotes transcription and DNA replication (Mermod *et al.* 1989) and prevents gene silencing through interactions with chromatin structures (Gaussin *et al.* 2012). The multiple roles of NF1 have been extensively reviewed, including those related to spinal cord development (Mason *et al.* 2009). Current data does not infer a role for this SNP in *GCH1* regulation, although further analysis may be prudent to determine whether CREB binding occurs in the absence of Tax (a viral oncoprotein), which are predicted binding partners with the major allele by MatInspector. Indeed, a plethora of evidence exists for a prominent role of CREB in modulating *GCH1* expression, including through nitric oxide and estradiol, leading to elevated BH_4 (Kumar *et al.* 2009, Sun *et al.* 2009), and with regards to animal models of neuropathic pain (Ma *et al.* 2001, Song *et al.* 2005).

3.4.5.3 rs8007267

A clear change in transcription factor binding was predicted. The DNA sequence consisting of the major allele for the 5' SNP highlighted an aryl hydrocarbon receptor (AhR)/ARNT heterodimer binding site with relatively high matrix similarity. In contrast, MatInspector analysis of the minor allele failed to predict AhR/ARNT binding due to a nucleotide change within the core sequence (Figure 3.21).

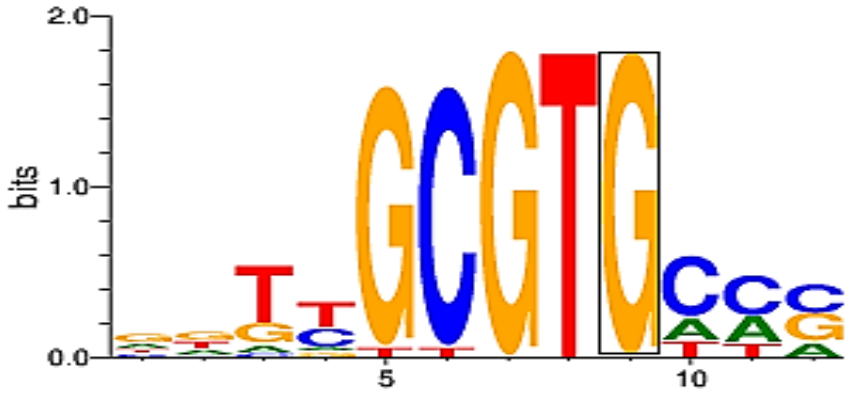

Figure 3.21: DNA binding motif illustrating the relative requirement of each nucleotide for AhR/ARNT binding

The DNA binding motif for AhR/ARNT illustrating the flanking and core (5' GCGTG) regions. Binding of ARNT occurs with the 3' half-site (5' GTG) whilst the AhR binds to the 5' half-site (5' T(C/T)GC). This is similar to the core binding sequence for HIF-1α (5' RCGTG). The nucleotide representing the investigated SNP is highlighted. Images were obtained from the MatInspector database.

The AhR has generally been associated with mediating the response to xenobiotics and is known to induce transcriptional changes after binding to the potent carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Fernandez-Salguero *et al.* 1996). The dioxin response element (DRE), xenobiotic responsive element (XRE)/AhRE (aryl hydrocarbon responsive element) are used interchangeably to describe DNA elements under the influence of the AhR (Beischlag *et al.* 2008). Deletion analysis of the *CYP1A1* promoter elucidated an AhR/dioxin enhancer region, termed the dioxin responsive element (Durrin *et al.* 1987).

Ligand binding to AhR, which requires HSP90 (Whitelaw *et al.* 1995), leads to nuclear translocation (Carlstedt-Duke *et al.* 1981). It has been suggested that phosphorylation of HSP90 may influence the transcription activity of the AhR (Ogiso *et al.* 2004). The core XRE was initially identified as 5′-T/GCGT**G**-3′ (emboldened text pertains to site of rs8007267 SNP), although the sequences flanking the core element were also shown to have great influence upon transcription in reporter assay studies (Denison *et al.* 1988, Denison *et al.* 1989). The putative XRE was subsequently expanded to incorporate flanking nucleotides (5′-TTGCGT**G**AGAA-3′) (Bacsi *et al.* 1995). Importantly, the binding of AhR to aryl hydrocarbon receptor nuclear translocator (ARNT; HIF-1β) precedes interaction with DNA, which may result in the recruitment of a multitude of

protein complexes, including co-activators and complexes relating to chromatin remodelling (Beischlag *et al.* 2008). It has been demonstrated that ARNT, which interacts with the 5′-G**T**G-3′ site within the core sequence (Swanson *et al.* 1995), also functions as a dimerisation partner for hypoxia inducible factors and single-minded 1 (SIM1) (Woods *et al.* 2002).

There are documented and hypothesised interactions between the AhR, which is widely expressed in the CNS (Cuartero *et al.* 2014), and inflammatory processes. Indeed, inflammation has been shown to modify the rate of drug metabolism, at least in part by modulation of CYP450 expression (Morgan 1997). Indeed, localisation and over-lapping of elements pertaining to C/EBPα, which has also been identified as a key enahancer for the regulation of *GCH1* (Liang *et al.* 2013), and AhR in the glutathione S-transferase Ya promoter (Pimental *et al.* 1993). Interactions are also suggested to occur between the AhR complex and C/EBPα in the *CYP1A1* promoter (Shin *et al.* 2005). A multitude of hypotheses have been reviewed which suggest that interactions occur between the AhR ligand TCDD, AhR/ARNT and inflammatory cytokines (Beischlag *et al.* 2008), including suggestions of convergence between inflammatory and AhR signalling pathways (Tian *et al.* 2002). Indeed, binding of AhR/ARNT to elements in the promoters of both IL-1β and IL-6 have been described (Lahoti *et al.* 2014).

Of particular interest is the potential for BH_4-independent feedback processes, thereby modulating BH_4 synthesis. Indoleamine 2,3-dioxygenase 1 (IDO1) functions as the rate-limiting enzyme in the degradation of tryptophan along the kynurenine pathway. IDO1 is upregulated by proinflammatory cytokines and exerts an immunosuppressive effect through kynurenic acid, thereby regulating the immune response (Mandi *et al.* 2012). Upregulation of IDO1 results in elevations in tryptophan metabolites, including L-kynurenine and xanthurenic acid (Haruki *et al.* 2015). Although a diverse range of environmental AhR 'activators' have been summarised (Beischlag *et al.* 2008), identification of endogenous AhR ligands has, until recently, proven somewhat elusive with suggestions that the AhR is capable of modulating transcriptional activity in the absence of ligand binding (Murray *et al.* 2005). However, the tryptophan metabolite, L-kynurenine, has been identified as an endogenous AhR ligand (Bessede *et al.* 2014) whilst another kynurenine pathway metabolite, xanthurenic acid, functions as a potent SPR inhibitor, thereby potentially bridging two pathways upregulated by inflammatory processes (Haruki *et al.* 2015). Moreover, L-kynurenine promotes the transcription of IL-6 through AhR/ARNT signalling, which leads to autocrine activation of IDO1, thereby representing a mechanism of maintenance of increased expression (Litzenburger *et al.* 2014, Wirthgen *et al.* 2015). It may therefore be

hypothesised that cytokine-induced upregulation of IDO1, which results in increased L-kynurenine levels, leads to activation and translocation of AhR. DNA interactions with the AhR/ARNT complex at the locus pertaining to the *GCH1* 5' SNP may then result in increased *GCH1* transcription, with variable magnitudes of upregulation in accordance to the genotype and the degree of IDO1 upregulation.

Given the plausible relationship between *GCH1* expression and the AhR/L-kynurenine, it is noteworthy that the observations made in this thesis are not necessarily supportive of this hypothesis, though they can not be discounted. In addition to the lack of variation observed with the EMSA, both MeBio and L-kynurenine failed to notably upregulate *GCH1*. However, of potential relevance, the AhR antagonist, CH-223191, downregulated *CYP1A1* expression and upregulated *GCH1* expression. Such observations suggest that the AhR antagonist may either increase unbound ARNT levels for binding with the appropriate ligand (e.g. HIF-1α, HIF-1β, SIM1 or SIM2) which in-turn regulates *GCH1* transcription, or alternatively, may prevent AhR/ARNT binding and increase the potential for the binding of an alternative transcription factor. In contrast, cobalt chloride upregulated both *VEGFA* and *GCH1*, although the EMSA utilising nuclear protein isolated from cells subject to normoxia and hypoxia illustrated strong specific binding with the probe representing the major allele when incubated with nuclear extract originating from normoxic conditions. This may suggest that hypoxic conditions reduced DNA-protein binding by depletion of unbound ARNT through cobalt chloride-induced stabailisation of complexation with HIF-1α, or that hypoxia may downregulate the protein implicated in DNA binding with the probe. Clearly, the qRT-PCR provides insight into whether *GCH1* may be regulated by the factors uncovered by MatInspector analysis, but does not elucidate whether such regulation is mediated through the given polymorphic locus (rs8007267). In contrast, the EMSA elucidated strongly specific and selective DNA-protein binding with the probe representing the major allele. This was seemingly reduced by the addition of HIF-1α antibody, although this is likely to be a consequence of changes to the composition of the binding reaction as a distinct shift was not observed. Further work towards the identification of the implicated transcription factor will undoubtedly aid in deciphering whether this SNP is implicated in *GCH1* transcription, whether the pathophysiological changes associated with CNP are likely to implicate transcription factor expression or activity and latterly, whether modulation of this transcription factor may facilitate 'mimicking' of the pain protective haplotype.

3.4.6 Regulation of tetrahydrobiopterin synthesis

Tetrahydrobiopterin synthesis is tightly regulated and differential expression of genes associated with BH_4 *de novo* synthesis and regeneration have been widely documented in animal models of neuropathic pain, including *Gch1, Dhrf, Spr and Qdpr* (Tegeder *et al.* 2006, Latremoliere *et al.* 2015b). Upregulation of the rate-limiting enzyme in *de novo* BH_4 synthesis, GTPCH, has consistently been observed in various experimental systems. A variety of studies have shown that upregulation occurs in the presence TNF-α (Milstien *et al.* 1993), IFN-γ, LPS (Kaneko *et al.* 2001), IL-1β (Franscini *et al.* 2003) and nerve growth factor (Hirayama *et al.* 1995). Both nitric oxide (Sun *et al.* 2009) and oestrogens (Serova *et al.* 2004) are thought to positively regulate *GCH1* through the cAMP/CREB pathway (Snider *et al.* 2002, Hannila *et al.* 2008), whilst the phosphatidylinositol-3 kinases (PI3K) pathway, which may contribute to the development of neuropathic pain (Xu *et al.* 2007), is activated by BH_4-induced Ca^{2+} influx via TRPV1/TRPA1 (Zhuang *et al.* 2004, Latremoliere *et al.* 2011) and has been shown to upregulate *GCH1* (Ishii *et al.* 2001). Indeed, stimulation of macrophages with LPS, acting via interactions with LPS binding protein, CD14, TLR4 and MD-2, leads to activation of multiple signalling cascades including p38/MAPK, JNK, MEK and NF-κB (Beutler 2000). There are clearly a multitude of mechanisms by which GTPCH can by differentially regulated, without considering post-transcriptional modifications (Li *et al.* 2016b), post-translational phosphorylation (Hesslinger *et al.* 1998, Lapize *et al.* 1998) and factors, such as melatonin (Jang *et al.* 2000) and leukocyte inhibitory factor (Stegenga *et al.* 1996), which downregulate *GCH1* expression.

Analysis of changes in reporter gene assay expression using the *GCH1* 5' region and first intron was conducted in order to elucidate regulatory elements which may contribute to differential regulation of *GCH1* in the event of nerve injury. Given the multitude of factors involved in modulating the expression of *GCH1,* it may be expected that a range of contrasting regulatory elements exist. Analysis of downstream interactions resulting from exposure of HUVECs with IFN-γ and TNF-α has previously highlighted NF-κB and STAT1/STAT3 as central signalling cascades resulting in *GCH1* upregulation (Huang *et al.* 2005). Furthermore, the transcription factors ATF-2 and NF-Y were shown to upregulate *GCH1*, through interaction with the 146 bp proximal promoter, after exposure of a neuroblastoma cell line to a cAMP analog (Hirayama *et al.* 2001). Similar conclusions were also reached by analysis of the rat *Gch1* 5' flanking region (Kapatos *et al.* 2000). Exploratory analysis of a small proportion of the *GCH1* first intron (~3 kb) has previously been conducted using various methodologies, including reporter assays,

culminating in the description of two highly conserved loci proposed as responsible for *GCH1* upregulation, evidenced by significantly increased relative luminescence in transfected RAW264.7 and HUVECs, exposed to LPS and TNF-α/IFN-γ, respectively (Liang *et al.* 2013). It was demonstrated that, in contrast to previous studies which have described the role of the *GCH1* 5' flanking region (Kapatos *et al.* 2000, Huang *et al.* 2005), no increase in luciferase activity was observed in LPS-treated RAW264.7 cells (Liang *et al.* 2013). Further analysis elucidated Ets and C/EBP binding motifs, within the identified loci, as integral to the effect of stimulation on luciferase reporter activity. Indeed, C/EBP-β is a transcription factor synonymous with regulating gene expression in response to immune activation (Pope *et al.* 1994). It has been documented that C/EBP-β and ATF-2 bind to the *GCH1* promoter (Kapatos *et al.* 2007), leading to suggestions of promoter-enhancer interactions mediated by these transcriptions factors, whilst the Ets-family members, Ets-1 and PU.1, may interact via their respectively binding domains within the *GCH1* intron (McNagny *et al.* 1998), which is particularly pertinent considering the identified Ets and C/EBP binding sites are separated by only ~100 bp (Liang *et al.* 2013).

In order to significantly expand on the scope of the work by Liang *et al* (Liang *et al.* 2013), cloning of 28.6 kb of the *GCH1* promoter and first intron was conducted. The impetus underpinning this approach was to broaden understanding of the regions or elements of potential regulatory function, contributing to differential *GCH1* regulation. Identification of enhancer regions using this approach may also guide the identification of SNPs which result in changes in transcription factor binding. This is pertinent as although data is suggestive that the SNPs within the pain protective haplotype may influence *GCH1* regulation, it is feasible that these SNPs may function as marker for another functional SNP(s) in strong linkage disequilibrium. For instance, the *GCH1* 3' SNP within the pain protective haplotype is in strong linkage with another SNP (rs841), which is situated within 3'UTR of *GCH1* (transcript variant 1) and with the intron of the other transcript variants. This SNP was found to be predictive of several cardiovascular parameters, and was associated with significantly reduced urinary nitrate (Zhang *et al.* 2007). Functional analysis using luciferase reporter assays also demonstrated reduced relative luciferase activity with the construct representing the minor allele (Zhang *et al.* 2007). It has been suggested that this SNP may mediate cytokine-induced alternative splicing (Golderer *et al.* 2001, Zhang *et al.* 2007). Interestingly, identification of potential transcription factor binding sites with this SNP illustrated binding of HIF-1α/ARNT only with the major allele, which again highlights ARNT as a key transcription factor.

Analysis of the pGL4.20-GCH1-3.4kb vector construct highlighted a significant reduction in relative luminescence in transfected cells exposed to cytokines. This was somewhat contrary to previous observations which have shown a lack of differential luminescence after transfection of vector constructs pertaining to the *GCH1* promoter, and subsequent cytokine stimulation (Liang *et al.* 2013). Indeed, cAMP and nerve growth factor, but not IFN-γ/TNF-α (Liang *et al.* 2013), have been shown to interact with the *Gch1* promoter in previous reporter gene assays (Kapatos *et al.* 2000, Hirayama *et al.* 2001). Subsequent analysis of the nine vector constructs consisting of sequential and overlapping segments of the *GCH1* intron showed similar results, with cytokine stimulation seemingly downregulating firefly reporter gene expression. This was particularly evident for constructs pGL4.20-GCH1-3.4kb-Int.1E to –Int1I. Although constructs pGL4.20-GCH1-3.4kb-Int.1B to –Int1.D similarly showed overall reductions in relative luminescence, albeit not meeting conventional statistical significance, it is perhaps noteworthy that the sole construct to buck this trend was pGL4.20-GCH1-3.4kb-Int.1A. Cells transfected with this vector construct and exposed to cytokines demonstrated a non-significant trend towards increased relative luminescence, thereby inferring that this region may be responsible for upregulation of *GCH1* in response to IFN-γ/TNF-α. Of particular note, this construct also contains the putative regulatory regions previously considered to be responsible for *GCH1* upregulation in response to IFN-γ/TNF-α (Liang *et al.* 2013). The rationale for the general downregulation of reporter gene expression in the presence of cytokines may be due to activation of repressor elements. Considering the apparent downregulation occurred amongst the majority of constructs, it may be suggested that the magnitude of the apparent increase in reporter gene expression observed with pGL4.20-GCH1-3.4kb-Int.1A in the presence of cytokines may be significantly greater, and similar to previous observations (Liang *et al.* 2013). Whilst the luciferase assay is a valuable tool for assessing potential DNA regulatory regions, it should also be considered that assay is incapable of replicating gDNA complexity. Therefore, DNA-transcription factor interactions that are influenced by epigenetic modifications, or promoter-enhancer interactions requiring specific chromatin structure, or those separated by large regions of DNA, are unlikely to be accurately represented in this assay. Variability may occur in relation to vector copy number due to multiple binding events with transcription factors, potentially depleting the availability of specific transcription factors.

3.4.7 Limitations and future considerations

The primary limitations include factors such as cohort size, which would increase the robustness of the study data, whilst also allowing categorisation of patients by diagnosis/phenotype. This is clearly a pertinent consideration due to the clear variation in results between both cohorts, which may be underpinned by distinct differences in pain aetiologies. In relation to the value of pterins and nitric oxide products as circulating biomarkers, there is no overwhelmingly clear association with CNP observed within this thesis, although further analysis allowing for categorisation of patients will undoubtedly add clarity. Considering the multitude of roles for nitric oxide, and the existing evidence for changes in circulating neopterin in various infections and diseases, the inclusion of disease controls would be a pertinent consideration to identify biomarker limitations. This would, for instance, seek to determine whether the level of specific biomarker (e.g. neopterin) was significantly differentiable between persons with CNP and those with CNP and a comorbidity associated with changes in neopterin, such as cardiovascular disease (Firoz *et al.* 2015).

In terms of experimental processes, there are several necessary avenues for further research. In relation to biomarker data, analysis of biopterins in the discovery/validation cohort is desirable, as this could not be completed due to lack of a functional electrochemical detection facility. This will allow further assessment of BH_4 as a biomarker, and to validate previous observations in relation to the BH_4/BH_2 ratio. Moreover, several avenues remain unexplored in relation to the pain protective haplotype. Considering the observed variability with the reporter gene assays, lack of clear influence for an AhR agonist and hypoxia with EMSAs, and the limitations of transcription factor binding prediction tools, further experimentation should be informed by analysis of transcription binding to the *GCH1* 5' locus pertaining to rs8007267, either by ChIP-seq (Mundade *et al.* 2014) or 2D-PAGE and subsequent mass spectrometry (Meleady 2011). Further work may encompass reporter gene transfections in the presence of AhR agonists or cobalt chloride, including the influence of the L-kynurenine pathway on both reporter gene expression and *GCH1* expression. Latterly, further analysis pertaining to the identification of intronic enhancer regions within *GCH1* may be modified or enhanced by achieving efficient transfection in a different cell line, such as RAW264.7, by simulation with mediators targeting alternative pathways of upregulation, and by further dissection of the cloned intronic region in order to identify the implicated transcription factor binding motifs, and whether such motifs are localised with SNPs.

3.5 Conclusion

Analysis of the tetrahydrobiopterin pathway and the regulatory influence of the pain protective haplotype has demonstrated mixed outcomes. There was a lack of discernible difference in plasma nitrite/nitrate and neopterin in both cohorts, although subsequent correlation analysis highlighted significantly stronger correlations in those with CNP. This suggests that whilst the regulation of the BH_4 pathway genes, in addition to nitrite/nitrate, may not function as biomarkers of CNBP or of CNP of mixed aetiologies, it raises the possibility of ongoing pathophysiological mechanisms pertaining to BH_4 synthesis. Further analysis of the pain protective haplotype using the luciferase reporter gene assay highlighted multiple results of potential functional significance, particularly with regards to the *GCH1* 5' SNP (rs8007267). *In silico* methods, qRT-PCR and EMSAs were using to assist in the prediction of transcription factor identification. Observations suggested that whilst the AhR/ARNT complex may not regulate *GCH1*, the use of an AhR antagonist differentiually regulated *GCH1* expression and manipulation of ARNT with a hypoxia mimetic notably decreases DNA-protein binding in the EMSA. Further analysis, preferably incorporating 2D-PAGE and mass spectrometry, would be required to facilitate identification of the relevant transcription factor and enable subsequent analysis of a role in *GCH1* regulation.

3.6 Summary points

- Analysis of *GCH1* transcriptional regulation, in conjunction with plasma nitrate and neopterin, did not highlight a potential biomarker of CNBP or of CNP of various aetiologies
- Plasma nitrate and neopterin did not clearly vary according to the pain protective haplotype, although *GCH1* was upregulated in haplotype carriers when considering all participants in the discovery/validation cohort
- Further analysis highlighted multiple correlations which became sequentially stronger in patients with CNP and in those with an S-LANSS score of ≥12, thereby inferring ongoing pathophysiological processes which when taken alone, may be difficult to distinguish from natural variation
- The BH_4/BH_2 ratio was notably lower in patients with CNBP, an indicator of oxidative stress and NOS uncoupling
- Several genes in the BH_4 synthesis pathway were differentially regulated in CNP patients, in particular *QDPR* and *GCHFR*

- Luciferase reporter assays highlighted significant reductions in relative luminescence with reference to the contsructs representing the *GCH1* 5' SNP (rs8007267) in HEK293, SH-SY5Y and cytokine-stimulated HUVECs
- In silico work using MatInspector, supported by qRT-PCR and EMSAs, highlighted that regulation of *GCH1* at the polymorphic locus (rs8007267) may occur through AhR or HIF-1α, in conjunction with ARNT, although results were inconclusive
- Analysis of the *GCH1* intronic region for enhancer regions provided the basis for future work. Despite unexplained increases in firefly luminescence across the majority of constructs, a trend towards elevated luminescence was observed within Int.1A, which corresponds to previous observations

Chapter 4 : Transcriptomic Biomarkers of Neuropathic Pain

4.1 Introduction

4.1.1 Microarrays

The study of the transcriptome, which is defined as the entire RNA component of a cell (Tang *et al.* 2011), has been greatly facilitated by revolutionary advances in genomic technologies which allow us to perform large-scale high-throughput analysis of the mRNA transcripts within a particular sample. The term 'microarrays' and their application to analyse patterns of gene expression was first coined by Brown *et al* in 1995, who determined differential expression of 45 Arabidopsis genes by two-colour fluorescence hybridisation (Schena *et al.* 1995), a feat that was to be superseded 2 years later with the use of microarray technology for yeast whole genome expression analysis (Lashkari *et al.* 1997). Sequencing of the human genome (Venter *et al.* 2001) and advances in microarray production technologies (Bumgarner 2013) have subsequently led to the widespread commercialisation and availability of microarray technologies with greater reproducibility and standardisation (Bammler *et al.* 2005). The GeneChip® Human Transcriptome Array 2.0 (HTA2.0) is indicative of such developments. It contains over 6 million probes targeting coding and non-coding transcripts, in addition to exon-exon splice junctions (Palermo *et al.* 2014).

4.1.2 qRT-PCR

The first documented use of PCR (Saiki *et al.* 1985), and the subsequent emergence of quantitative PCR (qPCR) as a method for nucleic acid quantification (Porcher *et al.* 1992) has provided the foundation for decades of sensitive quantification of RNA species wherein qRT-PCR remains the mainstay method for analysis. Early use of PCR for quantitative purposes used end-point analysis to determine mRNA abundance (Wang *et al.* 1989). Although progressive, such methodology is vulnerable to inherent variabilities in reaction efficiency, the emergence of factors limiting the PCR (such as dNTPs) and limitations in the sensitivity of post-PCR quantification. This methodology is now largely reserved for use when binary outcomes are sought, such as validation of gene knockout models (VanGuilder *et al.* 2008). However, the introduction of qRT-PCR subsequently permitted the analysis of transcript abundance by monitoring of accumulating fluorescent signal during the exponential phase of the reaction, therefore no longer necessitated post-amplification sample handling and the subsequent risk of carry-over contamination (Higuchi *et al.* 1993, Chiang *et al.* 1996, Heid *et al.* 1996). This method, with increased precision and a

wider dynamic range, is extensively used as the mainstay and gold-standard of small-to-medium scale gene expression studies, including validation of microarray data (Canales *et al.* 2006). In order to improve the reliability and consistency of reported qRT-PCR experiments, Bustin *et al* produced the 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments' (MIQE) guidelines (Bustin *et al.* 2009). These guidelines were subsequently summarised to describe a refined list of minimum required standards (Bustin *et al.* 2010).

4.1.2.1 Normalisation processes

Normalisation of gene expression data in qRT-PCR is an absolute requirement in order to account for multiple sources of experimental variation, ranging from RNA quantification and integrity, to the robustness of reverse transcription, including the presence of reaction inhibitors (van den Berg *et al.* 2015). Experimental and biological implications are also of consideration. For instance, equal volumes of blood acquired from different HIV patients may contain variable cell numbers in a given volume of blood, depending upon disease staging, and subsequently, variable RNA yields would be anticipated (Lorach *et al.* 2015). Whilst absolute quantification necessitates the use of a dilution series from a known entity to produce a standard curve, followed by interpolation of the unknown sample quantification cycle (C_q) value, relative normalisation considers the use of at least one reference (housekeeping) gene. This gene should be consistently expressed amongst all samples. Indeed, it is broadly accepted that the use of reference genes is the optimal approach to account for the aforementioned experimental variability (Huggett *et al.* 2005).

One of the most common strategies used for relative normalisation is the delta-delta-Ct (ΔΔCT) model (Livak *et al.* 2001). This method calculates the difference between the C_q values for the gene of interest and reference gene (which provides the ΔCT). Then, in the experimental scenario of a control and treatment/disease, the ΔCT value of the control is subtracted from that of the treatment/disease, yielding the ΔΔCT. The relative quantity value is then calculated based upon the $2^{-\Delta\Delta CT}$ equation (VanGuilder *et al.* 2008). Other data analysis methods have been proposed (Pfaffl 2001, Schefe *et al.* 2006). Although the Pfaffl model represented an improvement over the classical ΔΔCT model, it was unable to manage multiple reference genes required to perform accurate normalisation when seeking subtle changes in gene expression (Vandesompele *et al.* 2002).

4.1.2.2 Reference gene selection

The selection of a suitable reference gene relies on several criteria. These include that the candidate reference gene should be expressed in relative abundance, not be subject to co-regulation with the

gene of interest and should display minimal innate variability (Chervoneva *et al.* 2010). It is also critical that the expression of the selected reference gene(s) is consistent between different experimental groups with minimal variation between different tissue and disease states within an organism. On the other hand, the reference gene should also robustly account for technical variation in procedures, ensuring that such variation equally impacts upon the gene of interest and the reference gene (Kozera *et al.* 2013). Reference genes associated with the basic processes of cell survival were initially established as suitable candidate genes for normalisation, and termed 'housekeeping genes' (Thellin *et al.* 1999). However, use of the term 'housekeeping gene' is now discouraged, as many of these genes are not only involved in basic metabolic processes, and are not suitable for normalisation in all experimental scenarios (Bustin *et al.* 2009, Kozera *et al.* 2013).

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is a classic example of a commonly used reference gene (de Jonge *et al.* 2007). It is often used successfully to obtain reliable gene expression data, but contrary to the suggestion that 'housekeeping genes' are expressed at a constant level without influence from experimental procedures or disease, stark changes have been observed. For instance, an extensive analysis of *GAPDH* expression in 72 different human disease-free tissues showed up to 15 fold difference in mRNA levels between tissues, and also notable variation within the same tissue obtained from different donors (Barber *et al.* 2005). Aside for natural variation, one must also consider that the expression of a reference gene should not vary between different experimental parameters or control/disease groups in clinical studies. The expression of *Gapdh* has been shown to be upregulated in hypoxia both *in vivo* and *in vitro* (Yang *et al.* 2008, Higashimura *et al.* 2011). Such susceptibility for variation is exemplified by a study assessing *IL-4* expression in pulmonary tuberculosis which showed that the arbitrary selection of *GAPDH* as the sole reference gene led to false negative results for tuberculosis and failed to differentiate between treatment groups, when compared to the use of a validated reference gene (Dheda *et al.* 2005). Such research does not discredit the use of *GAPDH*, but emphasises the necessity of reference gene validation before qRT-PCR (Fink *et al.* 2008).

The stability of reference genes used within an experiment is therefore a critical consideration to ensure the outcome of normalisation accurately reflects changes in gene expression and importantly, the magnitude of such changes. This is of particular importance when generating conclusions based on relatively subtle fold changes. Many studies utilising qRT-PCR have selected a single reference gene for normalisation on a somewhat arbitrary basis, often based on previous literature and without validation (Kozera *et al.* 2013). Notwithstanding the lack of

informed reference gene selection, the use of a single gene, rather than multiple reference genes, may be associated with notable normalisation error (Vandesompele *et al.* 2002, Dmitriev *et al.* 2007) and was previously a highly prominent feature of qRT-PCR methodology (Mease *et al.* 2007, van den Berg *et al.* 2015). As eluded to, the selection of at least two validated reference genes is particularly poignant when seeking to determine potentially small changes in expression, as a distinguishable variation in reference gene expression may overwhelm potentially meaningful findings (Vandesompele *et al.* 2002, Dheda *et al.* 2005).

4.1.2.3 geNorm and qbase+

The selection of suitable, stably expressed, reference genes is clearly a necessary requirement for accurate data analysis. Vandesompele *et al* previously developed a measure of candidate reference gene stability based on non-normalised data, which relied on the principle that the expression ratio between two ideal candidate reference genes should not vary between control and experimental/disease groups. As such, increasingly contrasting ratios between two genes is indicative of unstable expression across the sample set. The pairwise variation is determined for a given candidate reference gene against all remaining candidate reference genes as the standard deviation of the logarithmically transformed expression ratios. Gene stability is subsequently assigned an *M* value depicting the pairwise variation of a candidate reference gene with the remaining candidate reference genes. Thus, genes with the lowest *M* values are most stably expressed across the sample set (Vandesompele *et al.* 2002). The degree of gene expression stability is therefore demonstrated using the gene stability values (*M*) and coefficients of variation (CV). For homogeneous samples (e.g. blood from healthy subjects) the *M* value and CV limits are 0.5 and 25%, respectively. When using heterogeneous samples (e.g. diseases tissues), the *M* value and CV limits are increased to 1 and 50%, respectively (Bennett *et al.* 2007c, Gardiner *et al.* 2007, Hellemans *et al.* 2007). Assessment of reference gene stability can be used prior to an experiment to inform and optimise reference gene selection, or after experimentation to verify the stability of selected reference genes (Bennett *et al.* 2007a). A geNorm analysis prior to experimentation should therefore cover a wide range of reference genes corresponding to a variety of functional groups and pathways in order to avoid co-regulated genes. The assessment should also include a sample set representative of that to be analysed. A software package, qbase+ (Biogazelle, Belgium), which incorporates the geNorm algorithm, is available. qbase+ is developed to facilitate qRT-PCR data analysis and data management. The calculations within qbase+ are based on the $2^{-\Delta\Delta CT}$ method, though modified to permit the use of multiple reference genes. The extensive

background calculations performed by qbase+ have been described in detail (Hellemans *et al.* 2007).

The transcriptomics component of this thesis therefore aims to elucidate a panel of potential CNP biomarkers, isolated from human blood, and to consider the functional relevance and potential of these molecules as therapeutic targets with expansion of gene expression analysis using qRT-PCR. In a subsequent chapter, where applicable, the genes identified here will be assessed as translational biomarkers in an animal model of neuropathic pain.

4.1.3 Aims and objectives

The overall aims and objectives of chapter 4 are as follows:

- Use microarrays to determine differentially regulated genes in the blood of patients with CNP, when compared to healthy controls, in two distinct patient cohorts
- Validate findings from microarray analysis across both cohorts using RT-PCR and ddPCR
- Use bioinformatic tools, including Ingenuity Pathway Analysis and DAVID, to analyse microarray data, thereby facilitating hypotheses pertaining to specific genes and their role in pain

4.2 Methods

All methods relating to this chapter are presented within chapter 2.

4.3 Results

4.3.1 Microarray analysis

Analysis of gene expression changes in the discovery cohort identified 515 differentially regulated genes in peripheral blood mononuclear cells of patients with CNBP, based on a minimum fold change of 1.2 and a *p* value of ≤0.05. Of which, 313 genes were downregulated. Similarly, using identical filtering criteria, a total of 332 genes were identified as significantly differentially regulated in the discovery/validation cohort. Of these genes, 105 were downregulated. The genes identified here are described under the file name 'Gene list' within the electronic supplementary data and were subsequently exported for analysis using various computational tools.

4.3.2 Candidate biomarker selection

Selected genes (section 4.3.1) were subject to further scrutiny and analysis in the SNL model of neuropathic pain by meeting pre-specified refinement criteria. These included a *p* value of ≤0.005

and a fold change of ≥1.5. However, should a given gene be identified within the gene-gene correlations (within the discovery cohort only), or have pre-existing associations with pain, statistical thresholds were relaxed to include genes with either a *p* value in the range of 0.005-0.05 and a fold change of ≥1.5, or a *p* value of ≤0.005 and a fold change in the range of 1.2-1.5. As a result of gene expression refinement, 15 genes (Table 4.1) in the discovery cohort were highlighted as potential biomarkers of CNBP. Similarly, a total of 12 genes (Table 4.2) satisfied these criteria within the discovery/validation cohort. These genes were subsequently carried forward for consideration as biomarkers of CNP, and to determine, where applicable, whether these genes represent translational biomarkers of CNP through analysis of gene expression in the DH after SNL

Table 4.1: Candidate biomarkers differentially regulated patients with CNP in the discovery cohort

Array ID	Accession Number	Gene Name	Gene Symbol	*p* value	FC in CNP	CA	Literature
7951385	NM_004347	Caspase 5	*CASP5*	0.045	↑2.23	No	(de Rivero Vaccari *et al.* 2008, Lukkahatai *et al.* 2013)
8149927	NM_001831	Clusterin	*CLU*	0.049	↑1.85	No	(Liu *et al.* 1995)
7941621	NM_005700	Dipeptidyl-peptidase 3	*DPP3*	0.003	↑1.50	No	(Lee *et al.* 1982, Sato *et al.* 2003, Barsun *et al.* 2007, Bezerra *et al.* 2012)
7908793	NM_004433	E74-like factor 3	*ELF3*	0.010	↑1.62	No	(Raju *et al.* 2014, Li *et al.* 2015)
7937707	NR_026643	Family with sequence similarity 99, member A	*FAM99A*	0.002	↑1.64	No	-
8070720	NM_015259	Inducible T-cell co-stimulator ligand	*ICOSLG*	7.00×10^{-4}	↑1.20	No	(Grace *et al.* 2012)
8065011	NM_024674	Lin-28 homolog A (C. elegans)	*LIN28A*	0.018	↓1.50	No	(Yue *et al.* 2014)
7998055	NM_002386	Melanocortin 1 receptor	*MC1R*	5.00×10^{-4}	↑1.40	No	(Liem *et al.* 2005, Mogil *et al.* 2005, Delaney *et al.* 2010, Juni *et al.* 2010, Andresen *et al.* 2011, Arout *et al.* 2015)
8051396	NM_021209	NLR family CARD domain-containing protein 4	*NLRC4*	0.044	↑1.99	No	(Lopes *et al.* 2015)
8157450	NM_000608	Orosomucoid 2	*ORM2*	0.023	↑1.97	Yes	-
7982287	NM_001039841	Rho GTPase activating protein 11B	*ARHGAP11B*	0.003	↑1.57	No	-
8075477	NM_152267	Ring finger protein 185	*RNF185*	0.003	↓1.68	No	-

7967972	NG_043316	RNA, U6 Small nuclear 76, pseudogene	*RNU6-76P*	0.005	↓1.54	No	-
8167185	NM_003254	TIMP metalloproteinase inhibitor 1	*TIMP1*	0.005	↑1.50	Yes	(Rodriguez Parkitna *et al.* 2006, Kawasaki *et al.* 2008a, Huang *et al.* 2011, Sandhir *et al.* 2011, Kim *et al.* 2012, Rojewska *et al.* 2014, McKelvey *et al.* 2015, Popiolek-Barczyk *et al.* 2015)
7924499	NM_003268	Toll-like receptor 5	*TLR5*	0.043	↑1.75	No	(Stokes *et al.* 2013)

Genes documented here and subsequently analysed in the SNL model either exhibited a *p* value (ANOVA) of ≤0.005 and a (linear) fold change (FC) of ≥1.5, or were present in our correlation analysis (CA)/literature search with a *p* value of 0.005-0.05 and a fold change of ≥1.5 or a *p* value of ≤0.005 and a fold change of 1.2-1.5. Microarray files are available in the electronic supplementary material within the folder entitled 'Microarray' and the sub-folder, 'Discovery'.

Table 4.2: Candidate biomarkers differentially regulated patients with CNP in the discovery/validation cohort

Array ID	Accession Number	Gene Name	Gene Symbol	*p* value	FC in CNP	Literature
TC03001304.hg.1	NM_001171174	Chemokine (C-X3-C motif) receptor 1	*CX3CR1*	0.002	↓1.42	(Verge *et al.* 2004, Zhuang *et al.* 2007, Staniland *et al.* 2010, Clark *et al.* 2011, Zhu *et al.* 2013, Clark *et al.* 2014, Old *et al.* , Bian *et al.* 2015, Liu *et al.* 2015, Li *et al.* 2016a)
TC22000722.hg.1	NM_000878	Interleukin 2 receptor subunit beta	*IL2RB*	0.002	↓1.31	(Yao *et al.* 2002, Rotty *et al.* 2006b, Uceyler *et al.* 2007, Nissenbaum *et al.* 2010)
TC12001202.hg.1	NM_002258	Killer cell lectin like receptor B1	*KLRB1*	0.002	↓1.63	-
TC01002764.hg.1	NR_031664	MicroRNA 1262	*MIR1262*	0.005	↑1.50	-
TC11001015.hg.1	NM_000615	Neural cell adhesion molecule 1	*NCAM1*	0.001	↓1.23	(Sakai *et al.* 2008a, Sakai *et al.* 2008c, Patil *et al.* 2011)
TC02002865.hg.1	NM_006056	Neuromedin U receptor 1	*NMUR1*	0.004	↓1.20	(Cao *et al.* 2003, Wang *et al.* 2014, Martinez *et al.* 2015)
TC04000410.hg.1	NM_002620	Platelet factor 4 variant 1	*PF4V1*	0.003	↑1.32	(Jin *et al.* 2013, Lukkahatai *et al.* 2013)
TC19001593.hg.1	NM_002659	Plasminogen activator, urokinase receptor	*PLAUR*	0.001	↑1.21	(Garcia-Monco *et al.* 2002, Rivellini *et al.* 2012)
TC14000305.hg.1	NM_000953	Prostaglandin D2 receptor	*PTGDR*	0.004	↓1.35	(Minami *et al.* 1996, Eguchi *et al.* 1999, Popp *et al.* 2009, Joo *et al.* 2012)
TC09000601.hg.1	NM_138554	Toll-like Receptor 4	*TLR4*	0.036	↑1.36	(Bettoni *et al.* 2008, Sorge *et al.* 2011, Jia *et al.* 2012, Lin *et al.* 2015, Jurga *et al.* 2016)
TC01002763.hg.1	NM_024911	Wntless Wnt ligand secretion mediator	*WLS*	0.038	↑1.51	(Petko *et al.* 2013, Herrero-Turrion *et al.* 2014)

TC01001469.hg.1	NM_002995	X-C motif chemokine ligand 1	*XCL1*	0.003	↓1.35	(Dawes *et al.* 2013, Zychowska *et al.* 2016)

Genes documented here and subsequently analysed in the SNL model either exhibited a *p* value (ANOVA) of ≤0.005 and a fold change (FC) of ≥1.5, or were present in a literature search with a *p* value of 0.005-0.05 and a fold change of ≥1.5 or a *p* value of ≤0.005 and a fold change of 1.2-1.5. Microarray files are available in the electronic supplementary material within the folder entitled 'Microarray' and the sub-folder, 'Discovery validation'.

4.3.3 Literature and general gene-gene correlations

TIMP1, *DPP3* and *MC1R* all exhibited a strong basis of literature supporting the role of these genes in pain pathways within the discovery cohort. Similar supportive literature was found in relation to several genes in the discovery/validation cohort, including *CX3CR1* and *TLR4*. In terms in gene-gene correlations (Figure 4.1), *TIMP1*, *ORM2* and *PROX1* were present in the correlation analysis, although *PROX1* did not meet any of our other refinement criteria.

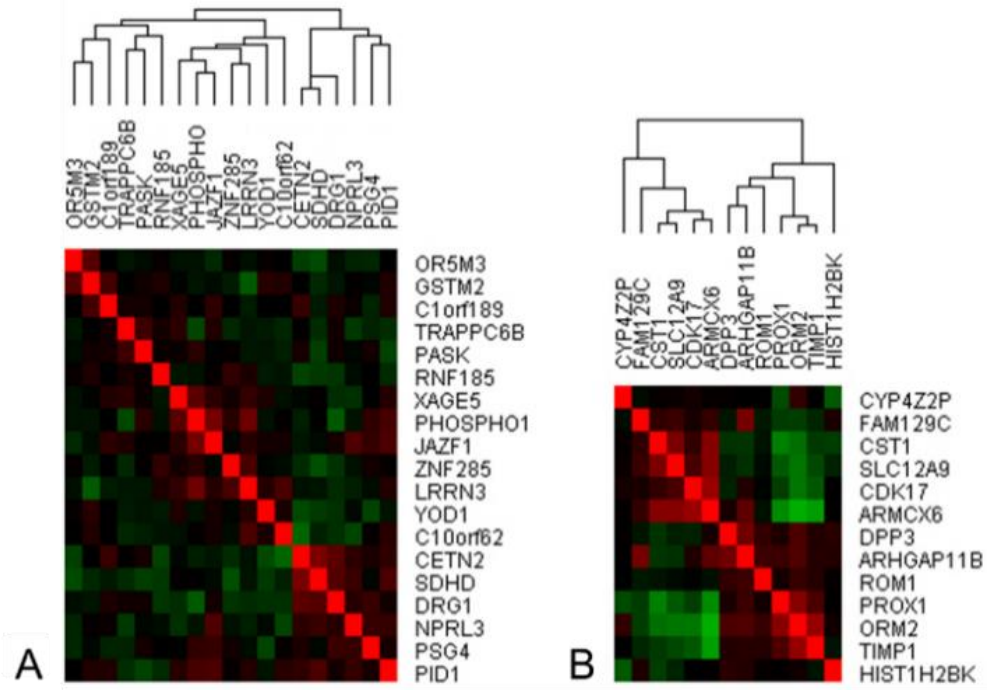

Figure 4.1: Prior transcriptional correlations between a subset of (A) highly downregulated and (B) upregulated genes in CNBP patients using 3,900 human 2-colour microarrays.

Using a matrix of transcriptional correlations derived from the analysis of 3,900 human 2-colour microarrays from NCBI's Gene Expression Omnibus (GEO), which includes data from a variety of control and experimental samples, gene-gene Pearson's correlation coefficients were determined. In the 3,900 microarrays used to perform gene-gene correlations, *PROX1*, *ORM2* and *TIMP1* were found to positively correlate with each other. In the 2-colour microarray analysis, several other upregulated genes, including *CST1*, *SLC12A9*, *CDK17*, *ARMCX6*, were usually negatively correlated (green) to *PROX1*, *ORM2* and *TIMP1* (the brightest red squares are the self-self comparisons along the diagonal). However, further analysis highlighted that both groups of genes were upregulated, thus providing evidence that *ORM2*, *TIMP1* and to a lesser degree, *PROX1*, which are highly correlated in previous experiments, may be associated with the pathophysiology of CNP and may function as CNP biomarkers. This work was conducted in collaboration with Dr J. Wren, an affiliate of the Oklahoma Medical Research Foundation, whom conducted the *in silico* data processing.

4.3.4 Microarray cross-validation

Cross-validation between the discovery and discovery/validation cohorts was performed in order to determine genes differentially regulated in both cohorts. Thresholds were maintained ($p = \leq 0.05$, fold change = ≥ 1.20) for the first cohort analysed and relaxed for the second cohort ($p = \leq 0.15$, fold change = ≥ 1.10). A total of 19 genes were differentially regulated across both cohorts with the discovery cohort as the first cohort (Table 4.3), of which 10 genes were differentially regulated in the same direction of fold change. When considering the discovery/validation cohort as the first cohort, 19 genes were differentially regulated across both cohorts (Table 4.4), of which 12 genes exhibited regulation in the same direction of fold change.

Table 4.3: Cross validation of potential CNP biomarkers in the discovery cohort with those observed in the discovery/validation cohort

				Discovery		Discovery/Validation	
Array ID	**Accession Number**	**Gene Name**	**Gene Symbol**	***p* value**	**FC in CNP**	***p* value**	**FC in CNP**
7982287	NM_001039841	Rho GTPase activating protein 11B	*ARHGAP11B*	0.003	1.57	0.037	1.12
7951385	NM_004347	Caspase 5	*CASP5*	0.045	2.23	0.001	1.41
8081214	NM_005290	G protein-coupled receptor 15	*GPR15*	0.043	-1.70	0.108	1.46
7918379	NM_000849	Glutathione S-transferase mu 3 (brain)	*GSTM3*	0.045	-1.43	0.139	1.14
8117594	NM_003521	Histone cluster 1, H2bm	*HIST1H2BM*	0.029	-1.54	0.067	1.14
8031344	NM_012312	killer cell immunoglobulin like receptor, two Ig domains and short cytoplasmic tail 2	*KIR2DS2*	0.031	-1.67	0.073	-1.31
8031344	NM_001242867	Killer cell immunoglobulin like receptor, three Ig domains and long cytoplasmic tail 2	*KIR3DL2*	0.031	-1.67	0.049	-1.33
8042283	NM_014181	Lectin, galactoside-binding-like	*LGALSL*	0.029	1.49	0.119	1.15
8054611	NR_024204	long intergenic non-protein coding RNA 152	*LINC00152*	0.034	1.48	0.085	1.10
7998157	NM_001077350	NPR3-like, GATOR1 complex subunit	*NPRL3*	0.010	-1.63	0.058	1.41
7971075	NG_032625	RNA, 7SK small nuclear pseudogene 1	*RN7SKP1*	0.017	3.62	0.036	1.12
8107857	NG_033689	RNA, 5S ribosomal pseudogene 191	*RNA5SP191*	0.003	1.38	0.062	1.20
7938070	NG_033478	RNA, 5S ribosomal pseudogene 329	*RNA5SP329*	0.026	-1.30	0.026	1.12
8025990	NG_033692	RNA, 5S ribosomal pseudogene 466	*RNA5SP466*	0.009	-1.27	0.044	1.12
8092763	NG_032598	RNA, U1 small nuclear 20, pseudogene	*RNU1-20P*	0.004	-1.20	0.033	1.19
7950810	NM_032943	Synaptotagmin like 2	*SYTL2*	0.040	-1.23	0.072	-1.16
8129608	NR_028511	Trace amine associated receptor 3	*TAAR3*	0.045	-1.69	0.131	1.11
7924499	NM_003268	Toll-like receptor 5	*TLR5*	0.043	1.75	0.053	1.14
7923967	NM_018566	YOD1 deubiquitinase	*YOD1*	0.006	-1.73	0.060	1.19

Genes described satisfied two criteria. Genes were differentially regulated in the discovery cohort according to a (linear) fold change (FC) of ≥1.20 and (ANOVA) *p* value of ≤0.05. Such genes were then identified in the discovery/validation cohort as differentially regulated with reduced statistical stringency consisting of a fold change of ≥1.10 and a *p* value of ≤0.15. Green text denotes differential regulation in the same direction of fold change in both cohorts.

Table 4.4: Cross-validation of potential CNP biomarkers in the discovery/validation cohort with those observed in the discovery cohort

Array ID	Accession Number	Gene Name	Gene Symbol	Discovery/Validation		Discovery	
				p value	FC in CNP	*p* value	FC in CNP
TC11003322.hg.1	NM_004347	Caspase 5	*CASP5*	0.010	1.41	0.045	2.23
TC06004150.hg.1	NM_004117	FK506 binding protein 5	*FKBP5*	0.047	1.24	0.061	1.39
TC04001226.hg.1	NM_001553	Insulin like growth factor binding protein 7	*IGFBP7*	0.021	-1.23	0.102	1.10
TC19002658.hg.1	NM_001242867	Killer cell immunoglobulin like receptor, three Ig domains and long cytoplasmic tail 2	*KIR3DL2*	0.049	-1.33	0.031	-1.67
TC07000288.hg.1	NR_037596	Long intergenic non-protein coding RNA 1061	*LINC01061*	0.004	1.40	0.057	1.40
TC03001450.hg.1	NR_029660	microRNA let-7g	*MIRLET7G*	0.032	1.21	0.089	1.13
TC20001202.hg.1	NM_006097	Myosin light chain 9	*MYL9*	0.028	1.27	0.072	1.77
TC05000307.hg.1	NM_004536	NLR family, apoptosis inhibitory protein	*NAIP*	0.025	1.46	0.064	1.34
TC19000886.hg.1	NM_004829	Natural cytotoxicity triggering receptor 1	*NCR1*	0.010	-1.20	0.118	1.23
TC11001015.hg.1	NM_000615	Neural cell adhesion molecule 1	*NCAM1*	0.001	-1.23	0.090	1.16
TC04000410.hg.1	NM_002619	Platelet factor 4	*PF4*	0.017	1.30	0.065	1.57
TC01001619.hg.1	NM_130782	Regulator of g protein signalling 18	*RGS18*	0.043	1.22	0.133	1.19
TC13000759.hg.1	NG_033512	RNA, 5S ribosomal pseudogene 33	*RNA5SP33*	0.030	1.24	0.060	-1.23
TC04000587.hg.1	NG_043463	RNA, U1 small nuclear 138, pseudogene	*RNU1-138P*	0.033	1.25	0.121	1.20
TC05001337.hg.1	NG_044479	RNA, U6 small nuclear 480, pseudogene	*RNU6-480P*	0.044	1.20	0.122	-1.12
TC11001303.hg.1	NG_045859	RNA, U6 small nuclear 1143, pseudogene	*RNU6-1143P*	0.029	1.21	0.088	-1.21
TC16000357.hg.1	NR_002966	Small nucleolar RNA, H/ACA box 30	*SNORA30*	0.003	1.23	0.093	1.14
TC0X000538.hg.1	NM_017698	Transmembrane protein 164	*TMEM164*	0.041	1.26	0.130	-1.52
TC01001469.hg.1	NM_002995	X-C motif chemokine ligand 1	*XCL1*	0.003	-1.35	0.105	-1.45

Genes described satisfied two criteria. Genes were differentially regulated in the discovery/validation cohort according to a (linear) fold change (FC) of ≥1.20 and (ANOVA) *p* value of ≤0.05. Such genes were then identified in the discovery cohort with reduced statistical stringency consisting of a fold change of ≥1.10 and a *p* value of ≤0.15. Green text denotes differential regulation in the same direction of fold change in both cohorts.

4.3.5 Microarray correlations with pain measures

Several genes were found to significantly correlate with general pain severity in the discovery/validation cohort, as assessed by a modified Chronic Pain Grade questionnaire. Of particular note, both *GCH1* and *CX3CR1* were present within the strongest correlates (Table 4.5). Both genes were anti-correlated with the accumulative score of the 5 pain severity measures. These genes, and their respective associations with CNP, have been discussed extensively within this thesis. Several transcripts were also found to significantly correlate with the S-LANSS score (Table 4.6).

Table 4.5: Correlations between gene expression and pain severity measures in the discovery/validation cohort

Rank	ProbeSet	Gene Symbol	Gene Name	Correlation Coefficient (*r*)	Parametric *p* value
1	TC10001535.hg.1	*MYOF*	Myoferlin	-0.945	3.81×10^{-5}
2	TC14002019.hg.1	*GCH1*	GTP cyclohydrolase 1	-0.926	1.18×10^{-4}
3	TC03001304.hg.1	*CX3CR1*	Chemokine (C-X3-C motif) receptor 1	-0.918	1.82×10^{-4}
4	TC06003759.hg.1*	*CNR1*	Cannabinoid receptor 1	0.916	1.98×10^{-4}
5	TC14002210.hg.1*			0.888	6.04×10^{-4}
6	TC6_mann_hap4000161.hg.1	*HLA-DRB4*	Major histocompatibility complex, class II, DR beta 4	-0.886	6.33×10^{-4}

Microarray data was uploaded into BRB Array Tools (section 2.1.2.6) alongside accumulative pain severity scores relating to the five measures of severity (questions 1-5) within the modified Chronic Pain Grade tool. Pearson correlations (*r*) were determined with a threshold of $p = 0.001$. *These transcripts are incompletely annotated within the Affymetrix database, UCSC Genome Browser and NCBI database. Annotations have been completed manually where possible.

Table 4.6: Correlations between gene expression and the S-LANSS score in the discovery/validation cohort

Rank	ProbeSet	Gene Symbol	Gene Name	Correlation Coefficient (*r*)	Parametric *p* value
1	TC07000989.hg.1*		Transfer RNA Cys	0.942	4.76×10^{-5}
2	TC06001905.hg.1*			-0.907	2.89×10^{-4}
3	TC09001017.hg.1*	*ANXA2P2*	Annexin A2 pseudogene 2 (ncRNA)	0.902	3.61×10^{-4}
4	TC09000770.hg.1*			0.901	3.78×10^{-4}
5	TC14001314.hg.1*			0.895	4.62×10^{-4}
6	TC12001810.hg.1*	*snoU13*		0.893	5.03×10^{-4}
7	TC21000582.hg.1	*ABCC13*	ATP-binding cassette, sub-family C (CFTR/MRP), member 13, pseudogene	0.890	5.55×10^{-4}
8	TC0X001192.hg.1	*MIR361*	microRNA 361	0.883	7.10×10^{-4}
9	TC06001402.hg.1*		Transfer RNA Ser	0.880	7.84×10^{-4}
10	TC10000866.hg.1	*MIR4681*	microRNA 4681	-0.877	8.62×10^{-4}
11	TC06002441.hg.1	*LTA*	Lymphotoxin alpha	0.873	9.65×10^{-4}
12	TC06000300.hg.1*		RNA, U6 small nuclear 930, pseudogene	0.872	9.93×10^{-4}

Microarray data was uploaded into BRB Array Tools (section 2.1.2.6) alongside the S-LANSS score. Pearson correlations (*r*) were determined with a threshold of $p = 0.001$. *These transcripts are incompletely annotated within the Affymetrix database, UCSC Genome Browser and NCBI database. Annotations have been completed manually when possible.

4.3.6 Ingenuity Pathway Analysis

A total of 515 transcripts within the discovery cohort were uploaded to IPA, of which 345 were successfully identified and mapped by the software. After generation of molecular networks both CD40 and PIK3CA featured multiple interactions (Appendix 7, Figure A). Upon addition of the appropriate molecules linked with CNP from the IPA database, miR-103-3p formed a central focus of molecular interactions (Appendix 7, Figure B). In the discovery/validation cohort, 332 transcripts were similarly uploaded, of which 157 were mapped by IPA. Formation of molecular networks between these molecules illustrated TLR4 as interacting, largely indirectly, with several other molecules (Appendix 7, Figure C). Latterly, the addition of molecules associated with CNP from the IPA database also resulted in multiple predicted interactions, again frequently featuring

miR-103-3p (Appendix 7, Figure D). Further scrutiny with regards to miR-103-3p in a similarly sized but unrelated cohort pertaining to multiple sclerosis also highlighted multiple interactions (data not shown). This suggests the observed frequency of molecular connections previously observed with miR-103-3p may not be underpinned by CNP.

4.3.7 DAVID bioinformatics and gene ontology analysis

4.3.7.1 Biological processes

Analysis of over-represented biological processes related to differentially expressed genes in the discovery cohort elucidated multiple core cellular processes, including mitosis, nuclear division and nucleosome assembly (Table 4.7). In contrast, immune response and cell surface receptor linked signal transduction were prominently over-represented processes in the discovery/validation cohort (Table 4.8). Both were also highly significantly associated, with each category comprising of 13.49% and 20.64% of the total gene input, respectively.

Table 4.7: Biological processes associated with the differentially regulated genes in the discovery cohort

Term	Percentage of Total Genes	*p* value	Fold Enrichment	Bonferroni
Category: GOTERM_BP_FAT				
GO:0006220~pyrimidine nucleotide metabolic process	1.12	0.015	7.59	1.000
GO:0048285~organelle fission	2.53	0.019	2.69	1.000
GO:0007018~microtubule-based movement	1.69	0.025	3.63	1.000
GO:0006334~nucleosome assembly	1.41	0.034	4.07	1.000
GO:0031497~chromatin assembly	1.41	0.038	3.93	1.000

This table has been reduced in size and detail and depicts the five most significant associations. A full table is available in the electronic supplementary material within the folding entitled 'DAVID' and has a file name of 'Discovery Biological Processes'.

Table 4.8: Biological processes associated with the differentially regulated genes in the discovery/validation cohort

Term	Percentage of Total Genes	*p* value	Fold Enrichment	Bonferroni
Category: GOTERM_BP_FAT				
GO:0006955~immune response	13.49	3.14×10^{-6}	4.02	0.003
GO:0007166~cell surface receptor linked signal transduction	20.64	6.52×10^{-5}	2.28	0.058
GO:0006968~cellular defense response	3.97	5.03×10^{-4}	13.36	0.369
GO:0006821~chloride transport	3.97	5.03×10^{-4}	13.36	0.369
GO:0015698~inorganic anion transport	3.97	0.002	8.76	0.893

This table has been reduced in size and detail and depicts the five most significant associations. A full table is available in the electronic supplementary material within the folder entitled 'DAVID' and has a file name of 'Discovery/validation Biological Processes'.

4.3.7.2 Cellular component

Allocation of differentially expressed genes to their reputed cellular function highlighted contrasting results between both cohorts. In the discovery cohort, over-represented cellular functions were similar to those presented in the biological processes analysis and included functions pertaining to DNA-protein complexes, chromatin and nucleosomes (Table 4.9). Genes pertaining to histones are a prominent feature amongst all over-represented cellular functions. Cellular functions over-represented in the discovery/validation cohort were highly associated with the plasma membrane (Table 4.10). The three most significant functions were representative of 36.51-43.65% of the differentially regulated gene imported to DAVID.

Table 4.9: Cellular components associated with the differentially regulated genes in the discovery cohort

Term	Percentage of Total Genes	*p* value	Fold Enrichment	Bonferroni
Category: GOTERM_CC_FAT				
GO:0032993~protein-DNA complex	1.97	0.001	5.95	0.239
GO:0000786~nucleosome	1.41	0.011	5.80	0.926
GO:0044427~chromosomal part	3.37	0.017	2.27	0.983
GO:0000785~chromatin	2.25	0.020	2.92	0.992
GO:0005694~chromosome	3.65	0.024	2.06	0.997

This table has been reduced in detail. A full table is available in the electronic supplementary material within the folder entitled 'DAVID' and has a file name of 'Discovery Biological Processes'.

Table 4.10: Cellular components associated with the differentially regulated genes in the discovery/validation cohort

Term	Percentage of Total Genes	*p* value	Fold Enrichment	Bonferroni
Category: GOTERM_CC_FAT				
GO:0005886~plasma membrane	36.51	3.35×10^{-7}	1.95	4.02×10^{-5}
GO:0031224~intrinsic to membrane	43.65	4.15×10^{-6}	1.60	4.98×10^{-4}
GO:0016021~integral to membrane	42.06	1.00×10^{-5}	1.60	0.001
GO:0031226~intrinsic to plasma membrane	16.67	3.48×10^{-5}	2.76	0.004
GO:0009897~external side of plasma membrane	6.35	8.43×10^{-5}	7.52	0.010

This table has been reduced in size and detail and depicts the five most significant associations. A full table is available in the electronic supplementary material within the folder entitled 'DAVID' and has a file name of 'Discovery validation Biological Processes'

4.3.7.3 Disease associations

Analysis of diseases associated with differentially expressed genes was performed using the two annotation categories within DAVID. Comparatively few diseases were featured as over-represented by differentially expressed genes in the discovery cohort (Table 4.11). Genes associated with susceptibility to multiple sclerosis showed the greatest statistical significance (p = 0.008) and fold enrichment (9.20). Identical analysis with the discovery/validation cohort elucidated multiple over-represented diseases, including asthma and HIV (Table 4.12). However, several of these diseases highlighted within this dataset are present by virtue of association with

only two genes. Caution is therefore required when interpreting the biological meaning of such associations.

Table 4.11: Diseases associated with the differentially regulated genes in the discovery cohort

Term	Percentage of Total Genes	*p* value	Gene Symbol	Fold Enrichment	Bonferroni
Category: GENETIC_ASSOCIATION_DB_DISEASE					
Oral cancer	1.12	0.011	*OGG, GSTM3, ITGA2, micA*	8.39	0.948
Crohn's disease ulcerative colitis	0.84	0.099	*TLR5, FCGR3A, TIMP1*	5.46	1.000
Category: OMIM_DISEASE					
Genome-wide association of susceptibility and clinical phenotype in MS	1.12	0.008	*FOXO3B, PDZRN4, SH3GL2, MXI1*	9.20	0.540

Table 4.12: Diseases associated with the differentially regulated genes in the discovery/validation cohort

Term	Percentage of Total Genes	*p* value	Gene Symbol	Fold Enrichment	Bonferroni
Category: GENETIC_ASSOCIATION_DB_DISEASE					
Asthma	6.35	0.004	*FCGR1B, GSTT1, MS4A2, CX3CR1, PTGDR, TLR4, FCER1A, TBX21*	3.69	0.747
Airway hyper-responsiveness atopy	1.59	0.031	*MS4A2, FCER1A*	61.17	0.999
HIV	3.18	0.045	*KIR3DL1, CX3CR1, TLR4, PRF1*	4.83	1.000
Lymphoma	2.38	0.081	*GSTT1, TLR4, PRF1*	6.12	1.000
Priapism	1.59	0.082	*F13A1, TGFBR3*	22.94	1.000
Bacteremia	1.59	0.082	*TLR4, TGFBR3*	22.94	1.000
Celiac disease; Wegener's granulomatosis; cervical cancer	1.59	0.091	*KIR3DL1, KIR3DL2*	20.39	1.000
Rheumatoid arthritis; preeclampsia; psoriasis; celiac disease; cervical cancer; psoriatic arthritis	1.59	0.091	*KIR3DL1, KIR3DL2*	20.39	1.000

4.3.7.4 Molecular functions

Allocation of differentially expressed genes into their respective molecular functions highlighted contrasting results between both cohorts. Molecular functions comprising of genes related to glutathione S-transferases represented the majority of associations within the discovery cohort (Table 4.13). However, a wide range of molecular functions were assigned as over-represented in the discovery/validation cohort (Table 4.14). These include carbohydrate binding and functions pertaining to the activity and receptor binding of cytokines and chemokines.

Table 4.13: Molecular functions associated with the differentially regulated genes in the discovery cohort

Term	Count	Percentage of Total Genes	*p* value	Gene Symbol	Fold Enrichment	Bonferroni
Category: GOTERM_MF_FAT						
GO:0004364~glutathione transferase activity	4	1.12	0.003	*GSTM3, GSTM5, GSTA5, GSTM2*	12.92	0.762
GO:0003777~microtubule motor activity	5	1.41	0.031	*KIF25, Kifc3, KIF14, STARD9, DNAH14*	4.19	0.999
GO:0016765~transferase activity, transferring alkyl or aryl (other than methyl) groups	4	1.12	0.040	*GSTM3, GSTM5, GSTA5, GSTM2*	5.27	1.000

Table 4.14: Molecular functions associated with the differentially regulated genes in the discovery/validation cohort

Term	Count	Percentage of Total Genes	*p* value	Gene Symbol	Fold Enrichment	Bonferroni
Category: GOTERM_MF_FAT						
GO:0030246~carbohydrate binding	11	8.73	5.41×10^{-5}	*PF4V1, PF4, PRPS1L1, KLRF1, CLEC4A, KLRB1, PTCH1, TNFAIP6, KLRD1, CLEC4D, TGFBR3*	5.04	0.013
GO:0019865~immunoglobulin binding	4	3.18	9.36×10^{-5}	*FCGR1B, MS4A2, FCGR1C, FCER1A*	43.28	0.022
GO:0019763~immunoglobulin receptor activity	3	2.38	2.18×10^{-4}	*FCGR1B, MS4A2, FCER1A*	121.72	0.051
GO:0008009~chemokine activity	4	3.18	0.003	*XCL2, PF4V1, PF4, XCL1*	14.11	0.478
GO:0042379~chemokine receptor binding	4	3.18	0.003	*XCL2, PF4V1, PF4, XCL1*	13.25	0.541

This table has been reduced in size and depicts the five most significant associations. A full table is available in the electronic supplementary material within the folder entitled 'DAVID' and has a file name of 'Discovery validation Molecular Functions'.

4.3.7.5 Pathway enrichment

Analysis of molecular functions amongst the differentially regulated genes again presented contrasting results between the cohorts. Pyrimidine and glutathione metabolism, in addition to drug metabolism, were featured in the discovery cohort (Table 4.15). Systemic lupus erythematosus was also prominently linked, primarily through association with the histone-related genes. In contrast, pathways over-represented in the discovery/validation cohort included natural killer cell mediated cytotoxicity, cytokine-cytokine receptor interaction and chemokine signalling (Table 4.16).

Table 4.15: Pathways associated with differentailly regulated genes in the discovery cohort

Term	Percentage of Total Genes	*p* value	Fold Enrichment	Bonferroni
Category: KEGG_PATHWAY				
hsa05322:Systemic lupus erythematosus	1.97	0.002	5.14	0.190
hsa00240:Pyrimidine metabolism	1.69	0.009	4.59	0.597
hsa00480:Glutathione metabolism	1.12	0.030	5.81	0.953
hsa00980:Metabolism of xenobiotics by cytochrome P450	1.12	0.047	4.84	0.993
hsa00982:Drug metabolism	1.12	0.051	4.69	0.995

This table has been reduced in detail. A full table is available in the electronic supplementary material within the folder entitled 'DAVID' and has a file name of 'Discovery Pathways'.

Table 4.16: Pathways associated with differentailly regulated genes in the discovery/validation cohort

Term	Percentage of Total Genes	*p* value	Fold Enrichment	Bonferroni
Category: KEGG_PATHWAY				
hsa04650:Natural killer cell mediated cytotoxicity	6.35	1.63×10^{-4}	6.51	0.009
hsa05332:Graft-versus-host disease	3.18	0.005	11.10	0.250
hsa04060:Cytokine-cytokine receptor interaction	5.56	0.029	2.89	0.816
hsa04062:Chemokine signaling pathway	3.97	0.088	2.89	1.000

This table has been reduced in detail. A full table is available in the electronic supplementary material within the folder entitled 'DAVID' and has a file name of 'Discovery validation Pathways'.

4.3.7.6 Transcription factor binding sites

Analysis of transcription factor binding site over-representation in the discovery cohort (Table 4.17) elucidated four transcription factors (BRN2, FREAC2, NKX61 and RSRFC4) exhibiting binding sites present within 25.28% and 38.48% of analysed genes. However, no transcription factors were statistically significant (p = 0.05) and no transcription factor exceeded a fold enrichment of 1.16. Similar results were apparent in the discovery/validation cohort (Table 4.18), with a maximum fold enrichment of 1.43, although eight genes were statistically significant. Over-represented transcription factor binding sites were present in a significant proportion of differentially regulated genes, ranging from 16.67% to 40.48%.

Table 4.17: Analysis of transcription factor binding site enrichment in the discovery cohort

Term	Count*	Percentage of Total Genes	*p* value	Fold Enrichment	Bonferroni
Category: UCSC_TFBS					
BRN2	137	38.48	0.053	1.12	0.999
FREAC2	90	25.28	0.072	1.16	0.999
NKX61	118	33.15	0.078	1.12	0.999
RSRFC4	127	35.67	0.083	1.11	1.000

*This refers to the number of genes shown to exhibit binding sites for the relevant transcription factor. This is expressed as a percentage in the following column.

Table 4.18: Analysis of transcription factor binding site enrichment in the discovery/validation cohort

Term	Count*	Percentage of Total Genes	*p* value	Fold Enrichment	Bonferroni
Category: UCSC_TFBS					
FREAC2	41	32.54	0.012	1.41	0.878
STAT	43	34.13	0.019	1.35	0.967
HFH3	47	37.30	0.036	1.27	0.998
AP1FJ	36	28.57	0.038	1.35	0.999
HNF3B	47	37.30	0.040	1.27	0.999
STAT5B	39	30.95	0.043	1.31	0.999
FOXD3	40	31.75	0.045	1.30	0.999
IRF1	31	24.60	0.050	1.37	0.999
NFKAPPAB65	27	21.43	0.067	1.38	0.999
MAX	22	17.46	0.076	1.43	0.999
GATA6	21	16.67	0.088	1.43	1.000
RORA2	51	40.48	0.092	1.18	1.000
CART1	51	40.48	0.097	1.18	1.000

*This refers to the number of genes shown to exhibit binding sites for the relevant transcription factor. This is expressed as a percentage in the following column.

4.3.8 qRT-PCR and geNorm analysis

4.3.8.1 geNorm

In order to determine the optimal reference genes for normalisation, geNorm analysis was performed within qbase+. A total of eight samples were used from each of the control and CNP groups in the discovery/validation cohort. Gene expression analysis was conducted as described (section 2.1.2.5). Twelve reference genes (*18S*, *ACTB*, *ATP5B*, *B2M*, *CYC1*, *EIF4A2*, *GAPDH*, *RPL13A*, *SDHA*, *TOP1*, *UBC* and *YWHAZ*) were initially considered. Several genes were not analysed within the geNorm calculations as they were not reliably detected by qRT-PCR (*ATP5B*, *B2M*, *EIF4A2*, *GAPDH* and *RPL31A*). It was highlighted that the geometric mean *CYC1* and *YWHAZ* (Figure 4.2) presented the optimal number of reference genes (geNorm V $\leq$0.15) with high reference gene stability (geNorm *M* $\leq$0.5).

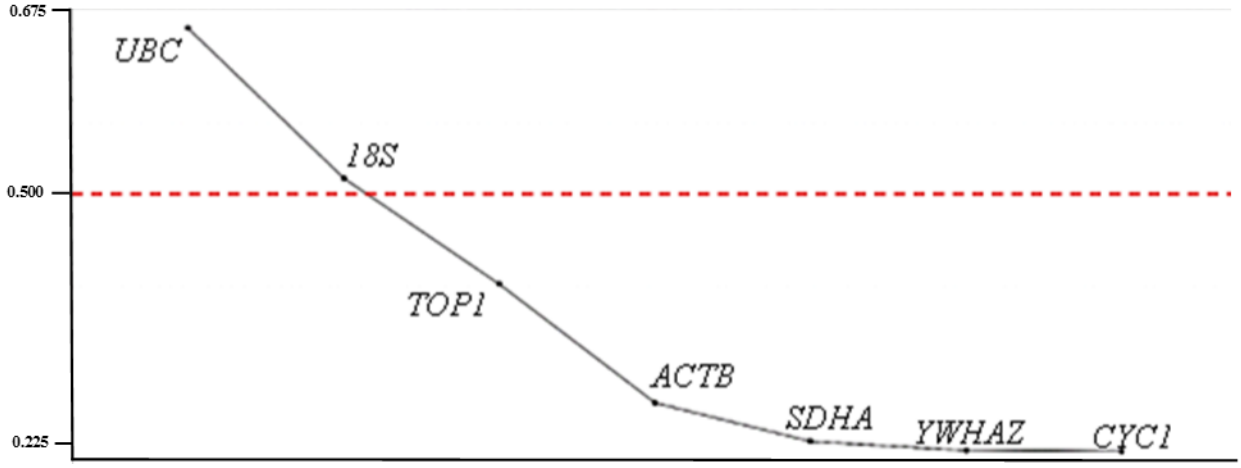

Figure 4.2: geNorm analysis illustrating reference gene stability in the discovery/validation cohort

Assessment of reference gene stability was conducted by qRT-PCR using the geNorm facility within qbase+. After removal of genes unsuitable for analysis, eight genes were analysed, of which *CYC1* and *YWHAZ* were the two reference genes deemed optimal for normalisation within this cohort (geNorm V ≤0.15, geNorm *M* ≤0.5). The geNorm *M* values are additive. For instance, the value corresponding to *ACTB* represents the accumulative stability when considering *ACTB*, *SDHA*, *YWHAZ* and *CYC1* together. The red line illustrates the limit of high reference gene stability (geNorm *M* ≤0.5). Data files are available in the electronic supplementary material within the folder path; qRT-PCR > Clinical samples > Discovery/validation > geNorm.

4.3.8.2 qRT-PCR

Genes found to be differentially regulated and depicted (Table 4.1 and Table 4.2) were subsequently analysed by qRT-PCR. Further analysis by qRT-PCR could not be carried out directly with the discovery cohort due to RNA availability. Therefore, genes arising from the discovery cohort were considered in the discovery/validation cohort, and genes originally differentially expressed in the discovery/validation cohort were analysed in the same cohort alongside the samples not previously studied using microarrays. Several genes in the discovery cohort were similarly differentially expressed in the discovery/validation cohort, particularly *CASP5*, *CLU*, *NLRC4* and *TLR5* (Table 4.19). The inclusion of all samples within the discovery/validation cohort and subsequent analysis by qRT-PCR yielded highly similar results to those observed using microarrays (

Table 4.20).

Table 4.19: qRT-PCR validation of discovery genes in the discovery/validation cohort

Accession Number	Gene Name	Gene Symbol	*p* Value	FC in CNP
NM_004347	Caspase 5	*CASP5*	$3.98x10^{-4}$	↑1.76
NM_001831	Clusterin	*CLU*	$1.30x10^{-8}$	↑2.29
NM_005700	Dipeptidyl-peptidase 3	*DPP3*	0.030	↑1.09
NM_015259	Inducible T-cell co-stimulator ligand	*ICOSLG*	0.099	↑1.16
NM_002386	Melanocortin 1 receptor*	*MC1R*	0.806	↓1.04
NM_021209	NLR family CARD domain-containing protein 4	*NLRC4*	$9.57x10^{-4}$	↑1.25
NM_000608	Orosomucoid 2	*ORM2*	0.080	↑1.67
NM_001039841	Rho GTPase activating protein 11B	*ARHGAP11B*	0.327	↑1.06
NM_152267	Ring finger protein 185	*RNF185*	0.002	↑1.22
NM_003254	TIMP metalloproteinase inhibitor 1	*TIMP1*	0.074	↓1.16
NM_003268	Toll-like receptor 5	*TLR5*	$4.99x10^{-8}$	↑1.68

Differentially regulated genes identified after refinement of microarray data in the discovery cohort were analysed in the discovery/validation cohort by qRT-PCR using all control (n = 24) and CNP (n = 24) samples. *Denotes analysis by ddPCR. Data files are available in the electronic supplementary material within the folder path; qRT-PCR > Clinical samples > Discovery/validation > Group 1/Group 2.

Table 4.20: qRT-PCR analysis of discovery/validation genes in the discovery/validation cohort

Accession Number	Gene Name	Gene Symbol	*p* Value	FC in CNP
NM_001171174	Chemokine (C-X3-C motif) receptor 1	*CX3CR1*	0.819	↑1.02
NM_000878	Interleukin 2 receptor subunit beta	*IL2RB*	$2.2x10^{-5}$	↓1.57
NM_002258	Killer cell lectin like receptor B1	*KLRB1*	0.001	↓1.47
NM_000615	Neural cell adhesion molecule 1	*NCAM1*	0.008	↓1.52
NM_006056	Neuromedin U receptor 1	*NMUR1*	$1.74x10^{-4}$	↓1.62
NM_002620	Platelet factor 4 variant 1	*PF4V1*	0.013	↑1.77
NM_002659	Plasminogen activator, urokinase receptor	*PLAUR*	0.032	↑1.21
NM_000953	Prostaglandin D2 receptor	*PTGDR*	0.160	↓1.17
NM_138554	Toll-like receptor 4	*TLR4*	$2.37x10^{-4}$	↑1.41
NM_024911	Wntless Wnt ligand secretion Mediator	*WLS*	$9.82x10^{-5}$	↑1.86
NM_002995	X-C motif chemokine ligand 1	*XCL1*	0.010	↓1.43

Differentially regulated genes identified after refinement of microarray data in the discovery/validation cohort were analysed in the same cohort by qRT-PCR using all control (n = 24) and CNP (n = 24) samples. Data files are available in the electronic supplementary material within the folder path; qRT-PCR > Clinical samples > Discovery/validation > Group 1/Group 2.

4.3.9 Plasma TIMP1 quantification

The mean (±SD) level of plasma TIMP1 in healthy control subjects was 157.34 (±33.24) ng/mL (range: 100.88-233.58 ng/mL). In contrast, the mean level in CNBP patients (±SD) was 278.48 (±131.44) ng/mL (range: 130.26-546.75 ng/mL). In patients with CIBP, the mean (±SD) was 147.82 (±75.55) ng/mL (range: 82.56-381.41 ng/mL). Plasma TIMP1 concentrations were therefore significantly elevated in patients with CNBP when compared to healthy controls (p = 0.043) and between patients with CNBP and CIBP (p = 0.027) (Figure 4.3). There was no significant change between controls and CIBP patients (Mann-Whitney test) (p = 0.668). When analysing controls, CNBP and CIBP patients together (Kruskal-Wallis test), significance was similarly observed (p = 0.043). Plasma TIMP1 levels for controls and CNBP patients were moderately positive correlated to *TIMP1* mRNA levels isolated from whole blood (r = 0.68, $p = \leq$ 0.05). Age (p = 0.498) and gender (p = 0.995) covariates did not significantly influence TIMP1 levels, as determined by ANOVA and unpaired t-test, repectively.

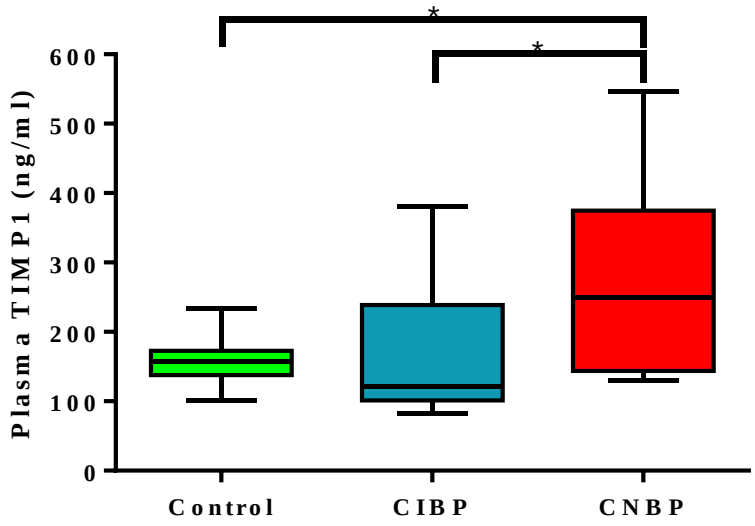

Figure 4.3: Plasma TIMP1 concentrations in healthy controls and patients with either CIBP or CNBP

Analysis of plasma TIMP1 concentrations in healthy controls (*n = 10*), CIBP patients (*n = 12*) and CNBP patients (*n = 10*) was carried out using an ELISA. Diluted plasma samples were exposed to human TIMP1 monoclonal antibody coated wells and treated with human TIMP1 antibody conjugated to biotin. After Streptavidin-Peroxidase treatment, addition of substrate allows for colourmetric detection at 450nm. *$p = \leq 0.05$ (Mann-Whitney). Data is available in the electronic supplementary material within the folder entitled 'ELISA' with the file name 'TIMP1 Discovery'.

The mean (±SD) level of plasma TIMP1 in healthy control subjects was 130.70 (±35.36) ng/mL (range: 73.48-187.30 ng/mL). In contrast, the mean level in CNP patients (±SD) was 116.10 (±40.53) ng/mL (range: 69.40-195.10 ng/mL). In patients with an S-LANSS score of <12, the mean (±SD) was 118.22 (±44.08) ng/mL (range: 71.22-195.10 ng/mL) whilst in those with an S-LANSS score of ≥12, the mean (±SD) was 114.30 (±39.41) ng/mL (range: 69.40-185.60 ng/mL). Plasma TIMP1 concentrations were not significantly different in patients with CNP when compared to controls ($p = 0.190$) and between patients with an S-LANSS score of <12 and ≥12 ($p = 0.801$) (Figure 4.4). There was also no significant change between controls and patients with an S-LANSS score of <12 ($p = 0.406$) and ≥12 ($p = 0.194$).

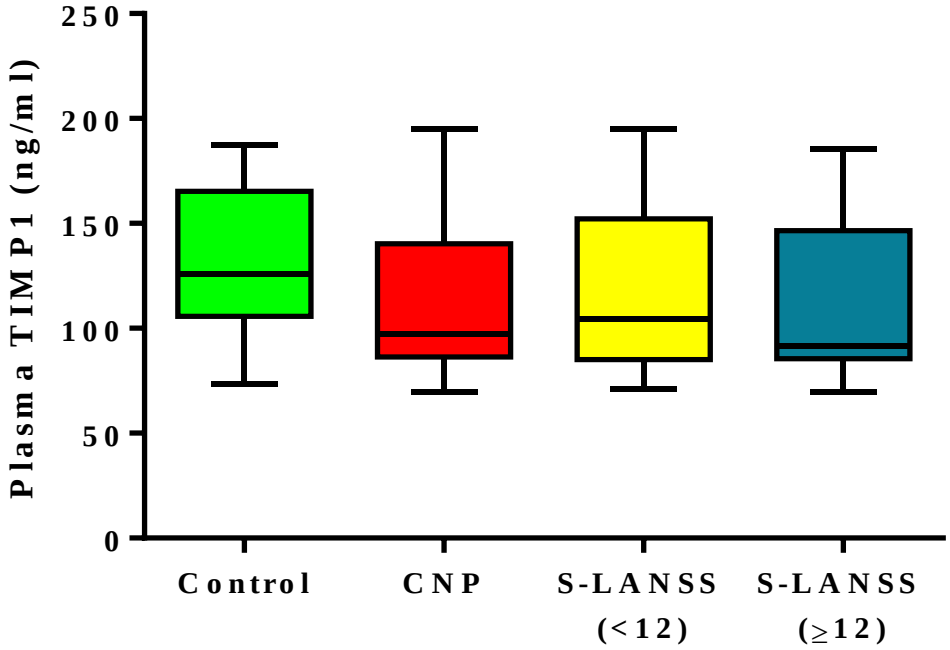

Figure 4.4: Plasma TIMP1 concentrations in healthy controls and patients with CNP with further consideration for the S-LANSS score
Analysis of plasma TIMP1 concentrations in healthy controls ($n = 24$) and CNP patients ($n = 24$) was carried out using an ELISA. Separation according to S-LANSS score included patients scoring below 12 ($n = 10$) and 12 or over ($n = 14$). Diluted plasma samples were exposed to human TIMP1 monoclonal antibody and treated with human TIMP1 antibody conjugated to biotin. After Streptavidin-Peroxidase treatment, addition of substrate allowed for colourmetric detection at 450nm followed by statistical analysis using an unpaired t-test. Data is available in the electronic supplementary material within the folder entitled 'ELISA' with the file name 'TIMP1 Discovery validation'.

4.4 Discussion

4.4.1 Microarray candidate biomarkers

The following candidate genes were differentially regulated in the blood of CNP patients and were selected based on their statistical significance, fold change, literature, and for the discovery cohort alone, correlation analysis with existing microarray data. Several genes are not discussed at length here due to the lack of literature evidence associating the gene with a relevant function or disease. *DPP3*, *MC1R* and *TIMP1* are discussed in chapter 5 considering their heightened potential as translational biomarkers of CNP.

4.4.1.1 Discovery cohort

4.4.1.1.1 Caspase 5

Caspase 5 (*CASP5*) was significantly upregulated in both the discovery (2.23 fold) and discovery/validation (1.41 fold) cohorts. Caspases are endoproteases and regulators of cell death and inflammation. In humans, caspases 1, 4, 5 and 12 are the subset of caspases categorised by their role in inflammation and innate immune responses, and are co-localised to chromosome 11 (q22.2-q22.3). In mice these equate to caspase 1, 11 and 12. It is considered that human *CASP5*, which shares notable sequence homology with *CASP1* (51%) and *CASP4* (74%) (Bian *et al.* 2011), originated from gene duplication in higher species (Nadiri *et al.* 2006). *CASP5* is also considered to be the human ortholog of murine *Casp11* (*Casp4*) (Martinon *et al.* 2002).

Caspases, which are synthesised as pro-enzymes, are activated following the detection of highly conserved pathogen-associated molecular patterns (PAMPs) by TLRs, and other pattern-recognition receptors. This results in the assembly of an inflammasome, a multiprotein complex and component of the innate immune system (Guo *et al.* 2015). Of the three NLR-subset inflammasomes, the NLRP1 inflammasome encompasses CASP5 along with NLRP1, Pycard and CASP1, and is involved in the proteolytic cleavage of pro-IL-1β into its mature form (Cerretti *et al.* 1992, Thornberry *et al.* 1992, Martinon *et al.* 2002, Vigano *et al.* 2015). Other factors can facilitate inflammasome assembly, in particular the NLRP3 inflammasome, including danger-associated molecular patterns (DAMPs). Examples of DAMPs include ATP mediated P2X7 receptor activation, toxins, UV-B, amyloid-β and monosodium urate (McIntire *et al.* 2009). Both amyloid-β and monosodium urate and their role in activation of the NLRP3 inflammasome have been linked to the pathophysiology of Alzheimer's disease (Halle *et al.* 2008, Heneka *et al.* 2013) and the sterile inflammation observed in gout (Busso *et al.* 2010), respectively.

Experimentally, CASP4 and CASP5 have been shown to mediate the release of IL-1α and IL-1β, via TLR4, from human monocytes exposed to LPS (Vigano *et al.* 2015). *In vitro* exposure to IFN-γ has also been shown to upregulate *CASP5* in a colon carcinoma (HT-29) cell line, whilst in human THP-1 cells, LPS-induced *CASP5*, but not *CASP1*, mRNA and protein (Lin *et al.* 2000). Indeed, considering that both *TLR4* and *CASP5* are upregulated within this study, the potential for TL4-mediated regulation *CASP5* in CNP should be considered. Unsurprisingly, accumulating evidence highlights a prominent role for IL-1β in CNP. Upregulation of IL-1β has been observed in the sciatic nerve, DH and poignantly, plasma, after SNI (Gui *et al.* 2016). The release of IL-1β has been attributed to Schwann cells and infiltrating macrophages, leading to reduced activation thresholds and subsequent contributions to spontaneous pain and hypersensitivity (Oliveira Júnior *et al.* 2016). Multiple specific mechanisms have been described, including IL-1β-mediated changes in voltage-gated sodium channels, increased NMDA activity due to receptor phosphorylation and reduced GABA/glycine in the spinal cord (Kawasaki *et al.* 2008b, Oliveira Júnior *et al.* 2016).

CASP4 and CASP5 have been associated with inflammation pertaining to inflammatory bowel disease and as tissue markers of colorectal cancer (Flood *et al.* 2015), with CASP5 also linked to psoriasis (Salskov-Iversen *et al.* 2011), lung cancer (Hosomi *et al.* 2003) and patients with cervical malignancy (Babas *et al.* 2010). As a biomarker of painful conditions, *CASP5* was upregulated in the blood of fibromyalgia patients reporting high pain, in contrast to those with low pain (Lukkahatai *et al.* 2013), and was upregulated in blood of patients with ankylosing spondylitis (Assassi *et al.* 2011). Further analysis is clearly necessary, both in order to clarify if *CASP5* is differentially expressed between patients with predominantly inflammatory and neuropathic pain, and whether attenuation of *CASP5* upregulation or modulation of the inflammasome complex, which has been previous considered (Ozaki *et al.* 2015), may be of therapeutic value in the treatment of pain.

4.4.1.1.2 Clusterin

The extracellular chaperone and complement inhibitor, clusterin (*CLU*), was 1.85 fold upregulated in the discovery cohort. CLU is constitutively expressed and is an abundant protein in various biological fluids, including plasma and CSF (Polihronis *et al.* 1993). CLU has previously been researched in relation to the neuropathology of several diseases, in particular Alzheimer's disease (Thambisetty *et al.* 2010, Schrijvers *et al.* 2011) and Parkinson's disease (Prikrylova Vranova *et*

al. 2010). Elevated levels of CLU have been detected in the CSF of patients with demyelinating neuropathology (Polihronis *et al.* 1993). Interestingly, upregulation of *clu* has been observed in a zebrafish model of neuron-specific cell death (Jeong *et al.* 2014), whilst CLU was also found to influence regenerative processes associated with sensory neurons after sciatic nerve transection (SNT) (Wright *et al.* 2014). Others have demonstrated that, after SNT, activation of the complement cascade occurs alongside increased *Clu* expression in the DH (Liu *et al.* 1995). Increased *Clu* expression was also observed after sciatic nerve crush injury which peaked at 7-14 days post injury and steadily declined towards day 28 (Bonnard *et al.* 1997). The role of chaperone related proteins after nerve injury in the PNS and CNS, including CLU, has been reviewed with multiple summarised studies unanimously demonstrating upregulation of clusterin, predominantly in neurons and glia, after axotomy (Ousman *et al.* 2017). Future work, particularly focused on nerve-blood expression correlations in animal pain models, in addition to validation of observations in larger cohorts of patients with CNBP, will undoubtedly add further clarity to the value of CLU as a pain biomarker.

4.4.1.1.3 Toll-like receptor 5

Toll-like receptor 5 (*TLR5)* was significantly upregulated (1.75 fold) in the discovery cohort and strongly trended ($p = 0.053$) towards upregulation in the discovery/validation cohort. TLRs are an integral constituent of the innate immune response and are highly conserved (Rock *et al.* 1998). Activation of TLRs occurs due to recognition of a specific PAMPs or DAMPs (Akira *et al.* 2004). TLR5 is traditionally considered to be activated by the virulence factor, flagellin, a component of bacterial flagella (Hayashi *et al.* 2001).

The molecular role of TLR5 in disease is emerging with increasing evidence of associations between TLR5 signalling and chronic inflammatory diseases. TLR5 has been identified as an anti-inflammatory target to reduce damage to the lung of patients with cystic fibrosis (Blohmke *et al.* 2008), whilst *TLR5* expression was also decreased in the mucosa of ulcerative colitis patients (Stanislawowski *et al.* 2009). Research has recently begun to unravel a role for TLR5 in neuropathic pain, including observations that *Trl5* knockout mice exhibited notably reduced tactile allodynia after L5 SNL (Stokes *et al.* 2013). One such rationale for the activity of TLR5 in the absence of flagellin is the DAMPs, such as those pertaining to nucleic acids, S100 proteins and high mobility group box 1 (HMGB1). These factors may be released after nreve injury, thereby triggering TLR signalling and subsequent increases in MyD88-depednent proinflammatory

signalling and neuroinflammation (Das *et al.* 2016). Indeed, HMGB1 has been shown to exert regulatory influences on gene expression, including implications on adaptive immunity and inflammatory responses (Lotze *et al.* 2005, Das *et al.* 2016). Both HMGB1 and flagellin have been shown to induce nitric oxide production in peripheral blood mononuclear cells, which was prevented by TH1020, a specific TLR5 inhibitor (Das *et al.* 2016, Yan *et al.* 2016).

Overexpression of *TLR4* and *TLR5* has been observed in the blood patients with ankylosing spondylitis, a chronic inflammatory condition implicating the vertebra of the spine (Assassi *et al.* 2011). This illustrates the potential for blood-based biomarkers of spinal disease, although further analysis would be required to clarify the discriminatory value of *TLR5* for differentiation of predominantly inflammatory and neuropathic pain of various aetiologies.

Although unrelated to the potential role of *TLR5* as a CNP biomarker, novel methodology has been described for TLR5-mediated targeting of A-fibres, which are implicated in neuropathic pain-associated mechanical allodynia (Campbell *et al.* 1988, Kingwell 2015, Xu *et al.* 2015). On the basis that TLR5 is co-expressed alongside neurofilament-200, a marker of myelinated A-fibres (Xu *et al.* 2015), it was observed that coadministration of the TLR5 ligand flagellin, with QX314, a lidocaine derivative, facilitated neuronal entry of the otherwise impermeable blocker of voltage-gated sodium channels with subsequent reductions in mechanical allodynia (Xu *et al.* 2015). Such methodology clearly demonstrates the potential value of biomarker discovery, wherein such targets may then be exploited in the development of novel therapeutics. This may be additionally advantageous should the target be upregulated of the target at the desired site action.

4.4.1.1.4 Other genes

Several other genes in the discovery cohort, although less thoroughly investigated and detailed within scientific literature, have noteworthy associations with pain. *ELF3* was significantly (1.62 fold) upregulated in the discovery cohort. Similarly to *CLU*, the use of the SNT model of neuropathic pain has also highlighted upregulation, in the DRG, of the transcription factor encoding gene, *Elf3*. *Elf3* expression was increased during the initial stress response (30 minutes post-injury) and maintained at comparatively lower levels until the final point of data collection at 14 days after surgery (Li *et al.* 2015).

The NLR Family CARD Domain-Containing Protein 4 (*NLRC4*) was 1.99 fold upregulated in the discovery cohort. NLRC4 is a key factor influencing the assembly of inflammasomes in response to pathogenic microorganisms, which has been discussed in detail with regards to *CASP5* (section

4.4.1.1.1). It has been demonstrated that mice deficient in NLRC4 inflammasomes showed attenuated carrageenan-induced mechanical and thermal hyperalgesia which coincided with reduced levels of IL-1β and CASP1 (Lopes *et al.* 2015). Taken together with the gene expression analysis, these findings suggest that upregulation of *NLCR4*, in addition to *Casp4*, may be useful indicators of injury and inflammatory processes, but further clarification is required to determine their specificity to CNP.

Rho GTPase Activating Protein 11B (*ARHGAP11B*) was upregulated in both the discovery (1.57 fold) and discovery/validation (1.12 fold) cohorts. *ARHGAP11B* is a human-specific gene and arose from a partial duplication of *ARHGAP11A* on the human lineage after the split from chimpanzee, and has been attributed to evolutionary expansion of the human neocortex and cortical reorganisation (Florio *et al.* 2015, Hillert 2015). Deletion of 15q13.3, which encompasses of several genes, including *ARHGAP11A* and *ARHGAP11B, is* associated with mental retardation and seizures (Sharp *et al.* 2008). An association has been postulated between a SNP (rs143536437), which is upstream of *ARHGAP11B*, and remission of epileptic seizures upon commencement of a medication (Speed *et al.* 2014). Aside from associations with epileptic seizures, which share a degree of physiological similarity to CNP, including the convergence of pharmacotherapy, there is little further literature evidence for the role of *ARHGAP11B* in CNP.

Latterly, the inducible T-cell costimulator ligand (*ICOSLG*) was 1.2 fold upregulated in patients with CNP. ICOSLG is expressed on antigen presenting cells with binding to inducible T-cell costimulator (ICOS) facilitating T-cell activation (Dong *et al.* 2001). It has been determined that ICOSLG expression in HUVECs was upregulated in response to TNF-α and IL-1β (Khayyamian *et al.* 2002). Perhaps most intriguing is that a previous study seeking to elucidate correlations in gene expression between blood and ipsilateral lumbar dorsal quadrant proposed *Icoslg* as a putative biomarker of neuropathic pain, considering a combination of gene expression correlations, signalling pathways and correlation to von Frey thresholds (Grace *et al.* 2012).

4.4.1.2 Discovery/validation cohort

4.4.1.2.1 Chemokine (C-X3-C motif) receptor 1

The chemokine (C-X3-C motif) receptor 1 (*CX3CR1*), 1.42 fold downregulated in CNP patients, is a g protein-coupled transmembrane chemokine receptor, abundantly expressed in peripheral blood leukocytes and microglia (Milligan *et al.* 2004). CX3CR1 is the sole receptor for CX3CL1 (fractalkine), a structurally unique chemokine and the only CX3CR1 ligand (Bazan *et al.* 1997,

Hesselgesser *et al.* 1999). CX3CL1 was initially described as a leukocyte adhesion molecule and as a chemoattractant, in a phase dependant (membrane bound or soluble) manner (Bazan *et al.* 1997), although CX3CL1/CX3CR1 signalling is now acknowledged as a key mechanism in neuropathic and central sensitisation (Milligan *et al.* 2004).

Analysis of CX3CL1 and CX3CR1 in the DRG and spinal cord under normal and neuropathic conditions showed that CX3CL1 localised to the extracellular surface of neurons, with no change in expression after induction of both sciatic inflammatory neuropathy and CCI (Milligan *et al.* 2004). A similar study contrasted the distribution of CX3CL1 and CX3CR1 during inflammatory (intra-plantar CFA) and neuropathic (L5 SNL) pain models. In naïve and CFA treated rats, CX3CL1 was localised to neurons, whereas after L5 SNL, localisation was also observed in astrocytes. Similarly, CX3CR1 immunoreactivity was unchanged in microglia of naïve and CFA treated rats, yet significant increases were observed in the DH after L5 SNL (Lindia *et al.* 2005). This suggests that CX3CR1 may be discriminatory between predominantly inflammatory and neuropathic pain.

Microglia-neuron interactions are an established factor critical to the establishment and maintenance of neuropathic pain (Beggs *et al.* 2010). Signalling of the CX3CL1/CX3CR1 pathway is a prominent feature of neuron-microglia communication in both homeostasis and pathological processes (Harrison *et al.* 1998). Cathepsin S, also known to be upregulated in the DH after peripheral nerve injury, has been identified a key component for the maintenance neuropathic pain and microglia activation. It has been suggested that under neuropathic conditions, recurrent stimulation of primary afferent fibres causes activation of the P2X7 receptor, resulting in the release of cathepsin S from microglia. Cathepsin S then mediates cleavage of CX3CL1, which induces p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation in microglia via CX3CR1 binding, thereby leading to the release of pro-nociceptive mediators (Clark *et al.* 2007, Zhuang *et al.* 2007, Clark *et al.* 2009, Clark *et al.* 2010). Interestingly, metalloproteinases have also been described as potential mediators of CX3CL1 cleavage, including MMP-2 (Bourd-Boittin *et al.* 2009) and MMP-9 (Gao *et al.* 2010). Clearly, upregulation of TIMP1 (section 4.3.9 and 5.4.2.3), potentially leading to changes in the MMP-9/TIMP1 axis, may lead to modulation of CX3CL1 cleavage, resulting in implications on pain states.

The use of animal models has provided further evidence for CX3CL1/CX3CR1 signalling in neuropathic pain. Administration of CX3CL1 to the DRG was hypernociceptive, elevating the

production of TNF-α, IL-1β and prostanoids, through activation of satellite glial cells, thereby contributing to the maintenance of inflammatory pain (Souza *et al.* 2013). CX3CR1 deficient and knockout mice display limited behavioural signs of neuropathic pain, including reduced mechanical allodynia and thermal hyperalgesia (Staniland *et al.* 2010), whilst both mechanical allodynia and thermal hyperalgesia were delayed by the administration of CX3CR1 neutralising antibody in sciatic inflammatory neuropathy and CCI models (Milligan *et al.* 2004). Upregulation of *Cx3cl1* was also observed in a rat models of disc herniation (Park *et al.* 2011), a common cause of radicular type pain (Likar *et al.* 2012).

Pharmacological targeting of the CX3CL1/CX3CR1 pathway has previously been identified as having significant potential in the treatment of pain and inflammation (D'Haese *et al.* 2012). The CX3CR1-mediated analgesic effect of exogenous compounds, including resveratrol (Cheng *et al.* 2014) and curcumin (Zheng *et al.* 2011), has been demonstrated. Moreover, gabapentin administration was shown to reduce the degree of CX3CR1 upregulation after intra-articular injection of CFA, reducing spinal microglia activation in the DH (Yang *et al.* 2012). Moreover, a clinical trial of dilmapimod, a p38 MAPK inhibitor, demonstrated efficacy in the treatment of neuropathic pain (Anand *et al.* 2011b). The role of p38 MAPK signalling has been described in CX3CR1-mediated hyperalgesia (Ding *et al.* 2015), and as a mediator of CX3CR1 upregulation after CCI (Lee *et al.* 2010).

An interesting hypothesis pertaining to CX3CR1, which may also extend to other differentially expressed genes in these cohorts, and has been previously eluded to (Old *et al.* 2014, Kulkarni *et al.* 2016), is that differential regulation in blood may be reflective of leukocyte migration to the site of injury. For instance, research analysing vascular inflammation in HIV led to suggestions that apparent monocyte downregulation of CX3CR1 may be a consequence of cells with higher CX3CR1 expression migrating into the vascular wall, thereby leaving monocytes with low CX3CR1 expression to remain within the circulation (Kulkarni *et al.* 2016). Indeed, previous work has defined monocyte populations according to the expression of CX3CR1 (Landsman *et al.* 2009) and affirmed their role in immune-related trans-endothelial migration (Auffray *et al.* 2007, Clark *et al.* 2014). Considering immune cell infiltration is a key contributor to neuroinflammation after nerve injury, changes in cell migration in this scenario may result in apparent downregulation in peripheral blood mononuclear cells, whilst contrasting observations of apparent upregulation in the injured nerve. It is perhaps noteworthy that *CX3CR1* expression anti-correlated with measures of pain severity in the discovery/validation cohort. This may be underpinned by elevated pain

intensity due to increased contributions from infiltrating cells expressing high levels of *CX3CR1*. Simultaneous analysis of blood and nerve gene expression within the same animal model, in order to derive blood-nerve gene expression correlations, would undoubtedly add necessary clarity.

4.4.1.2.2 Neural cell adhesion molecule 1

Neuronal cell adhesion molecule 1 (*NCAM1*) was significantly downregulated (1.23 fold) in the discovery/validation cohort. In contrast, a non-significant ($p = 0.090$) trend towards upregulation (1.16 fold) was observed in the discovery cohort. NCAM1 is glycoprotein localised to the surface of various cells, including neurons and glia, and possesses a variety of functions centred around cell-cell interactions, including cell adhesion, guidance, differentiation and synapse formation during neuronal growth (Senkov *et al.* 2006, Stoenica *et al.* 2006, Weledji *et al.* 2014).

NCAM1 exhibits immunoglobulin-like extracellular domains, which are involved in homophilic binding with NCAM1, and fibronectin type III domains, which have been shown to contribute to neurite outgrowth through interactions with the fibroblast growth factor receptor (FGFR) (Kiselyov *et al.* 2005) (Weledji *et al.* 2014). Further recruitment of neuronal cadherin to the NCAM1-FGFR complex leads to signalling cascades promoting neurite outgrowth (Cavallaro *et al.* 2004). However, NCAM1 has also been shown to interact with glial derived neurotrophic factor (GDNF). The GDNF ligand family consist of neurotrophic growth factors influencing neuronal survival, neurite outgrowth, and differentiation (Airaksinen *et al.* 1999, Baloh *et al.* 2000, Paratcha *et al.*). Signalling-induced by a GDNF family ligand is generally mediated by RET, a receptor tyrosine kinase (RTK), in conjunction with a GDNF family receptor (e.g. GFRα1) (Goodman *et al.* 2014). However, the expression of GFRα receptors in regions of the CNS and PNS wherein RET is not expressed has long been suggested as indicative of alternative GDNF signalling mechanisms, independent of RET (Trupp *et al.* 1997, Trupp *et al.* 1999, Paratcha *et al.*). There is robust evidence that NCAM1, in conjunction with GFRα receptors, function as a signalling receptor for GDNF ligands independent of RET (Paratcha *et al.* 2003a), wherein interactions between NCAM1 and GFRα1 result in conformational changes that favour binding with GDNF ligands rather than homophilic NCAM1 interactions (Paratcha *et al.* 2003a). Indeed, molecules necessary for GDNF-derived signalling exhibit widespread expression within nociceptive pathways of the nervous system, with evidence of differential regulation after nerve injury (Nolte *et al.* 1999, Bennett *et al.* 2000).

The molecular function NCAM1, which is highly expressed in the superficial DH (Sakai *et al.* 2008b), and the related implications of downstream signalling, are both diverse and complex. Associations between NCAM1 and CNP have been described with particular focus on role of NCAM1 in conferring the analgesic properties of GDNF after nerve injury (Sakai *et al.* 2008b). The potent analgesic effects of GDNF in neuropathic pain (Boucher *et al.* 2000, Wang *et al.* 2003, Sakai *et al.* 2008a) are thought, at least partially, to be underpinned by the reversal of changes that occur after nerve injury, including GDNF-induced changes in sodium channel, $P2X_3$ purinoreceptor and neuropeptide expression (Bradbury *et al.* 1998, Costigan *et al.* 2003, Wang *et al.* 2003). Furthermore, administration of antisense oligonucleotide reduced *Ncam1* expression after CCI and abolished the analgesic effect of GDNF (Sakai *et al.* 2008b) whilst intrathecal administration of GDNF in animal models of neuropathic pain successfully reversed heightened sensitivity to nociceptive stimuli to basal levels (Nagano *et al.* 2003).

NCAM1 intracellular signalling is calcium dependent (Kiryushko *et al.* 2006) and as previously described, is thought to be mediated by the FGFR, a member of the receptor tyrosine kinase (RTK) family. As such, inhibitors of RTK or protein tyrosine kinases (e.g. Src), may impact upon NCAM1 signalling. Indeed, lavendustin A, an RTK and protein tyrosine kinase inhibitor, prevented Ca^{2+} influx and neurite outgrowth (Kiryushko *et al.* 2006), which has previously been described as potential method of supressing sympathetic spouting and thereby ameliorating CNP (Todoroki *et al.* 2004). Unsurprisingly, modulation of GDNF signalling has also been highlighted as a potential avenue for the treatment of CNP (Boucher *et al.* 2001, Dinah *et al.* 2005). Administration of Cd3, a NCAM1 mimetic, ameliorated pain severity after CCI (Sakai *et al.* 2008b) whilst agonist-induced enhancement of GDNF family receptor-α (*GFRα*) indicated promise in disease models of small-fibre neuropathy (Hedstrom *et al.* 2014).

4.4.1.2.3 Neuromedin U receptor 1

Neuromedin U receptor 1 (*NMUR1*) was significantly downregulated (1.20 fold) in the discovery/validation cohort. NMUR1, similarly to NMUR2, is a g protein-coupled receptor with a range of physiological functions, including roles in smooth muscle contraction, blood pressure, gastric acid secretion and cancer (Brighton *et al.* 2004). There are two neuromedin U receptor ligands, with somewhat contrasting receptor affinities. Neuromedin U is a highly conserved and ubiquitously expressed neuropeptide, originally discovered in porcine spinal cord (Minamino *et*

al. 1985). In contrast, neuromedin S, a structurally similar peptide to neuromedin U, exhibits considerably greater affinity for NMUR2 (Mori *et al.* 2005).

High neuromedin U-like immunoreactivity has previously been observed in the spinal cord, with particularly notable levels in the DH, in addition to the DRG (Domin *et al.* 1987), suggesting a role of neuromedin U in sensory pathways (Domin *et al.* 1987). Subsequent analysis of the expression patters of neuromedin U receptors have led to the summarisation that NMUR1 and NMUR2 are predominantly expressed in the periphery and CNS, respectively (Brighton *et al.* 2004). However, *Nmur1* expression has been described within small/medium diameter neurones in the rat DRG (Yu *et al.* 2003) and widely throughout the CNS, particular in the cerebellum, spinal cord and DRG, although the levels observed here remain significantly lower than those observed in peripheral tissues (Raddatz *et al.* 2000, Szekeres *et al.* 2000). Studies have also unveiled neuromedin U binding sites localised to lamina I and the outer section of lamina II in the DH, although it was unclear whether such binding pertained to NMUR1 or NMUR2 (Yu *et al.* 2003).

Considering the localisation of neuromedin U receptors, multiple studies have sought to detail a potential role for these receptors in pain sensitivity. Intrathecal administration of neuromedin U to the rodent has been shown to increase behavioural signs of elevated nociception, including thermal hyperalgesia (Cao *et al.* 2003, Yu *et al.* 2003). Systemic administration of neuromedin U resulted in electrophysiological changes in the DH, suggestive of elevated basal activity and heightened responsiveness to noxious stimuli (Cao *et al.* 2003). Subsequent research using rodents with natural or induced deficiencies in neuromedin receptors have provided a degree of clarity relating the role of each receptor in sensory pathways. The serendipitous discovery of a polymorphism in the rat genome resulting in non-functional NMUR1 initially provided a degree of insight. Rats homozygous for the *Nmur1* variant allele failed to exhibit membrane bound NMUR1, suggesting a failure in protein trafficking (Panetta *et al.* 2013). As such, in homozygous variant rats, peripheral administration of neuromedin U no longer caused thermal hypersensitivity, whilst central administration to the spinal cord also failed to induce increases in spinal excitability when compared to wild-type rats (Panetta *et al.* 2013). Methodology compromising of gene knockout models has, however, produced mixed outcomes. It has been demonstrated that knockout of *Nmur2*, rather than *Nmur1*, led to a reduction in behavioural responsiveness to thermal stimuli (hot plate), reduced thermal hyperalgesia after capsaicin injection and resilience to pain during the formalin test (Torres *et al.* 2007). In contrast, the use of wild-type and *Nmur2* knockout mice failed

to demonstrate an influence for NMUR2 on hypersensitivity after both spared tibial nerve injury and CFA-induced inflammation (Gilbert *et al.* 2013).

Efforts to elucidate an antagonist of the neuromedin U receptors have, thus far, seen modest progress. Despite high binding site conservation between NMUR1 and NMUR2, an inhibitor derived by Liu *et al* was found to exhibit >200 fold selectivity for NMUR2 (Liu *et al.* 2009). It was determined that subsequent inhibition of exogenous neuromedin U was incomplete, leading suggestions that limited efficacy may be attributable to lack of NMUR1 inhibition (Liu *et al.* 2009).

4.4.1.2.4 Plasminogen activator receptor

The urokinase-type plasminogen activator receptor (*PLAUR*) was 1.21 fold upregulated in CNP patients. Multiple studies have been conducted elucidating various pathophysiological roles for PLAUR, including those with potential relevance to neuroinflammation and pain. Soluble PLAUR has been associated with disruption of the BBB, facilitating chemotaxis of inflammatory/immune cells (Garcia-Monco *et al.* 2002) and has been shown to be elevated in cancer, infection, and to a lesser degree, demyelinating disease (Garcia-Monco *et al.* 2002). Further analysis subsequently demonstrated upregulation of *Plaur* in relation to intervertebral disc degeneration (Krock *et al.* 2014), whilst *Plaur* knockout mice displayed impaired nerve regeneration after sciatic nerve crush injury (Rivellini *et al.* 2012). Perhaps conversely, reduced soluble PLAUR levels in patients with peripheral neuropathies when compared to the levels observed in separate cohorts of health individuals (Brunner *et al.* 1999).

4.4.1.2.5 Toll-like receptor 4

Toll-like receptor 4 (*TLR4*) was 1.36 fold upregulated in the discovery/validation cohort. TLR4 is a cell surface receptor which is activated by the exogenous ligand, LPS, and has a key role in the induction of cytokines (Kaisho *et al.* 2002). Unlike TLR5, TLR4 exhibits a range of adaptor proteins including Myd88, Tirap, TRIF and TRAM. Evidence suggests that, in addition to responsiveness to PAMPs such as LPS, TLR4 signalling may be initiated by endogenous factors such as peptidoglycans and the heat shock protein family (Vabulas *et al.* 2002, Tsan *et al.* 2004, Gay *et al.* 2007, Liu *et al.* 2012). TL4-mediated intracellular signalling is also divided into two somewhat convergent pathways, regarded as either MyD88 dependent or independent, leading to activation of interferon regulatory factor 3 (IRF-3) and NF-κB (Bettoni *et al.* 2008). This results

in transcriptional changes, including upregulation of genes encoding proinflammatory cytokines, *PTGS2* and *NOS2* (Bettoni *et al.* 2008).

The expression of *Tlr4* has been described within the rodent CNS (Eklind *et al.* 2001, Laflamme *et al.* 2001) with further research identifying TLR4 expression localised to microglia (Lehnardt *et al.* 2002, Lehnardt *et al.* 2003). It is suggested that after L5 nerve transection, activation of TLR4 precedes enhancement of neuroimmune interactions and cytokine upregulation, with potential contributions to pain hypersensitivity (Tanga *et al.* 2005). Clearly the sterile inflammation associated with nerve injury negates a role for LPS in TLR4 signalling. There have been several postulated activators of TLR4, including saturated fatty acids released after nerve injury, reactive oxygen species and DAMPs (Ma *et al.* 2002, Lee *et al.* 2003, Lee *et al.* 2004, Tanga *et al.* 2005, Li *et al.* 2014).

Multiple studies, predominantly using rodent models of neuropathic pain, have demonstrated various associations between TLR4 and CNP. Upregulation of *Tlr4* was observed in the lumbar spinal cord after transection of the L5 spinal nerve, which peaked at 14 days post-injury and returned to basal levels by 28 days (Tanga *et al.* 2004). Moreover, the influence of spinal TLR4 on behavioural measures of neuropathic pain was subsequently affirmed by observations of attenuated behavioural signs. These included reduced expression of microglial markers and cytokines in both *Tlr4* knockout models and mice treated with *Tlr4* antisense oligodeoxynucleotide (Tanga *et al.* 2005).

In addition to reductions in proinflammatory cytokine expression, decreased *TLR4* expression has been shown to coincide with reduced expression of β_2-integrins, including *ITGAM*, thereby compromising the innate immune response by hampering leukocyte adhesion and migration in response to inflammatory stimuli (Tanga *et al.* 2005). Interestingly, activated leukocyte adhesion molecule (*ALCAM*) and epithelial cell adhesion molecule (*EPCAM*) were both significantly downregulated in the discovery cohort. Intracellular adhesion molecule-1 (*ICAM1*) and *NCAM1* trended towards upregulation in the discovery cohort, with the latter significantly downregulated in the discovery/validation cohort. Clearly further research is necessary to determine if the patterns of expression between *TLR4* and integrins are of pathophysiological importance. Such mechanisms, encompassing the regulation of integrins, may also be of importance in the apparent changes in *CX3CR1* expression.

TL4 has also been identified as a potential mediator of chemotherapy-induced neuropathic pain (CINP), with particular reference to paclitaxel. TLR4 has been shown to induce a similar pattern of cytokine regulation in response to specific chemotherapeutic agents when compared to LPS (Li *et al.* 2014), thereby implicating TLR4 signaling in the induction and maintenance of paclitaxel-related CIPN (Li *et al.* 2015). Research has shown upregulation of TLR4 in the DRG, alongside the associated signalling molecules, MyD88 and TRIF, in a model of CINP, with subsequent antagonism of TLR4 leading to attenuation of mechanical allodynia (Li *et al.* 2014).

Pharmacological targeting of TLR4 commenced with endeavours to synthesise lipid A analogues, which is based upon the integral role of the phosphorylated disaccharide core in the activity of LPS, which resulted in the development of two TLR4 antagonists (Kawata *et al.* 1999, Mullarkey *et al.* 2003). Antagonism of TLR4 in rodent models of neuropathic pain has since demonstrated attenuation of injury-induced allodynia and hyperalgesia by preventing NF-kB activation and subsequent upregulation of proinflammatory cytokines (Bettoni *et al.* 2008, Lin *et al.* 2015). However, the risk of potentially serious immunosuppression should be considered with regards to systemic TLR4 inhibition (Jordan *et al.* 2008).

4.4.1.2.6 Chemokine (C motif) ligand

The chemokine (C motif) ligand (*XCL1*) was significantly downregulated (1.35 fold) in the discovery/validation cohort. Both XCL1 and XCL2 (which was also significantly downregulated in the same cohort) interact with a single g protein-coupled receptor (X-C motif chemokine receptor 1; XCR1) (Yoshida *et al.* 1998). A high degree of homology is observed between XCL1 and XCL2, which differ by only two amino acids (Yoshida *et al.* 1996, Lei *et al.* 2012).

The role of the XCL-XCR axis in the immune response has been extensively reviewed (Lei *et al.* 2012). Upregulation of *Xc1*, alongside simultaneous increases in CD4+ and CD8+ T cell populations within the prostate have been observed 30 days after induction of experimental autoimmune prostatitis, an animal model of chronic prostatitis/chronic pelvic pain syndrome (Quick *et al.* 2012). XCL1 has also been investigated in rheumatoid arthritis (Blaschke *et al.* 2003) and was upregulated in tissue of patients with Crohn's disease, which was attributed to associated T-cells, mast cells and dendritic cells (Middel *et al.* 2001).

Akin to other chemokines, XCL1 exhibits chemotactic properties, promoting the recruitment of lymphocytes through interaction with its receptor, XCR1 (Obara *et al.* 2013), which suggests that XCL1 may influence neuroimmune interactions after nerve injury. Multiple studies have

highlighted potential roles for the XCL-XCR axis in neuropathic pain. Dense localisation of XCR1 has been observed in laminae I and II within the DH, which infers that XCR1 may be expressed at terminals of excitatory interneurons within the DH, or on small diameter primary afferent fibres (Obara *et al.* 2013). Indeed, a role for XCL1 in central sensitisation has been discussed (Iannitti *et al.* 2014). *In vitro* analysis has previously highlighted upregulation of *Xcl1* in primary microglial cultures exposed to LPS (Zychowska *et al.* 2016), whilst *in vivo* studies have shown enhanced nociceptive transmission after XCL1 administration in naïve mice (Zychowska *et al.* 2016). Moreover, minocycline-induced microglial inhibition following implementation of a streptozotocin-induced mouse diabetic neuropathic pain model has been shown to reduce allodynia and hyperalgesia, alongside attenuated XCL1 and XCR1 upregulation (Zychowska *et al.* 2016). The potential role of the XCL-XCR signalling in CNP, as both a biomarker and therapeutic target, is therefore undoubtedly worthy of further exploration.

4.4.1.2.7 Other genes

Several other genes were also differentially regulated in the discovery/validation cohort, although current evidence associating them with CNP is somewhat anecdotal. Platelet factor 4 variant 1 (*PF4V1*) was 1.32 fold upregulated in patients with CNP. PF4V1 is a member of the chemokine family with antiangiogenic (Sarabi *et al.* 2011) and chemotactic activity, mediated through CXCR3 (Struyf *et al.* 2011). Evidence for associations with pain is limited to observations of differential regulation in whole blood of fibromyalgia patients demonstrating a high degree of pain catastrophising (Lukkahatai *et al.* 2013), and in patients with complex regional pain syndrome (CRPS) (Jin *et al.* 2013). This does however, illustrate potential as a peripherally accessible pain biomarker, although further research is necessary to determine its value as a biomarker of CNP.

The prostaglandin D2 receptor (*PTGDR*) and the Interleukin 2 receptor subunit beta (*IL2RB*) were downregulated in CNP patients, 1.35 and 1.31 fold, respectively. Evidence for literature associations with CNP are largely indirect. Prostaglandins contribute to sensitisation of peripheral and central neurons (Yaksh *et al.* 1999, Popp *et al.* 2009) and have been implicated in allodynia (Minami *et al.* 1994, Yaksh *et al.* 1999). Similarly, research has demonstrated upregulation of *IL-2* in the blood of patients with painful neuropathy when compared to both healthy controls and patients with painless neuropathies (Uceyler *et al.* 2007). Current evidence also suggests a role for IL-2 in the treatment of CNP. *IL-2* gene therapy has been considered in animal models (Yao *et al.*

2002), whilst a case report highlighted considerable therapeutic benefit to subcutaneous IL-2 in PHN (Rotty *et al.* 2006a).

The Wntless Wnt ligand secretion mediator (WLS) was 1.51 fold upregulated in CNP. *WLS* is a highly conserved gene encoding an opioid receptor (OPRM1) interacting protein (Wayman *et al.* 2008). Zebrafish models have demonstrated differential regulation of a range of genes after exposure to morphine, including downregulation of *wls* (Herrero-Turrion *et al.* 2014). Evidence suggest that exposure to morphine-induced a shift towards cell membrane localisation of WLS, promoting WLS and OPRM1 dimerisation, thereby limiting the availability of WLS for WNT signalling and increasing membrane bound OPRM1 availability for morphine binding (Reyes *et al.* 2012). Unsurprisingly, WLS has been proposed as a potential target in the treatment of opiate addiction and pain (Reyes *et al.* 2012) whilst prominent roles for WNT signalling in rodent models of neuropathic pain have been described (Zhang *et al.* 2013, Itokazu *et al.* 2014).

4.4.2 Gene ontology analysis

4.4.2.1 Biological processes

Analysis of over-represented biological processes within the discovery cohort highlighted multiple associations pertaining to core biological processes. The association of these biological processes to CNP is largely unclear. Pyrimidine nucleotides have previous been considered as a therapy for diabetic polyneuropathy, with modest benefit in a small scale trial (Muller 2002), although the study was insufficiently robust to derive meaningful conclusions. This is in stark contrast to the disease associations within the discovery/validation cohort wherein a plethora of biological processes were over-represented. Both immune response and cell surface receptor linked signal transduction were prominently, and highly significantly, featured. Neuro-immune interactions are an established mechanism and feature of neuropathic pain (Calvo *et al.* 2012).

Within the genes association with immune response, research seeking to elucidate the apparent association between TLRs and CNP has garnered significant interest (Kim *et al.* 2009b, Liu *et al.* 2012) and has been discussed at length in this chapter (section 4.4.1.1.3 and 4.4.1.2.5). Similarly to the case of the TLRs, consideration for the role of chemokines in CNP is becoming increasingly prominent (White *et al.* 2007). In addition to downregulation *CX3CR1*, similar downregulation regulation of both *XCL1* and *XCL2* was observed in patients with CNP in the discovery/validation cohort. Again, the reputed contributions of these genes has been discussed in this chapter (Zychowska *et al.* 2016).

Genes associated cell surface receptor linked signal transduction have frequently been associated with pain. Analysis of peripheral blood gene expression in fibromyalgia patients previously identified *SERPING1*, in addition to *CASP5*, as significantly differentially regulated between patients reporting high and low pain severity, whilst both *PF4V1* and *CLEC4D* were differentially expressed between high and low pain catastrophising groups (Lukkahatai *et al.* 2013). Moreover, analysis of peripheral blood gene expression in patients with CRPS highlighted differential regulation of *CD160*, *FOXO3* and *PF4V1* (Jin *et al.* 2013).

Interestingly, a study seeking to elucidate the role of RGS18 in platelet function found that *Rgs18* knockout mice displayed no observable differences to wild-type mice, apart from behavioural changes suggestive of increased pain sensibility (Delesque-Touchard *et al.* 2014). Upregulation of *Rgs4* has been observed in the lumbar spinal cord after partial sciatic nerve ligation with evidence that overexpression also leads to morphine insensitivity (Garnier *et al.* 2003). Indeed, the potential for pharmacological modulation of g protein signalling (RGS) proteins has been discussed in relation to the treatment of CNS diseases (Roman *et al.* 2011, Turner *et al.* 2012) with RGS inhibitors proposed as a means of potentiating opioids, allowing dose reduction and reducing opioid-induced side effects (Neubig *et al.* 2002).

It is also noteworthy that, in addition to *CX3CR1*, *NMUR1*, *MS4A2* and *NCAM1*, which are discussed in this chapter, the IFN-inducible GTPase, *Gbp2*, was previously shown to be upregulated after sciatic nerve injury, inferring elevated IFN-γ signalling. Interestingly, cannabinoid receptor 2 knockout mice demonstrated significantly greater *Gbp2* upregulation, interfering cross-talk between cannabinoid and IFN signalling (Racz *et al.* 2008).

4.4.2.2 Cellular component

Over-represented categories within the cellular component analysis predominately identified core biological processes, including DNA-protein complexes, chromatin and nucleosomes, within the discovery cohort. Genes relating to histones were a common feature of all significant cellular component analysis outputs. The consequences of DNA methylation often result in changes to transcription factor binding and the docking of methyl-CpG-binding domain proteins (MBDs), leading to the recruitment of transcriptional repressors or activators (Liang *et al.* 2015). It has been shown that changes in DNA methylation after nerve injury may evoke, or contribute to, neuropathic pain with *Mbd2* expression in the lumbar spinal cord of the rat decreased 14 days after CCI (Wang *et al.* 2016). Moreover, studies have also shown that nerve injury induces acetylation

of histone H3 and H4 in the brain-derived neurotrophic factor (*Bdnf*) promoter, leading to *Bdnf* upregulation in the DRG (Uchida *et al.* 2013). Indeed, there is plethora of literature associating BDNF with CNP (Geng *et al.* 2010, Siniscalco *et al.* 2011, Chen *et al.* 2014).

Epigenetic changes to histones in infiltrating macrophages have also been shown to facilitate the upregulation of the chemokine ligands, *Ccl2* and *Ccl3,* in the sciatic nerve after partial sciatic nerve ligation (Kiguchi *et al.* 2013). Intrathecal administration of histone deacetylase inhibitors have been shown to attenuate both CFA-induced inflammatory hyperalgesia (Bai *et al.* 2010) and neuropathic pain after CCI (Kukkar *et al.* 2014), with reduced thermal and mechanical hypersensitivity observed in traumatic nerve injury and drug-induced peripheral neuropathy model of neuropathic pain (Denk *et al.* 2013).

Cellular component analysis of genes in the discovery/validation cohort demonstrated that genes pertaining to the plasma membrane were highly significantly over-represented, which includes multiple genes discussed in detail within this thesis, including *CX3CR1* (section 4.4.1.2.1), *NCAM1* (section 4.4.1.2.2), *NMUR1* (section 4.4.1.2.3) and *TLR4* (section 4.4.1.2.5). Such over-representation is perhaps an indicator of continuing cell-cell interaction and cell signalling cascades. Similar observations have also been made after sciatic nerve injury in the rat. Analysis of differentially regulated genes in the DRG using DAVID determined that the majority of differentially regulated genes were associated with the cytoplasm and cellular membrane (Raju *et al.* 2014).

4.4.2.3 Disease associations

Disease association analysis highlighted that four genes in the discovery cohort were associated with multiple sclerosis susceptibility and/or clinical phenotype with SNPs in *PDZRN4* and *SH3GL2* also significantly associated with multiple sclerosis susceptibility (Baranzini *et al.* 2009). Forkhead box O3 (FOXO3) has been linked with neuroinflammation through modulation of CD4 T-cell differentiation, with FOXO3 deficiency ameliorating the severity of autoimmune encephalomyelitis, an animal model of multiple sclerosis (Stienne *et al.* 2015). Indeed, infiltration of T-cells and subsequent signalling has been observed in the spinal cord after peripheral nerve injury (Costigan *et al.* 2009a). Moreover, research has highlighted *Sh3gl2* to be predominantly expressed in neuronal tissue with expression detailed within the DH and DRG, thereby suggesting a potential role in nociceptive pathways (Fortin *et al.* 2010). A quantitative trait locus spanning 14

mb and consisting of 39 genes, including *Sh3gl2*, were also found to be associated with pain sensitivity (Fortin *et al.* 2010).

Both asthma and HIV feature as over-represented by association with the differentially regulated genes in the discovery/validation cohort. The presence of asthma, which showed the greatest degree of over-representation of associated molecules, is perhaps indicative of a commonality in the underlying inflammatory responses associated with asthma and CNP; both of which feature chemokines and proinflammatory cytokines (Ishmael 2011, Ramesh *et al.* 2013). Furthermore, rhe granulocyte specific genes, *FCER1A* and *MS4A2*, were upregulated in CNP patients, assigned as associated with asthma in this analysis, and have previously shown to be downregulated in blood of patients with fibromyalgia (Jones *et al.* 2016). Similarly, it is uncertain whether the over-representation of genes associated with multiple sclerosis and HIV is indicative of the mechanisms associated with neuropathic pain, as both multiple sclerosis and HIV are synonymous with neuropathic pain (Foley *et al.* 2013, Liu *et al.* 2016).

4.4.2.4 Molecular functions

Analysis of molecular functions associated with the discovery cohort highlighted glutathione transferase activity as enriched. The role of glutathione S-transferases (GSTs) is established in the metabolism of xenobiotics (Hinson *et al.* 2010). Specific rationale for the upregulation of GSTs in patients with CNP is uncertain, though modest evidence of an association exists. GST activity was shown to increase after CCI, and after subsequent treatment with N-acetylcysteine (NAC), a precursor to L-cysteine in the biosynthesis of glutathione, behavioural measures of hyperalgesia returned to baseline, whilst simultaneously reducing levels of nitric oxide metabolites (Horst *et al.* 2014). In contrast, treatment with glutathione has demonstrated modest efficacy in the prevention of diabetic neuropathy in streptozotocin-induced diabetic rats (Bravenboer *et al.* 1992). After administration of NAC prior to CCI, Wallerian degeneration was attenuated through a glutathione-mediated mechanisms. This led to suggestions that pre-emptive treatment with NAC may reduce the risk or intensity of post-surgical painful nerve injury (Wagner *et al.* 1998). Inducers of GSTs include a plethora of xenobiotics and chemicals, often influencing gene expression via antioxidant and xenobiotic-response elements (Hayes *et al.* 1995, Hodges *et al.* 2015). It is plausible that exposure to specific xenobiotics or chemicals, which contrast between the controls and CNP patients, contributed to the observed differential regulation of GSTs.

Molecular function analysis pertaining to the discovery/validation cohort highlighted multiple functions contrasting to those determined in the discovery cohort. The association between carbohydrate binding and chronic neuropathic pain is perhaps less apparent than other significantly associated functions, such as chemokine activity and chemokine receptor activity. Studies have previously described a potential role for galectins, a family of carbohydrate binding proteins with various biological functions (Camby *et al.* 2006). For instance, galectin-1 has been associated with nociceptive neuronal development, changes in thermal sensitivity (McGraw *et al.* 2005) and was found to potentiate neuropathic pain after peripheral nerve injury (Imbe *et al.* 2003). Although galectins were not differentially expressed in the analysis, a potential role in CNP is suggested.

4.4.2.5 Pathway enrichment

Analysis of enriched pathways associated with the discovery cohort highlighted similar associations to molecular function analysis. Prominently over-represented pathways include glutathione and xenobiotic metabolism, by virtue of differentially expressed GSTs. Systemic lupus erythematosus, an autoimmune disease, represented the pathway with greatest association within the differentially expressed genes list. This was underpinned by range of differentially regulated histone-related genes.

Pathways highlighted in the discovery/validation cohort were also similar to previous gene ontology analysis, with cytokine-cytokine receptor interaction and chemokine signalling both over-represented. Other enriched pathways pertained to graft-versus-host disease (GVHD) and natural killer cell mediated cytotoxicity. Peripheral neuropathy has been described as a severe manifestation of GVHD (Greenspan *et al.* 1990) and an accumulation of natural killer cells in the sciatic nerve has been observed in rat models of mononeuropathy (Cui *et al.* 2000). In contrast to the discovery cohort, over-represented pathways in this cohort point to prominent cytokine/chemokine involvement, reaffirming previous studies illustrating both their role in CNP and their value, albeit not equivocal, as peripherally accessible biomarkers of CNP (Backonja *et al.* 2008, Ramesh *et al.* 2013, Bäckryd 2015).

4.4.2.6 Transcription factor binding sites

Analysis of over-represented transcription factor binding sites pertaining to the differentially regulated genes in the discovery cohort highlighted four modestly enriched transcription factors, although none achieved statistical significance. The forkhead-related activator 2 (FREAC2) is a transcriptional activator (Rossetti *et al.* 2007) which interacts with TATA-binding protein and transcription factor II B (TFIIB). Intestinal FREAC2 has been associated with murine Wnt

signalling and extracellular matrix production (Takada *et al.* 2007), but definitive associations with CNP are lacking. There is also little evidence pertaining to the other transcriptions factors, although differential regulation of *Brn2* has been observed in remyelinating Schwann cells, regenerating transected nerve and in Schwann cells following upregulation of cAMP (Sim *et al.* 2002).

Transcription factor binding site enrichment analysis was similarly conducted with genes in the discovery/validation cohort. A total of 13 transcription factor binding sites were identified as enriched amongst the differentially expressed genes. In contrast to previous findings, over-represented transcription factors in this cohort tended to have more defined associations with inflammatory processes and CNP. Evidence regarding the role of JAK/STAT signalling in pain has been extensively reviewed (Busch-Dienstfertig *et al.* 2013). Research has demonstrated that SNL triggers upregulation of *Il-6* in the DRG and DH, resulting in elevated JAK/STAT3 signalling in spinal microglia and subsequent contributions to CNP (Dominguez *et al.* 2008).

In addition to the widely documented role of NF-κB and related factors in neuroinflammation (Shih *et al.* 2015), research has also demonstrated marked upregulation of IRF-8 in the spinal cord after nerve injury. It has been suggested that IRF-8 regulates the expression of various genes responsible for the transition of microglia towards a reactive phenotype (Masuda *et al.* 2012), thereby representing a potentially key mediator of CNP development. Further research has also highlighted that upregulation of *Irf-1*, in the ipsilateral spinal cord after peripheral nerve injury, is IRF-8-dependent (Masuda *et al.* 2015). It was subsequently deduced that IRF-8-mediated Il-1β upregulation is IRF-1-dependent, with multiple other genes, including *Cx3cr1* and *Tlr2*, also considered as regulated by IRF-1/IRF-8 (Masuda *et al.* 2012, Masuda *et al.* 2015). The transcription factor IRF-1 is therefore potentially integral to the contributions of microglia to neuropathic pain, and may underpin the observed differential regulation of several genes in this study.

4.4.3 Limitations and future considerations

The clearest limitation of the presented study is cohort size. Considering the purpose of this preliminary research was to elucidate a group of genes differentially regulated in the blood, further research with larger cohorts are both planned and undoubtedly necessary in order to validate findings and fully unravel the potential clinical value of the putative biomarker(s) described here. However, in terms of the discovery/validation cohort, due to time constraints and patient

recruitment rates, data represented here pertains to approximately 25% of the total target cohort size. Further increasing cohort sizes would also remedy another limitation of the study. It would permit categorisation of patients by diagnosis/phenotype. For instance, patients with post herpetic neuralgia, TGN and painful diabetic neuropathic, in addition to those with CNBP underpinned by specific aetiology, could be analysed separately. This would inevitably assist with the discovery of biomarkers associated with a specific diagnosis/phenotype, which may be lost within a cohort of mixed diagnosis/phenotype. Clearly, such variability may explain the differences in gene regulation observed between the discovery cohort (neuropathic back pain presentation/diagnosis) and the discovery/validation cohort (mixed presentation/diagnosis). Disease controls could also be considered as valuable additions to the study. This may herald valuable insights and potential biomarkers of CNP, by including patients who for instance, have painless diabetic neuropathy, thereby enabling the discovery of transcriptomic disparities when compared with those exhibiting painful diabetic neuropathy.

Another probable source of variability is the reliability of the self-assessment pain tools, primarily due to the subjective nature of pain sensitivity/severity. This may be ameliorated by the inclusion of additional questionnaires pertaining to CNP diagnosis, such as NPQ and DN4. In order to increase the scope of analysis consideration could be given to performing QST, and with increased cohort sizes, consideration could also be given to markers of treatment efficacy. This would allow assessment of correlations between gene expression and QST measurements and between gene expression and changes in pain severity resulting from pharmacological intervention. Longitudinal aspects to a future study, which involve transcriptomic analysis at various time-point from diagnosis, would be an intriguing inclusion, although acceptability to patients would be a consideration due to multiple required phlebotomy procedures. Such study dynamics may be more suitable to patients already requiring frequent blood tests, such as patients with relapsing-remitting multiple sclerosis receiving IFN-β (with and without CNP). The range of analytical procedures undertaken with the obtained samples could also be expanded to incorporate extracellular vesicles, including exosomes and microvesicles. These membrane bound vesicles, secreted into various bodily fluids including urine and blood, contain a plethora of biologically actives molecules, from proteins to mRNA, miRNA and other ncRNA. Exosomes, though previous disregarded, are of increasing interest as circulating biomarkers (Goetzl *et al.* 2015, Hornick *et al.* 2015) and are beginning to be considered in both animal models (Li *et al.* 2017) and cases of CINP (Chen *et al.* 2015).

4.5 Conclusion

Transcriptomic analysis of blood in two separate cohorts of patients with CNP initially yielded a plethora of differentially regulated genes, which were then subject to further refinement. A total of 27 genes were then carried forward for further scrutiny. Multiple supportive literature associations were observed, in addition to qRT-PCR validation of microarray data and the observation of upregulated TIMP1 in the plasma of patients with CNBP. Gene ontology analysis also demonstrated strong evidence for ongoing immune related processes in the discovery/validation cohort, predominantly involving chemokines and cytokines. A degree of cross-validation was also observed between the two cohorts, including *CASP5*, *TLR5* and *XCL1*. However, considering the variability in patient disease characteristics between the two cohorts, further expansion and validation is necessary to add clarity to the potential function of these genes as biomarkers of CNP.

4.6 Summary points

- After refinement of microarray data a total of 15 genes in the discovery cohort and 12 genes in the discovery/validation cohort were selected for further analysis
- *TIMP1* demonstrated significant potential as a biomarker of CNBP. It was strongly upregulated, supported by extensive literature evidence, present with correlation analysis and was strongly correlated with increased circulating TIMP1 levels
- Despite the heterogeneous aetiologies of CNP, cross-validation between microarrays highlighted multiple genes differentially expressed or trending strongly in the same direction of fold change, including *CASP5*, *TLR5* and *XCL1*
- Apparent downregulation of *CX3CR1* may be indicative of changes in circulating mononuclear cell populations, potentially due to extravasation and migration in response to neuroinflammation. *CX3CR1* also negatively correlated with the accumulative pain severity score, leading to consideration that lower blood *CX3CR1* levels (due to the rationale described), may be indicative of increased leukocyte migration and resulting contributions to pain signalling and ultimately, measures of severity
- Gene ontology analysis in the discovery/validation cohort highlighted strong associations with immune response, chemokine signalling pathways and cytokine-cytokine receptor interactions as major over-represented functions

Chapter 5 : Translational Biomarkers of Chronic Neuropathic Pain

5.1 Introduction

Traditionally, animal models of disease, and their subsequent use in drug candidate screening, have formed the cornerstone of preclinical studies. This follows the conventional approach, which seeks to utilise animal models in order to elucidate answers, or predictions, to clinical questions that could not, ethically, involve human subjects. Such methodology has numerous advantages for the purposes of safety in candidate drug screening, allowing for validation of effect, monitoring of serious adverse events and rapid optimisation of suitable dosing in preparation for human trials (Vandamme 2014). The representability of animal disease models to reflect their human equivalents is not equivocal (Seok *et al.* 2013). To date, the vast majority of transcriptomic studies of neuropathic pain have followed the traditional preclinical pathway for screening and discovery with the use of a multitude of animal models (LaCroix-Fralish *et al.* 2011). These have ranged from chronic constriction injury (CCI) (Bennett *et al.* 1988) and spinal nerve ligation (SNL) (Kim *et al.* 1992) to the spared nerve injury (Decosterd *et al.* 2000) and drug-induced neuropathy models (Jaggi *et al.* 2011). Such studies have identified extensive groups of genes exhibiting changes in expression after nerve injury and have provided valuable insights into the mechanisms underpinning the development and maintenance of neuropathic pain, yet little has been translated to advances in diagnostic biomarkers of CNP in humans.

In an attempt to improve the potential outcome and subsequent clinical utilisation of novel biomarkers of neuropathic pain, a top-down approach to biomarker discovery is herein considered (Sapunar *et al.* 2009). Using this strategy, candidate transcriptomic biomarkers from peripheral blood mononuclear cells of patients with CNP and healthy controls were analysed for changes in expression in the DH of Sprague Dawley and Wistar Kyoto rats after SNL. This is beneficial in several ways. Clearly, differential gene regulation in blood may be different to that observed in the injured nerve, DRG or DH during the development and maintenance phases of neuropathic pain. As such, transcriptomic changes may be highlighted that have not previously been determined using animal models alone. Additionally, the prioritisation of human blood as a source biomarkers ensures that the search for novel biomarkers is undertaken in actual clinical cases of CNP, which has unrivaled relevance for the detection of accessible biomarkers of CNP, but relies

on the presumption that CNP may lead to transcriptomic changes or variations in cell populations within the blood. In addition, the chronicity associated with neuropathic pain presents notable variation between the reputed 3-6 months duration for classification of pain as 'chronic' in humans, which contrasts to the vast majority of animal models of neuropathic pain, wherein animals are often sacrificed after just days or weeks after surgery. Such models are rarely maintained for several months (Berge 2014).

5.1.1 Animal models of neuropathic pain

5.1.1.1 The need for animal models

The capacity for human subjects to facilitate the analysis of the molecular intricacies of CNP within the nervous system is somewhat limited. In addition to the challenges relating to patient recruitment, particularly pertaining to the less common painful neuropathies, such as TGN and PHN, there is a great reliance placed upon the accuracy of a previous diagnosis. This is undoubtedly entwined within the paradigm of a lack of diagnostic methodology in the clinic, which subsequently impedes research requiring CNP of specific aetiologies, which in-turn results in a lack of tangible research outcomes translated to the clinic. Undoubtedly, there is also significant potential for variability associated with the subjective nature of pain and the potential interference from comorbidities and the associated use of pharmacological interventions, which themselves may exert a broad range of implications, ranging from changes in gene expression to implications on QST and self-reported pain descriptors (e.g. S-LANSS). As CNP often results from irreversible damage, there is also a distinct lack of research methods applicable to human subjects, which are generally limited to the non-invasive application of stimuli to the skin. The use of animal models, despite their limitations, does permit modelling of human neuropathic pain, producing characteristic, reproducible and measurable sensory deficits, including allodynia, hyperalgesia and spontaneous pain (Sapunar *et al.* 2009). As such, there is a clear impetus for the use animal models to provide further insight into pathophysiological mechanisms and the subsequent assessment of novel biomarkers and pharmacotherapies (Jaggi *et al.* 2011).

Notwithstanding, CNP is inherently complex, results from variable aetiology and manifests with a diverse range of symptoms. Despite the clear value of animal models of neuropathic pain, there is no single animal model capable of accurately mimicking the complex pathophysiological mechanisms and symptoms associated with these diverse aetiologies. As such, researchers have, over time, now documented around 40 different animal models of neuropathic pain, including those with subsequent modifications (Jaggi *et al.* 2011). Several of the most commonly used

animal models of neuropathic pain are illustrated (Figure 5.1), summarised (Table 5.1) and described below.

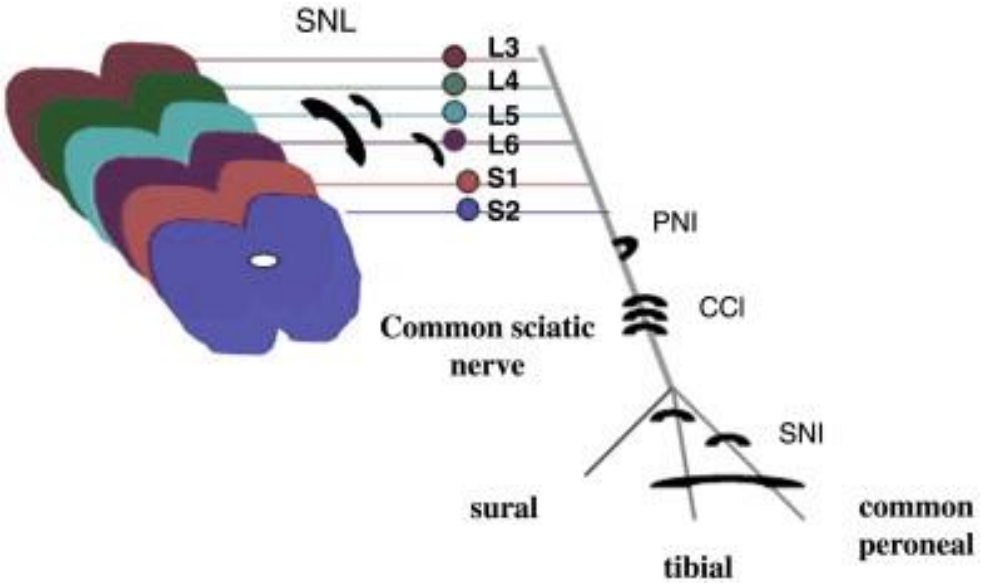

Figure 5.1: An illustration depicting the common animal models of neuropathic pain

Spinal nerve ligation (SNL) involves ligatures applied to either the L5, L6 or L5 and L6 nerve roots. Partial nerve injury (PNI) uses ligatures applied to approximately half of the sciatic nerve diameter. Chronic constriction injury (CCI) involves the application of three to four loose ligatures to the sciatic nerve. The spared nerve injury (SNI) model involves the use of a tight ligature applied to different braches of the sciatic nerve. Permissions were obtained for the use of this figure (Colleoni *et al.* 2010).

5.1.1.2 Common models of peripheral neuropathic pain

In the 1970s, Wall and co-workers broke new ground in the field of neuropathic pain research with the development of a model of CNP caused by injury to a peripheral nerve. This technique involved complete transection of the sciatic nerve at mid-thigh level, which is typically followed by the formation of a neuroma at the proximal nerve stump, consisting of a mass of sprouting regenerative nerves and connective tissue (Wall *et al.* 1979, Beizberg 2006). The resulting *anesthesia dolorosa*, a deafferentiation pain, is typified by loss of feeling alongside painful sensations within the implicated area. The degree of autotomy was originally postulated as a measure of pain severity, but it is now considered that this is a likely consequence of excessive grooming resulting from a lack of sensory feedback (Kauppila 1998, Jaggi *et al.* 2011). The major drawback of this model is that complete nerve transection is relatively uncommon, and is typically associated amputation (phantom limb pain). It is also noteworthy that ethical considerations are of heightened poignancy

due to the excessive autotomy associated with this technique (Bridges *et al.* 2001, Jaggi *et al.* 2011).

5.1.1.2.1 Chronic constriction injury

The chronic constriction injury (CCI) model of peripheral mononeuropathy was developed by Bennett and Xie (Bennett *et al.* 1988). After anesthesia and exposure of the common sciatic nerve at mid-thigh level, three or four loose ligatures are applied to the sciatic nerve proximal to trifurcation (Jaggi *et al.* 2011). Observed behavioral changes included hyperalgesia, allodynia and signs of spontaneous pain. Hyperalgesia in response to radiant heat was evident for over 8 weeks post-surgery. Nocifensive behaviours to innocuous stimuli, and in the absence of stimuli, inferred the presence of allodynia and spontaneous pain, respectively (Bennett *et al.* 1988). It was noted that the CCI model produced behavioral signs akin to those of causalgia and CRPS in man (Bennett *et al.* 1988, Jaggi *et al.* 2011).

5.1.1.2.2 Partial sciatic nerve injury

The partial sciatic nerve injury (PSL/PNI) model was developed by Seltzer *et al* (Seltzer *et al.* 1990). After exposure of the sciatic nerve at upper-thigh level, a tight ligature is applied to around half of the sciatic nerve. Rats subsequently displayed guarding of the ipsilateral hind paw, inferring the presence of spontaneous pain. Thresholds to von Frey hairs were significantly reduced, whilst also demonstrating evidence of allodynia, thermal hyperalgesia and mechanical hyperalgesia. The PSL model has been considered as an appropriate mimic of causalgiform pain disorders (Seltzer *et al.* 1990).

5.1.1.2.3 Spared nerve injury

The spared nerve injury (SNI) model was developed by Decosterd and Woolf (Decosterd *et al.* 2000). After exposure of the sciatic nerve and its terminal branches (the common peroneal, sural and tibial nerves), a tight ligature is applied to the common peroneal and tibial nerves followed by axotomy of these nerves. The sural nerve is not implicated, though this method has been modified using different combinations of axotomised nerves, in each instance leaving at least one nerve spared (Bourquin *et al.* 2006). This model leads to behavioral changes present at 6 months post-surgery, including increased von Frey hair and pinprick sensitivity (Decosterd *et al.* 2000). One such benefit of the SNI model is that it allows behavioral testing to be conducted on non-injured regions adjacent to those which are denervated (Decosterd *et al.* 2000, Jaggi *et al.* 2011).

5.1.1.2.4 Spinal nerve ligation

The spinal nerve ligation (SNL) model of peripheral neuropathy was developed by Kim and Chung (Kim *et al.* 1992). After anesthesia, the left L5, or L5 and L6 spinal nerves, are exposed. A ligature is then applied distal to the DRG using a silk suture. The sole use of the L5 nerve for ligature application is highly reproducible and is associated with comparatively less damage to surrounding tissues than the L5 and L6 model. The L4 spinal nerve is avoided as ligature application to this nerve leads to severe motor deficits with implications on behavioral tests (Jaggi *et al.* 2011). In addition to signs of spontaneous pain, the model was found to produce persistent hyperalgesia and mechanical allodynia, of at least 5 and 10 weeks duration, respectively (Kim *et al.* 1992). These animals also tended to exhibit pronounced responsiveness (or reduce thresholds) to von Frey hairs (Kim *et al.* 1997).

Table 5.1: Advantages and disadvantages associated with various animal models of neuropathic pain

Model	Model Description	Induced Behavioural Changes	Advantages	Disadvantages	Reference
Peripheral Nerve Injury Models					
Axotomy model	Complete transection of the sciatic nerve at the mid-thigh level combined with lesioning of the saphenous nerve to induce complete denervation of the hind limb. A neuroma develops at the proximal nerve stump.	Pain is produced in the absence of any sensory input to the area (*anaesthesia dolorosa*). Autotomy is also a feature.	Reproducibility.	Complete nerve transection is uncommon in humans, and is typically seen after amputation. Notable ethical issues are associated with autotomy.	(Wall *et al.* 1979)
Chronic constriction injury	Three or four loose ligatures are applied to the sciatic nerve. The procedure leads to intraneural oedema, focal ischaemia, and Wallerian degeneration.	Mild to moderate autotomy, guarding, excessive licking, and altered weight bearing are observed. Features persist for at least 7 weeks after surgery. Mechanical and thermal hyperalgesia, and cold allodynia, are present.	Clinical features are similar to CRPS in humans.	Variability associated with the CCI model is of particular relevance and may be attributed to tightness of the ligatures placed around the nerve.	(Bennett *et al.* 1988)

Partial sciatic nerve ligation	Tight ligation of one-third to half of the sciatic nerve using a single ligature.	Paw guarding and licking which are not associated with autotomy.	Immediate onset and long-lasting duration of allodynia and hyperalgesia with similarities to causalgia in humans.	(Seltzer *et al.* 1990)
Spinal nerve ligation	The L5 or L5 and L6 spinal nerves are exposed followed by the application of a silk suture. Sole application to the L5 spinal nerve is highly reproducible and leads to less damage of surrounding tissue.	Persistent hyperalgesia, mechanical allodynia and signs of spontaneous pain are observed.	Notable consistency is observed with this model, particularly in regards to the site and degree of ligation.	(Kim *et al.* 1992)
Spared nerve injury	The tibial and common peroneal nerves are tightly ligated followed by axotomy of the distal nerve, the sural nerve remains undamaged. Modifications have been described.	See above.	Allows simultaneous investigation of changes in both injured and uninjured sensory neurons.	(Decosterd *et al.* 2000)
Sciatic inflammatory neuritis	A catheter is implanted under the sciatic nerve (under anaesthesia), which is used to inject zymosan (a yeast based immune activator) in awake, freely moving rats.	See above.	Human neuropathies are commonly caused by inflammation or infection rather than trauma. This model may better mimic for these conditions.	(Chacur *et al.* 2001)
Cuffing of the sciatic nerve	Placement of a polyethylene cuff around the sciatic nerve.	See above.	High reproducibility.	(Mosconi *et al.* 1996)

Central pain models					
Weight drop or contusion model	The spinal cord is exposed at the thoracolumbar level and a given mass is dropped on the spinal cord to induce injury.	Severe paraplegia and complete segmental necrosis. Dysthesia, spontaneous and evoked pain are observed.		Ethical considerations due to associated paraplegia.	(Siddall *et al.* 1995)
Excitotoxic spinal cord injury	Intraspinal injections of excitatory amino acids to simulate injury-induced elevations in neuronal activity.	See above.	Progressive pathological changes associated with the injection closely resemble those induced by ischaemic and traumatic spinal cord injury.		(Aanonsen *et al.* 1989)
Drug-induced neuropathy models					
Drug-induced neuropathy	Systemic injection of drugs that induce neuropathy such as vincristine and cisplatin.	Drug-induced neuropathy with clinical features determined by the implicated nerve(s).	Non-surgical.	Drugs often produce concurrent effects on the health of the animal which may confound pain assessment.	(Authier *et al.* 2003)
Disease-induced neuropathy models					
Diabetes-induced neuropathy	Diabetes is induced by the administration of a pancreatic β-cell toxin, such as streptozotocin.	Induction of peripheral neuropathy with associated signs of neuropathic pain.	Clinical signs associated with painful diabetic neuropathy in humans may be replicated.	Animals develop other metabolic changes associated with diabetes that can confound pain assessment.	(Courteix *et al.* 1993)

Cancer pain models, e.g., bone cancer pain	Bone cancer is induced by, for instance, inoculation of cancer cells into the femur.	Produces behavioural changes attributed to neuropathic and inflammatory pain.	Demonstrates the distinct aspects of cancer pain, suggesting the involvement of inflammatory, neuropathic and tumorigenic components in the pathogenesis of pain.	May induce systemic changes in the animal associated with tumour growth that can compromise well-being and confound pain assessment.	(Schwei *et al.* 1999)

Table adapted with permissions (Jaggi *et al.* 2011, McKune *et al.* 2015).

5.1.1.3 Transcriptomics in animal models of neuropathic pain

Analysis of gene expression changes after experimentally induced nerve injury has largely encompassed either the DRG or spinal DH (LaCroix-Fralish *et al.* 2011). Injured adult DRG neurons do not tend to die after axonal injury (Tandrup *et al.* 2000) at least in-part due to changes in the regulation of genes implicated in cell survival, including *Gadd45a* (Chen *et al.* 1998) and *Tspo* (Bono *et al.* 1999). Regeneration of neurons with axons in the peripheral nervous system may also occur after nerve injury, which is underpinned by injury-induced differential regulation of regeneration-associated genes (Skene 1989). Such transcriptomic changes may facilitate the typical maladaptive responses associated with the production of ectopic spontaneous activity towards the CNS, including increases in excitability, reduced inhibitory function within the DH and the inappropriate generation of synaptic contacts (Woolf *et al.* 1999, Costigan *et al.* 2002).

The development of microarray technology pertaining to the rat and mouse genomes has subsequently permitted high-throughput and highly detailed analysis of transcriptomic changes after experimentally induced nerve injury in the rodent. In 2002, Costigan *et al* used high-density rat genome oligonucleotide microarrays, a significant advance on previous studies using immunohistochemistry and *in situ* hybridisation (Hökfelt *et al.* 1994, Woolf *et al.* 2000) to elucidate 240 differentially regulated genes (based on a fold change of >1.5) in the DRG, three days after SNT (Costigan *et al.* 2002). Further analysis of gene expression patterns in the DRG at various time-points after SNT was also conducted by Xiao *et al* (Xiao *et al.* 2002), which deduced that half of the markedly regulated genes pertained to neurotransmission, including those related to synaptic transmission, neuropeptides and ion channels. Interestingly, differential regulation of such genes was found to persist for over 28 days in 80% of instances (Xiao *et al.* 2002, Zhang *et al.* 2005). A plethora of studies have since been undertaken to further assess transcriptomic changes relating to experimentally induced pain in rodents, many of which have been subject to meta-analysis (LaCroix-Fralish *et al.* 2011). In more recent years the depth of scrutiny has increased with the analysis of changes in miRNA expression in the DH after CCI (Genda *et al.* 2013).

It is established that few genes are differentially regulated in both the DH and DRG after nerve injury (Zhang *et al.* 2005). Such transcriptomic differences are primarily associated with neuropeptides (which are generally regulated in the DRG) and molecules pertaining to signal

transduction (which are more frequently regulated in the DH) (Zhang *et al.* 2005). This encompasses changes in the expression of genes associated with a multitude of signal transduction pathways, such as PKCα, JNK and p38 MAPK (Zhang *et al.* 2005). One such example of a gene which breaks this trend is the Ca^{2+} channel α2/δ1 subunit, which is transiently upregulated in the DRG and persistently upregulated in the DH after nerve injury. This lends evidence to the suggestion that the long-term therapeutic benefit of gabapentin is founded upon Ca^{2+} channel α2/δ1 subunit binding in the DH (Zhang *et al.* 2005).

The use of animal models has also highlighted transcriptomic variation between different phases of neuropathic pain, an early phase concerned with the onset and development of neuropathic pain, and a late phase attributed to the transition towards, and maintenance of, neuropathic pain (Ru-Rong 2008). Indeed, inhibition of p38 MAPK in spinal microglia is more effective in reducing pain in the early phase rather than the later phase, mimicking the pattern of pattern of microglial activation (Jin *et al.* 2003). In contrast, activation of astrocytes is a persistent feature which is maintained in the late phase, alongside associated upregulation of astrocyte signalling molecules (e.g. phosphorylated JNK), and therefore represents a potentially effective target in the treatment of CNP (Zhuang *et al.* 2006). Such variation, demonstrating dynamic transcriptomic changes, is clearly an important consideration for the determination of translation biomarkers of CNP.

5.1.2 Aims and objectives

The overall aims and objectives of chapter 5 are as follows:

- To determine whether genes pertaining to BH_4 *de novo* synthesis, salvage or recycling are differentially regulated in the dorsal horn in an animal model of neuropathic pain
- To determine whether the genes differentially regulated in human cohorts (chapter 4) are similarly differentially regulated in the rat dorsal horn after nerve injury, thus highlighted potential translational biomarkers of neuropathic pain

5.2 Methods

Specific methods relating to this chapter are detailed within chapter 2.

5.2.1 Method development

5.2.1.1 Droplet digital PCR

Analysis of gene expression changes after SNL was initially undertaken using qRT-PCR. However, low gene expression, coupled with a relatively low RNA input, meant that multiple

genes could not be reliably quantified due to late (35-40) and erratic C_q values. A lack of total RNA was somewhat ameliorated by the use of the QuantiTect Whole Transcriptome Kit. However, such amplification relies on the assumption that the copy number of the target transcript is of sufficient abundance prior to whole transcriptome amplification as to avoid stochastic problems introduced through amplification of very low copy number transcripts. This may also be exacerbated by the need to perform multiple post-amplification dilutions to prevent immediate saturation the qRT-PCR detection system. As such, droplet digital PCR (ddPCR) was considered for quantification of selected genes. This method utilises water-in-oil droplet formation as a basis for the PCR. In contrast to the conventional single well reaction setup of qRT-PCR, an entire 20 µL reaction mix, including cDNA, is partitioned into ~20000 droplets. Each droplet containing the target cDNA then undergoes PCR amplification and subsequent binding of EvaGreen for fluorescent detection. After PCR, the droplets are analysed by flow cytometry to determine the proportion of PCR-positive droplets. Data is then analysed using Poisson statistics to calculate the concentration of the original DNA template. This technique features high precision and sensitivity, allowing reliable analysis of lowly expressed genes and has been successfully utilised for single-cell gene expression analysis (Karlin-Neumann *et al.* 2014). The use of ddPCR successfully enabled the assessment of gene expression in multiple cases where qRT-PCR was distinctly suboptimal, such as *Arhgap11a* (Figure 5.2), although some genes (e.g. *Xcl1*) remained unsuitable for analysis due to gene expression in the region of 0-3 transcripts per 20 µL reaction.

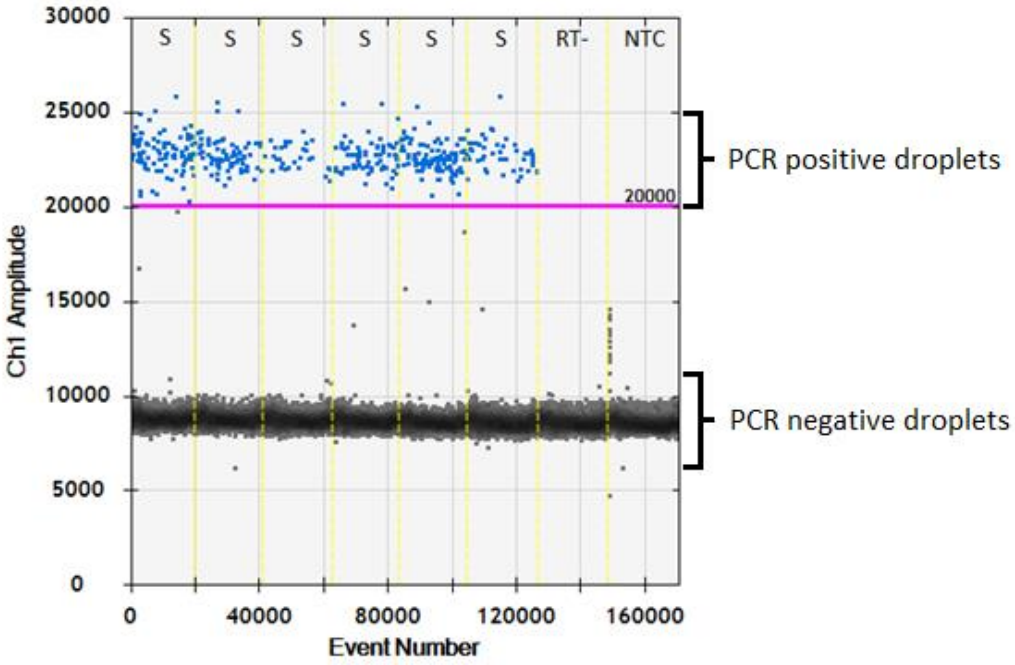

Figure 5.2: Representative ddPCR data output for *Arhgap11a*

A graphical representation of a typical data output pertaining to 5 samples (S), a reverse transcriptase control (RT-) and a no template control (NTC). Positive droplets are blue and exhibit higher signal amplitude. Ch1 Amplitude and Event Number related to the level of fluorescent signal and the accumulative number of analysed droplets, respectively.

5.3 Results

5.3.1 geNorm analysis

In order to determine the optimal reference genes for normalisation, geNorm analysis was performed within qbase+. A total of four samples from each of four groups were used, incorporating Sprague Dawley and Wistar Kyoto (sham and SNL) rats. Gene expression analysis was conducted as described (section 2.3.2 and 2.3.3). Twelve reference genes (*18s*, *Actb*, *Atp5b*, *B2m*, *Canx*, *Cyc1*, *Gapd*, *Mdh1*, *Rpl13*, *Top1*, *Ubc* and *Ywhaz*) were considered (PrimerDesign, UK). Reference gene stability is illustrated for both rat strains (Figure 5.3). The optimal number of reference was determined as two (geNorm V ≤0.15), of which *Atp5b* and *Ubc* were highly stable (geNorm *M* ≤0.5) in both strains.

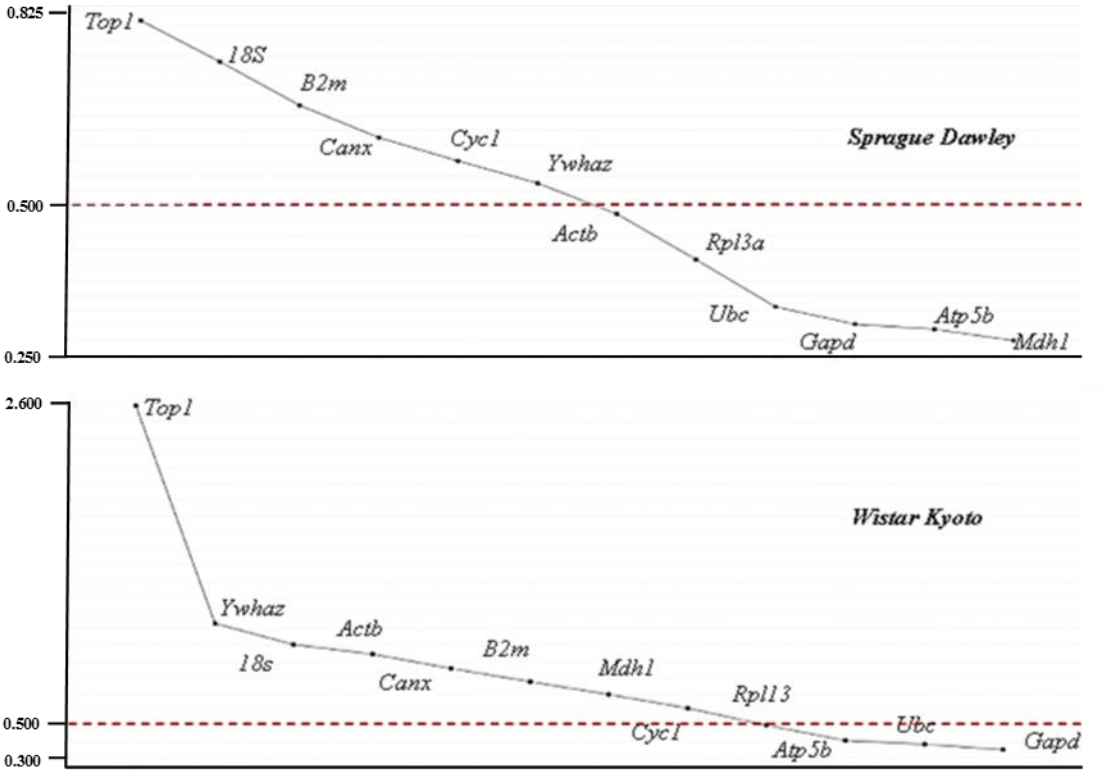

Figure 5.3: geNorm analysis illustrating reference gene stability in the different rat strains treated as sham or subject to SNL

Assessment of reference gene stability was conducted by qRT-PCR using the geNorm facility within qbase+. Two reference genes were deemed optimal for normalisation (geNorm V ≤0.15). Both *Atp5b* and *Ubc* were highly stable across both rat strains (geNorm *M* ≤0.5) and were therefore selected for future normalisation. The red line illustrates the limit of high reference gene stability. Raw data is presented in the supplementary electronic material with the following file path; qRT-PCR > Translational > geNorm.

5.3.2 Tetrahydrobiopterin and SNL

Differential regulation of genes pertaining to BH_4 in the DH of Sprague Dawley rats after SNL elucidated only *Gchfr* ($p = 0.035$) to be significantly downregulated after nerve injury (

Table 5.2). A non-significant trend towards upregulation of *Spr* ($p = 0.167$) was observed, although no other gene analysed notably varied between sham and SNL Sprague Dawley rats. Analysis in the DH of Wistar Kyoto rats found no single gene to be significantly differentially regulated.

Table 5.2: Analysis of changes in the expression of genes relating to BH_4 synthesis in the rat DH after SNL

			Sprague Dawley		Wistar Kyoto	
Accession Number	**Gene Name**	**Gene Symbol**	***p* value**	**FC in SNL**	***p* value**	**FC in SNL**
NM_130400	Dihydrofolate reductase	*Dhfr*	0.945	↑1.07	0.574	↑1.34
NM_024356	GTP cyclohydrolase I	*Gch1*	0.663	↑1.35	0.379	↑2.00
NM_133595	GTP cyclohydrolase I feedback regulator	*Gchfr*	0.035	↓3.70	0.843	↑1.13
NM_001007601	Pterin-4 alpha-carbinolamine dehydratase 1	*Pcbd1*	0.497	↓1.36	0.883	↓1.06
NM_017220	6-pyruvoyltetrahydropterin synthase	*Pts*	0.498	↓1.22	0.780	↑1.11
NM_022390	Quinoid dihydropteridine reductase	*Qdpr*	0.976	↑1.00	0.463	↓1.21
NM_019181	Sepiapterin reductase	*Spr*	0.167	↑1.90	0.759	↑1.10

Gene expression analysis was conduct by qRT-PCR. Data was processed in qbase+ and normalised to *Act5b* and *Ubc*. $p = 0.05$ considered statistically significant (unpaired t-test). Raw data is presented in the supplementary electronic material with the following file path; qRT-PCR > Translational > Sprague or Wistar.

5.3.3 Translational biomarkers in the discovery cohort

Genes found to be differentially regulated in the blood of patients with CNP in the discovery cohort were subsequently analysed in the DH of Sprague Dawley rats subjected to L5 SNL, in conjunction with their sham counterparts (Table 5.3). Upregulation of *Timp1* ($p = 0.006$) was observed alongside a strong trend for upregulation of *Mc1r* ($p = 0.085$) and to a lesser degree, *Casp4* ($p = 0.195$). Further analysis in the DH of Wistar Kyoto rats also highlighted upregulation of *Timp1* ($p = 0.057$) and *Mc1r* ($p = 0.008$). Downregulation of *Dpp3* ($p = 0.027$) and *Rnf185* ($p = 0.039$) was also observed. Normalisation of gene expression was performed using both *Atp5b* and *Ubc*. Of 12 reference genes analysed with the rat geNorm kit (Primerdesign, UK), both were found to be comparably highly stable ($M = 0.419$, CV = 0.145).

Table 5.3: **Analysis of differentially regulated genes from the discovery cohort in the rat DH after SNL**

			Sprague Dawley		Wistar Kyoto	
Accession Number	**Gene Name**	**Gene Symbol**	***p* value**	**FC in SNL**	***p* value**	**FC in SNL**
NM_053736	Caspase 4*	*Casp4*	0.195	↑1.22	0.529	↓1.11
NM_053021	Clusterin	*Clu*	0.999	1.00	0.895	↑1.02
NM_053748	Dipeptidyl-peptidase 3	*Dpp3*	0.467	↓1.16	0.027	↓3.92
XM_006256260	Inducible T-cell co-stimulator ligand	*Icoslg*	0.792	↓1.16	ND	ND
NM_001109269	Lin-28 homolog A	*Lin28a*	0.786	↑1.30	ND	ND
XM_006255795	Melanocortin 1 receptor*	*Mc1r*	0.085	↑2.72	0.008	↑1.61
NM_001309432	NLR family CARD domain-containing protein 4*	*Nlrc4*	0.524	↑1.15	0.298	↓1.41
NM_001168524	Rho GTPase activating protein 11A*	*Arhgap11a*	0.852	↓1.03	0.458	↑1.12
NM_001024271	Ring finger protein 185	*Rnf185*	0.390	↓2.18	0.039	↓3.47
NM_053819	TIMP metalloproteinase inhibitor 1	*Timp1*	0.006	↑2.19	0.057	↑2.17
NM_001145828	Toll-like receptor 5	*Tlr5*	0.682	↑1.51	ND	ND

*Denotes analysis by ddPCR. Where appropriate, the ortholog, or closely related gene with high sequence similarity was selected. Genes not described here are either not present within the rat genome with no apparent ortholog, or were not reliably detected for robust statistical analysis. Data pertaining to qRT-PCR was processed in qbase+ and normalised to *Act5b* and *Ubc*. Data pertaining to ddPCR was processed in GraphPad Prism 6.0 and normalised to the geometric mean of *Rpl13a* and *Ubc*. *Rpl13a* was stably expressed and unlike *Atp5b*, allowed for reliable separation of PCR-positive and negative droplets by flow cytometry. $p = 0.05$ considered statistically significant (unpaired t-test). ND: Could not be reliably detected for sufficiently robust analysis. Raw data is presented in the supplementary electronic material with the following file paths, qRT-PCR > Translational > Sprague or Wistar, alternatively ddPCR > Translational > SD WK ddPCR data.

5.3.4 Translational biomarkers in the discovery/validation cohort

Analysis of differentially expressed genes identified in the discovery/validation cohort in both Sprague Dawley and Wistar Kyoto rats showed no significant change in the DH after SNL (Table 5.4). However, several genes could not be reliably detected in the DH tissue due a combination of low expression, limited RNA and a lack of continued ddPCR facility.

Table 5.4: Analysis of differentially regulated genes from the discovery/validation cohort in the rat DH after SNL

			Sprague Dawley		Wistar Kyoto	
Accession Number	**Gene Name**	**Gene Symbol**	***p* value**	**FC in SNL**	***p* value**	**FC in SNL**
NM_133534	Chemokine (C-X3-C motif) receptor 1**	*Cx3cr1*	0.738	↑1.17	0.541	↓1.14
NM_013195	Interleukin 2 receptor subunit beta*	*Il2rb*	0.489	↑1.25	0.484	↑1.18
NM_031521	Neural cell adhesion molecule 1**	*Ncam1*	0.268	↑1.32	0.231	↓1.10
NM_019178	Toll-like receptor 4*	*Tlr4*	0.142	↑1.23	0.825	↓1.03
NM_199408	Wntless Wnt ligand secretion mediator**	*Wls*	0.849	↓1.04	0.692	↑1.08

*Denotes analysis by ddPCR. **Denotes analysis by qRT-PCR using cDNA derived using the Verso cDNA synthesis procedure, as described (section 2.1.2.5), to minimise inconsistency between replicates observed with lowly expressed genes after whole transcriptome amplification. When necessary, the ortholog or closely related gene with high sequence similarity was selected. Genes not described here are either not present within the rat genome with no apparent ortholog or were not reliably detected for robust statistical analysis. Data pertaining to qRT-PCR was processed in qbase+ and normalised to the geometric mean of *Act5b* and *Ubc*. Data pertaining to ddPCR was processed in GraphPad Prism 6.0 and normalised to *Rpl13a* and *Ubc*. $p = 0.05$ considered statistically significant (unpaired t-test). Raw data is presented in the supplementary electronic material with the following file paths: qRT-PCR > Translational > Sprague or Wistar, alternatively ddPCR > Translational > SD WK ddPCR data.

5.4 Discussion

In order to elucidate translational transcriptomic biomarkers of neuropathic pain, genes pertaining to BH_4 synthesis, or to those found to be differentially regulated in the blood of patients with CNP, were subsequently analysed in the DH of the rat spinal cord 35 days after SNL. Genes pertaining to both BH_4 synthesis, and several genes previously described as differentially expressed in the

human cohorts, were subsequently identified as regulated in the DH after SNL. Although differential expression of several genes was not observed in the both human cohorts, this may be attributed to the invariable presentation of CNBP in the discovery cohort, compared to various neuropathic presentations within the discovery/validation cohort. It should also be considered that studies using animal models of neuropathic pain have shown significant differences between rat strains in relation to pain-related behaviors (Lovell *et al.* 2000, del Rey *et al.* 2012), cytokine expression (del Rey *et al.* 2012) and DH gene expression after experimentally induced nerve injury (Le Coz *et al.* 2014). Such differences may indeed account for a proportion of the variability observed between Sprague Dawley and Wistar Kyoto rats.

5.4.1 Tetrahydrobiopterin synthesis pathway

Analysis of changes in the expression of genes pertaining to the BH_4 synthesis pathway demonstrated only downregulation of *Gchfr* in the Sprague Dawley DH as significantly differentially expressed, although similar downregulation of *GCHFR* was observed in patients in the discovery/validation cohort. Should such observations be validated in further experimentation, this is perhaps contrary to previous observations that upregulation of *Gch1*, rather than downregulation of *Gchfr*, is responsible for altering the stoichiometry of GTPCH activity regulation, leading to increased BH_4 synthesis (Tegeder *et al.* 2006, Latremoliere *et al.* 2011).

It is poignant to consider that contrasting observations have been made during various *in vitro* and *in vivo* studies seeking to elucidate the influence of GFRP on BH_4 synthesis. Incubation of HUVECs with IFN-γ has been shown to upregulate *GCH1* and downregulate *GCHFR* (Gesierich *et al.* 2003), leading to changes in protein expression which favour BH_4 synthesis. Similar observations have been made following the application of hydrogen peroxide to vascular endothelial cells (Ishii *et al.* 2005). Further investigation again reaffirmed these findings through intraperitoneal injection of LPS, leading to downregulation of *Gchfr* (Werner *et al.* 2002). However, contrasting results were obtained using the *tet*-regulated *GCH1* expression system. Both exposure to doxycycline and the overexpression of GFRP failed to elucidate a role for GFRP in the modulation of BH_4 levels (Tatham *et al.* 2009), which is particularly surprising considering that overexpression of GFRP has been shown to attenuate cytokine-induced increases in nitric oxide production (Nandi *et al.* 2008).

The expression of *Gchfr* exhibits significant variation within the CNS. A relative abundance of *Gchfr* has been described in the serotonergic neurons of the dorsal raphe nuclei within the

brainstem, but could not be identified within the dopaminergic neurons of the midbrain (Kapatos *et al.* 1999). This illustrates the potential for a variable influence of GFRP on the regulation of GTPCH activity. Indeed, contrasting feedback mechanisms are apparent with regards to L-phenylalanine, wherein L-phenylalanine increases BH_4 levels in serotonergic neurons, but not in dopaminergic neurons (Kapatos *et al.* 1999). It is also noteworthy to consider that such variation in expression may have implications for pharmacological inhibition of BH_4 synthesis, particularly as GFRP is a prerequisite for the activity of the GTPCH inhibitor, DAHP. As such, the activity of DAHP, or a novel GTPCH inhibitor which functions via a similar mechanism of inhibition, may exhibit reduced efficacy when GFRP is either expressed at low basal levels, or is downregulated.

5.4.2 Validated translational biomarkers

5.4.2.1 Dipeptidyl-peptidase 3

Dipeptidyl-peptidase 3 (*DPP3*), a zinc-dependent aminopeptidase and single member of the M49 family of metallopeptidases, was upregulated 1.50 fold in the blood of CNP patients in the discovery cohort and downregulated 3.92 fold in the DH of Wistar Kyoto rats after SNL. There was no difference in the expression of *Dpp3* in the DH of Sprague Dawley rats.

DPP3 was initially isolated from bovine pituitary (Ellis *et al.* 1967) and cleaves the N-termini of various peptides, including enkaphalins (Prajapati *et al.* 2011). The involvement of DPP3 in several pathophysiological processes has been considered. Initial research elucidated a potential role for DPP3 in gynecological malignancy, wherein DPP3 expression and activity was increased (Simaga *et al.* 1998). Of particular relevance, research has also elucidated that DPP3 is involved in the degradation of enkephalin, endomorphins, hemorphins and exorphins (Barsun *et al.* 2007), thereby inferring a potential role in pain modulatory systems. The activity of DPP3 in human neutrophils has also been the subject of previous study, with assertions that such activity may relate to a role in the enkephalinergic system and modulation of localised inflammatory responses (Hashimoto *et al.* 2000). Perhaps such activity may be pathophysiologically relevant to CNP due to the potential for leukocyte infiltration into the injured nerve and surrounding inflamed tissue. Further evidence supporting a role for DPP3 in pain pathways was suggested after observations that DPP3 activity in human CSF was reduced in subjects with acute pain, when compared to pain-free subjects (Sato *et al.* 2003).

DPP3, alongside other enkephalinases, such as aminopeptidase N and neutral endopeptidase, have previously been considered as therapeutic targets for the pharmacological management of pain using both endogenous peptides (Yamamoto *et al.* 2002, Chiba *et al.* 2003) and synthetic

compounds (Le Guen *et al.* 2003, Thanawala *et al.* 2008). Spinorphin, an inhibitor of enkephalinases, was first isolated from bovine spinal cord (Nishimura *et al.* 1993) and demonstrated analgesia in mice (Ueda *et al.* 2000), although it was later determined that the more potent endogenous N- and C-terminal truncated form, tynorphin, exhibited selectivity for DPP3 inhibition (Yamamoto *et al.* 2000). Another enkephalinase inhibitor, thiorphan, the active metabolite of racecadtril, has also shown antinociceptive activity in mice (Roques *et al.* 1980). Kelatorphan and RB101, also enkephalinase inhibitors, have been studied for their analgesic value with neither molecule associated with respiratory depression, a classical side effect of excessive opioid administration (Boudinot *et al.* 2001). However, the activity of RB101 has been attributed to inhibition of aminopeptidase N and neutral endopeptidase, rather than DPP3. RB101 has also demonstrated antidepressant and anxiolytic properties (Jutkiewicz *et al.* 2006, Jutkiewicz 2007) in addition to inhibition of opiate withdrawal syndrome (Maldonado *et al.* 1995), which illustrates the potential for pharmacological inhibition of enkephalin degradation. Whether this can achieved through inhibition of DPP3 remains to be determined.

In addition to enkephalins and endomorphins, angiotensin II, III and IV also function as DPP3 substrates, whilst the dipeptide cleavage products of DPP3-mediated substrate hydrolysis also function as angiotensin converting enzyme (ACE) inhibitors (Prajapati *et al.* 2011). This provides strong evidence for DPP3 in modulating activity within the renin-angiotensin system. Aside from the potential role of DPP3 in blood pressure control, the use of animal models have affirmed a role for components of the renin angiotensin system in neuropathic pain (Ogata *et al.* 2016). Indeed, significant progress has been made with angiotensin II type 2 receptor antagonists with documented efficacy in both rodent models of peripheral neuropathic pain, and more recently, in human trials for the alleviation of PHN (Rice *et al.* 2015). The reputed efficacy of angiotensin II type 2 receptor blockade in neuropathic pain is thought to be underpinned by inhibition of downstream p38 MAPK and p44/p42 MAPK activation, which are widely accepted as key components in signalling cascades associated with CNP (Smith *et al.* 2013, Smith *et al.* 2015). To date, phase II clinical trials of an orally administered angiotensin II type 2 receptor antagonist, EMA401, have described a well-tolerated treatment exhibiting superior efficacy to placebo (Rice *et al.* 2014). Further trials are currently pending (Keppel Hesselink *et al.* 2017). The structural conformations of DPP3 and the subsequent changes upon substrate binding have also recently been elucidated, giving additional impetus for furthering the development of novel DPP3 inhibitors (Bezerra *et al.* 2012, Kumar *et al.* 2016). This, alongside the need for further validation

of DPP3 as a biomarker, will clearly provide further clarity on the therapeutic potential of DPP3 modulation.

5.4.2.2 Melanocortin 1 receptor

The melanocortin 1 receptor (*MC1R*) was upregulated 1.40 fold in patients with CNP in the discovery cohort. There was no significant difference in *MC1R* expression in the discovery/validation cohort. After SNL, *Mc1r* was also notably upregulated in the DH in both Sprague Dawley (2.72 fold) and Wistar Kyoto (1.61 fold) rats.

MC1R, a G protein-coupled receptor, is a key regulator of pigment formation through the melanin biosynthesis pathway. The expression of MC1R has been described in a multitude of distinct cell types, providing evidence of biological functions beyond melanogenesis (Catania *et al.* 2004), including, although not exclusive to, macrophages (Star *et al.* 1995), neutrophils (Catania *et al.* 1996) and astrocytes (Wong *et al.* 1997). Point mutations resulting in dysfunctional MC1R are often cited as responsible for the red-haired and fair-skinned phenotype, due to an excessive accumulation of phaeomelanin and/or reduced eumelanin in skin and hair. The regulation of phaeomelanin and eumelanin is influenced by alpha melanocyte stimulating hormone (α-MSH) via its receptor, MC1R (Burchill *et al.* 1993, Hunt *et al.* 1995, Valverde *et al.* 1995, Schioth *et al.* 1999). The α-MSH tridecapeptide is derived from post-translational processing of proopiomelanocortin (POMC), which is also a precursor to β-endorphin. Other melanocortins, including adrenocorticotropic hormone (ACTH), β-MSH and γ-MSH are similarly derived from POMC. These peptides have wide ranging effects which diversify from the traditional functions in adrenal stimulation and pigmentation, and include modulatory effects on inflammatory responses (Catania *et al.* 2004).

There is growing evidence that α-MSH has a significant role in inflammation. *In vitro* application of α-MSH has previously demonstrated inhibition of nitric oxide production in a stimulated murine macrophage cell line (RAW264.7) exposed to LPS and IFN-γ (Star *et al.* 1995). Furthermore, modulation of the inflammatory response was suggested to occur by an autocrine mechanism with increased α-MSH release after TNF-α exposure leading to attenuation of the inflammatory response via the MC1R (Star *et al.* 1995). Others have made similar observations, including the α-MSH mediated attenuation of increases in neopterin after *in vitro* IFN-γ and TNF-α exposure using the THP-1 cell line (Rajora *et al.* 1996). MC1R agonists have also been shown to attenuate the expression of vascular cell adhesion molecule (*VCAM1*) and intercellular adhesion molecule

(*ICAM1*) upon TNF-α exposure (Brzoska 1999), reducing the recruitment of inflammatory cells. Of note, *ICAM1* trended towards upregulation in both the discovery (p = 0.094) and discovery/validation (p = 0.141) cohorts. A number of hypotheses have been documented pertaining to the rationale behind the anti-inflammatory properties of α-MSH, including inhibition of TNF-mediated NF-κB activation (Manna *et al.* 1998), prevention of IκB-α degradation (which is bound with inactive NF-κB in the cytoplasm) (Ichiyama *et al.* 1999) and α-MSH-mediated increases in IL-10, a cytokine with anti-inflammatory properties (Bhardwaj *et al.* 1996, Delgado *et al.* 1998). Further work to confirm the necessity of MC1R in mediating the anti-inflammatory role of α-MSH used LPS stimulated RAW264.7 cells alongside siRNA transfection targeting *Mc1r*. It was demonstrated that after *Mc1r* knockdown, the α-MSH-mediated attenuation of nitric oxide and TNF-α production was notably reduced (Li *et al.* 2008), providing further evidence for the role of MC1R in modulation the production of nitric oxide, a reputedly key component of the pathophysiological changes contributing to the development of CNP (Levy *et al.* 2004).

Plasma levels of α-MSH are tightly regulated, but increases have been observed in inflammatory disorders and at localised regions of inflammation (Lipton *et al.* 1997), including the synovial fluid of patients with rheumatoid arthritis (Catania *et al.* 1994a), the CSF of patients with bacterial meningitis (Ichiyama *et al.* 2000) and in the plasma of patients with HIV (Catania *et al.* 1994b). It has been suggested that α-MSH may function as a compensatory mechanism to the inflammatory response, which is based upon the observation that low α-MSH may be indicative of poor prognosis (Catania *et al.* 2000).

The influence, and mechanisms underpinning, the MC1R in pain sensitivity remain relatively poorly defined. It is suggested that *MC1R* expression is influenced by its endogenous ligands α-MSH and ATCH (Abdel-Malek *et al.* 2000, Catania *et al.* 2004), which may explain the apparent upregulation of *MC1R*, considering that increased α-MSH has been frequently observed as elevated in regions of localised inflammation (Catania *et al.* 1994a, Lipton *et al.* 1997). Research has shown that *Pomc* is not differentially expressed in the sciatic nerve, DRG or spinal cord after nerve crush injury (Plantinga *et al.* 1992). Indeed, the differential expression of POMC convertases across different tissues is thought to control the generation of specific POMC cleavage products, with *PCSK2* expression promoting the production of α-MSH and β-endorphin (Day *et al.* 1992). Further analysis has provided additional clarity on the impact of α-MSH on nerve injury and inflammation. Firstly, *in vitro* analysis of cultured fetal spinal cord slices showed that application of α-MSH or ACTH promoted axonal outgrowth (van der Neut *et al.* 1992, Bar *et al.* 1993),

although it has been suggested that such observations may been attributed to MC4R binding (Adan *et al.* 1996). Intriguingly, centrally administered α-MSH has also been shown to reduce peripheral inflammation (Lipton *et al.* 1991). The mechanisms by which this occurs are incompletely understood. It is thought that α-MSH may facilitate descending inhibitory activity, thereby attenuating the neurogenic component of inflammation. Such activity may subsequently inhibit of the release of substances such as histamine and substance P, both of which increase vascular permeability and ultimately, evoke pain (Macaluso *et al.* 1994).

The role of the melanocortin system in pain sensitivity has been the subject of multiple studies yielding variable outcomes. Female mice lacking *Mc1r* were more tolerant to noxious heat and exhibited reduces responsiveness to formalin-induced inflammatory pain, although no difference was found in relation to *Mc1r* genotype and neuropathic pain after CCI (Delaney *et al.* 2010). Red-haired females have been shown to exhibit greater sensitivity to thermal pain, in addition to observations of reduced efficacy to subcutaneous lidocaine in pain tolerance tests (Liem *et al.* 2005). Moreover, females with two variant *MC1R* alleles also tended to experience enhanced analgesia with pentazocine, a κ-opioid receptor agonist (Mogil *et al.* 2003),

Dynorphin, an opioid peptide, is able to antagonise melanocortin receptors at physiologically relevant concentrations (Quillan *et al.* 1997). It has been suggested that sex-dependant influences on dynorphin and κ-opioid receptors may be founded upon genetic linkage with *MC1R* (Chartoff *et al.* 2015). Sex-specific influences have also been shown to underpin opioid-induced hyperalgesia, which is facilitated by the NMDA receptor and the MC1R in male and female mice, respectively (Juni *et al.* 2010). The determinants for gender-specific control of opioid-induced hyperalgesia have therefore been attributed to oestrogen and progesterone (Mogil 2012).

Given the apparent associations between MC1R and inflammatory conditions, consideration for MC1R as a therapeutic target has been given (Catania *et al.* 2004). Endogenous agonists of MC1R have been shown to exert anti-inflammatory action after systemic or local administration (Luger *et al.* 2007), whilst hypersecretion of the endogenous MC1R antagonist, agouti signaling protein, has been shown to augment the inflammatory response upon LPS challenge (Ollmann *et al.* 1998, Lipton *et al.* 1999). Studies encompassing both rodents and human participants have subsequently reaffirmed these conclusions with improved recovery rates observed in rats treated with either the ACTH analogue, ORG2766, or α-MSH, after sciatic nerve crush injury (Bijlsma *et al.* 1983, Van der Zee *et al.* 1988), whilst ORG2766 also attenuated cisplatin-induced neuropathy in human

subjects (van der Hoop *et al.* 1990). Evidence therefore suggests complex and diverse mechanisms by which MC1R influences pain sensitivity and considered evidence of *MC1R* upregulation in CNBP patients and in the dorsal horn after SNL, further validation is paramount to assess the value of MC1R as a translational biomarker.

5.4.2.3 TIMP metalloproteinase inhibitor 1

TIMP metalloproteinase inhibitor 1 (*TIMP1*), an inducible, soluble and secreted glycoprotein with cytokine-like properties (Ries 2014), was 1.50 fold upregulated in the blood of CNP patients within the discovery cohort, highlighted during correlation analysis and was also supported by a plethora of publications pertaining to pain (Table 4.1). Subsequent analysis of *Timp1* expression in the DH after SNL demonstrated a 2.17 fold and 2.19 fold upregulation in Wistar Kyoto and Sprague Dawley rats, respectively.

TIMPs function to inhibit the matrix metalloproteinases (MMPs), which are categorised as either collagenases, gelatinases, stromelysins and membrane-bound MMPs, at a 1:1 stoichiometric ratio (Gomis-Ruth *et al.* 1997). The MMPs represent a group of calcium-dependent and zinc-containing endopeptidases involved in extracellular matrix degradation, and have several consequential roles in cell-cell interactions, migration and cell proliferation (Lorenzl *et al.* 2003). Multiple other roles for MMPs have been described, including caspase-independent activation of pro-IL-1β (Schonbeck *et al.* 1998), cleavage of Interleukin 2 receptor subunit alpha (Sheu *et al.* 2001) and cleavage-mediated release of TNF-α from activated microglia (McCawley *et al.* 2001, Lee *et al.* 2014). The MMPs have a diverse range of physiological and pathophysiological roles, including cancer progression (Egeblad *et al.* 2002), bone remodeling (Paiva *et al.* 2014) and cardiovascular disease (Agewall 2006). As a circulating prognostic marker, the endogenous MMP-9 inhibitor, TIMP1, has been scrutinised in gastric cancer (Wang *et al.* 2006), breast cancer (Thorsen *et al.* 2013), inflammatory bowel disease (Kapsoritakis *et al.* 2008) and as a marker of pre-diagnosis pancreatic ductal adenocarcinoma (Jenkinson *et al.* 2015). TIMP1 levels were also significantly higher in the CSF of patients with Parkinson's disease, Alzheimer's disease, Huntington's disease and motor neuron disease (Lorenzl *et al.* 2003).

Considering that both MMP-9 and TIMP1 are closely associated, it is unsurprising that many researchers have sought to determine disease associations with the MMP-9/TIMP1 ratio, which is thought to provide greater insight into MMP-9 activity than either component alone. High serum MMP-9 and low TIMP1 levels were associated with brain lesion formation in relapsing-remitting

MS (Waubant *et al.* 1999), and a decrease in the ratio was associated with IFN-β treatment in patients with MS (Avolio *et al.* 2005). However, others have found the ratio to be an unreliable indicator of MMP-9 activity (Tarr *et al.* 2011). An alternative concept to the MMP/TIMP axis is that MMPs may effectively inhibit alternative roles of TIMPs by binding their endogenous ligand and reducing the availability of free TIMP1 (Cleutjens 1996, Moore *et al.* 2012). Such assertions may reflect the importance of TIMP1 in reputed MMP-independent roles (Moore *et al.* 2012).

The observed upregulation of *Timp1* in Sprague Dawley and Wistar Kyoto ipsilateral DH supports its potential role in the mechanisms underpinning the development or maintenance of neuropathic pain. Such upregulation has also been observed in the L4 and L5 DRG 28 days after SNT (Huang *et al.* 2011) and in a rat spinal cord injury model 28 days after surgery, again leading to suggestions that *Timp1* may be involved in the maintenance phase of neuropathic pain (Sandhir *et al.* 2011). Moreover, this was observed in the absence of significantly upregulated MMP-9, which again supports growing evidence that TIMPs may have MMP-independent functions (Chirco *et al.* 2006, Stetler-Stevenson 2008). In addition, *Timp1* was previously found to be upregulated in the spinal cord after CCI (Rodriguez Parkitna *et al.* 2006). Interestingly, this upregulation was not observed in rats with CFA-induced inflammatory pain, which suggests that *Timp1* expression may be discriminatory between neuropathic and inflammatory pain, thereby lending further support to the observations that TIMP1 was significantly higher in the plasma of CNBP patients, than those with CIBP (section 4.3.9).

Microglial activation is a central component pertaining to the mechanisms underpinning chronic pain after nerve injury (Hulsebosch 2008) and has been demonstrated in both rodent models of neuropathic pain (Miller *et al.* 2013) and in the post-mortem of three patients with spinal cord contusion (Chang 2007). It is suggested that microglia activation, which contributes to hyperalgesia, is present for weeks or months in rodents, but may become protracted, persisting for years in patients with spinal cord injury, thereby contributing to phenotypic remodelling (Chattopadhyay *et al.* 2009, Sandhir *et al.* 2011). Given that activated microglia release proinflammatory cytokines, which leads to *Mmp-9* upregulation, this is somewhat supportive of the role of the MMP/TIMP axis in pain, suggesting it may have value as a translational biomarker of CNP (Brew *et al.* 2000, Chattopadhyay *et al.* 2007). MMP-9, which is produced by resident and infiltrating cells, including Schwann cells, endothelial cells and macrophages (Shubayev *et al.* 2002), has been shown to facilitate demyelination after nerve injury by degrading myelin basic protein (Chattopadhyay *et al.* 2007). Crucially, MMP-induced elevations in blood-spinal cord

barrier disruption may occur, leading to increased immune cell infiltration. Subsequent interactions with resident microglia and astrocytes may then lead to lesion expansion and potential exacerbation of neuropathic pain (Goussev *et al.* 2003, Ahmed *et al.* 2011, Qin *et al.* 2016).

Novel molecular mechanisms pertaining to TIMP1 regulation have also been considered, namely between cannabinoid receptors and the MMP system (Finnerup *et al.* 2006). It is established that factors such as reactive oxygen species and mechanical stimuli are capable of activing transcription factors such as AP-1 and NF-κB, leading to direct increases in the transcription of inflammatory mediators. Indeed, both MMP-9 and TIMP1 are upregulated by AP-1 (Ahmed *et al.* 2011). It has been considered that activation of the cannabinoid receptors leads to induction of MAPK pathways, which in-turn actives AP-1 resulting in *MMP-9* and *TIMP1* upregulation (Ahmed *et al.* 2011). This represents a bridging between two distinct pathways and considers novel downstream implications of pharmacological modulation of the CB1 and CB2 (cannabinoid) receptors, which are both upregulated in the DRG and spinal cord after nerve injury (Walczak *et al.* 2006).

Clearly, pharmacological modulation of MMP activity may provide a valuable therapeutic avenue for the treatment of CNP. Broad spectrum inhibition of MMPs reduces the infiltration of immune cells after nerve injury, decreases caspase-mediated apoptosis and inhibits astrocyte activation (Kobayashi *et al.* 2008). It has also been reported that broad inhibition of MMPs downregulates two key genes commonly targeted in the treatment of CNP, *Scn9a* (encoding $Na_v1.7$) and *Scn10a* (encoding $Na_v1.8$) (Kim *et al.* 2012). However, the clinical utilisation of MMP inhibitors is impeded by the diverse range of functions of the MMPs, with inhibition of certain functions, such as TNF-α release, resulting in severe side effects (Solorzano *et al.* 1997, Wojtowicz-Praga 1999). Indeed, this is exemplified by the MMP-9 inhibitor, minocycline, which resulted in less-favourable outcomes when compared to placebo in patients with motor neurone disease (Gordon *et al.* 2007). However, it remains noteworthy that administration of another MMP-9 inhibitor, atorvastatin, which is traditionally used in the treatment of hypercholesterolemia, reduced interruption of the blood-spinal cord barrier after SCI, leading to reduced infiltration of immune cells and subsequent reductions in tissue damage and improvement in functional recovery (Pannu *et al.* 2007). Indeed, these are similar to the reputed benefits of TIMP1 administration (Ahmed *et al.* 2011). Atorvastatin has been shown to successfully reduce neuropathic pain in rats after CCI (Pathak *et al.* 2014) and is documented in case reports describing human subjects experiencing unexpected analgesia as a consequence of treatment for hypercholesterolemia (Gillon *et al.* 2013). The potential for MMP-

independent roles for TIMP1 should not however, be disregarded, as MMP inhibition may fail to unravel the potential benefits of pharmacologically modulating TIMP1 activity.

5.4.3 Limitations and variability in animal models

The implementation of an animal model of neuropathic pain is often proceeded by the assessment of response to external stimuli. It is important to determine that altered behavioral measurements are truly reflective of neuropathic pain and are not a result of non-specific changes in sensory responsiveness and non-neuronal injury (Sluka *et al.* 2001). The same principle therefore applies to the use of animal models for the analysis of gene expression after nerve injury. Measurements should be reflective of neuropathic pain, differentially observed in sham animals and when possible, occur predominantly ipsilateral to the injury site (Sapunar *et al.* 2009).

Multiple parameters must remain tightly controlled during animal studies. The requirement for environmental consistency is a profound consideration when assessing behavioral changes in animal models of pain. The time of day, humidity and cage density are all examples of parameters that are known to interfere with behavioral measurements. Isolation may also have permanent species-specific implications on behaviour for the rat, but not mouse, gerbil or guinea pig (Einon *et al.* 1981). Consideration must also be given to group-housing of adult male mice, which may lead to displays of dominance and aggression. This has been shown to have implications for baseline nociceptive thresholds (Miczek *et al.* 1982). Clearly, animals injured as a result of this, which could be unknown to the experimenter, may exhibit reduced tolerance or thresholds to sensory tests (Wilson *et al.* 2001) with additional implications on the analysis of changes in gene expression.

After SNL, behavioral measurements have been shown to vary both between different rat strains obtained from the same source, and between the same strains obtained from different sources (Yoon *et al.* 1999). This emphasises the potential for variability. Indeed, an analysis of baseline thermal nociceptive sensitivity highlighted that the experimenter conducting the study was responsible for greater variation on behavioral measurements than mouse genotype (Chesler *et al.* 2002). Such variation may be reflected in the CCI model, which in contrast to SNL, showed greatest variability in behavioral measurements between animals (Kim *et al.* 1997). This has been attributed to variability in the tightness of the applied sutures (Ro *et al.* 1993). Moreover, other factors such as the choice of suture material have also been acknowledged as contributing to potential variation (Austin *et al.* 2012).

5.4.4 Future directions

Although several genes were found to be differentially expressed in the DH after SNL, it cannot be concluded that the remaining genes are either invalid biomarkers of CNP, or that they do not function as translational biomarkers. This is particularly noteworthy considering the previously discussed variations, both between the human cohorts and between different strains of rat. In order to further elucidate whether the genes differentially regulated in human blood also represent translational biomarkers of CNP, several aspects of the translational study could be expanded to encompass further parameters. These include the analysis of gene expression changes in the injured nerve and DRG, particularly as research has shown that there is little overlap between the gene expression changes in the DRG and DH after experimentally induced nerve injury (Zhang *et al.* 2005). In addition, the use of the contralateral side to the injury when assessing changes in gene expression is a useful consideration. Another pertinent consideration for future work would be to analyse transcriptomic changes over variable durations of time after nerve injury, in order to detect changes in gene that occur, and persist, over the first days, weeks and months after nerve injury. Previous similar studies using the L5/6 SNL model have shown that after 2 days, 309 genes were differential expressed (>2 fold) when compared to the contralateral side, which reduced further to 224 genes after 2 weeks and further to 54 genes after 5 months (Kim *et al.* 2009a). Clearly, should the transcriptomic profile in the DH exhibit similar dynamic changes after nerve injury, a single analysis of gene expression after 35 days may not detect changes synonymous with the early phase of neuropathic pain. Although it may be anticipated that changes in gene expression present at 35 days post-injury may reflect a degree of chronicity, further analysis after several months would provide valuable information pertaining to genes which may be involved in the maintenance of CNP, thereby potentially representing true translational CNP biomarkers (Ru-Rong 2008). In addition to consideration for other models such as SNI, CCI, and those pertaining to inflammation for comparative purposes (Rodriguez Parkitna *et al.* 2006), the use of data relating to behavioural responses to nerve injury may be beneficial with regards to the selection of animals for transcriptomic analysis. This may ameliorate variations resulting from experimentally induced nerve injury by distinguishing animal that have failed to demonstrate clear behavioural responses indicative of neuropathic pain. Latterly, the inclusion of the rat blood transcriptomic profile, in addition to the DRG and DH (Grace *et al.* 2012), would undoubtedly prove a powerful tool in the clarification of translational biomarkers of CNP.

5.5 Conclusion

Despite the acknowledged limitations of animal models of neuropathic pain, particularly in relation to the ability of a single pain model to reflect the complex pathological and transcriptomic changes in humans with CNP, their value in the determination of translational biomarkers and to provide insight into changes in the nervous system after injury is unrivalled. Indeed, *Timp1*, *Mc1r* and to a lesser extent, *Dpp3*, all exhibited significant differential regulation suggestive of a translational biomarker of neuropathic pain. Moreover, considering the plethora of future considerations for the expansion of animal work, with particular focus on blood-nerve transcriptomic correlations, the potential for the identification and subsequent investigation of further translational biomarkers is vast.

5.6 Summary points

- Downregulation of *Gchfr* was observed both in Sprague Dawley rats after SNL, and in the discovery/validation cohort, thereby suggesting a role for the regulation of GFRP in *de novo* BH_4 synthesis
- The use of ddPCR successfully enabled assessment of changes in gene expression in the DH
- *Timp1*, *Mc1r* and *Dpp3* were differentially regulated and may represent novel translational biomarkers of CNP
- Multiple future considerations are given for the expansion of translational biomarker studies

Chapter 6 General Discussion

6.1 Current deficiencies in CNP diagnosis

Chronic neuropathic pain is a major healthcare challenge, with prominent deficits in both diagnosis and treatment. It also tends to be protracted, with potentially severe implications on physical health, mental health and quality of life. In comparison to several other similarly prominent diseases, the current diagnostic capability for neuropathic pain is starkly lacking and often relies on verbal descriptors and questionnaire based screening tools to aid diagnosis. Moreover, when considering the modest specificity and selectivity associated with these case identification tools, it should be considered that clinician diagnosis, the gold standard to which these tools are graded against, may fall considerably short of generally acceptable accuracy due to a lack of routine and acceptable diagnostic methodology. Indeed, the paradigm of a lack of diagnostic capability subsequently impeding the development of novel diagnostic methodology is clear, and was a recurring theme throughout this thesis. As previously eluded to, the current state of diagnostic capability is exemplified by the advocating of a 'possible, probable and definite' CNP case categorisation (Treede *et al.* 2008), which if suggested in other long-term conditions, such as Parkinson's disease and cystic fibrosis, would clearly be unpalatable. This clear shortcoming, a near unmet clinical need, provided the impetus for this thesis which aimed to identify potential biomarkers of CNP, with additional consideration for their apparent value as pharmacological targets and for the translation of findings in an animal model of neuropathic pain.

6.2 Pterins and CNP

After the development of a suitable method, analysis of neopterin was achieved for both cohorts. Although the quantification of pterins was not entirely comprehensive due to lack of data pertaining to (reduced) biopterin(s) in the discovery/validation cohort, when analysed alone, the accrued data does not indicate an abundantly clear role for pterins as biomarkers of CNP. The predominant reservations pertaining to the value of circulating pterins in CNP diagnosis are multifactorial. The ability of neopterin to differentiate between predominantly neuropathic and non-neuropathic pain is questionable, particularly considering the suggestion that BH_4 may be implicated in pathways that converge between pain of predominantly neuropathic or inflammatory origin (Tegeder *et al.* 2006). Moreover, there is also a general lack of evidence pertaining to the maintenance of *Gch1* upregulation in the injured nerve, DRG or DH after nerve injury. Indeed,

animal models have frequently demonstrated differential regulation of genes pertaining to *de novo* BH_4 synthesis and regeneration after nerve injury, although their ability to reflect persisting changes in gene expression is a notable shortcoming within current literature (Tegeder *et al.* 2006, Latremoliere *et al.* 2015a).

Despite these reservations, a proportion of data relating to pterins is of particular interest. Although neopterin was undifferentiable between controls and CNP patients in the discovery cohort, differences were observed in the discovery/validation cohort, with particular focus on observations after categorisation of patients according to S-LANSS score. Whilst statistical significance was not observed, the imperfect specificity/selectivily of the S-LANSS may be considered as a contributing factor to this. Similarly, mixed outcomes were also observed in relation to biopterins, with decreased BH_4 and marginally elevated BH_2 in patients with CNP. Whilst it is thought that the BH_4 pathway induces pain through nitric oxide synthases (Tegeder *et al.* 2006), the BH_4/BH_2 ratio is a key consideration which also circumvents a degree of variability in the interpretation of either pterin alone. Low ratios, such as those observed in inflammatory conditions (Mikkelsen 2016) and within the CNP group in the discovery cohort, may be a consequence of NOS uncoupling and subsequent superoxide and peroxynitrite production. This may be exacerbated by resulting peroxynitrite-mediated oxidation of BH_4 to BH_2, further augmenting BH_2 abundance relative to that of BH_4 (Bendall *et al.* 2014). Strategies for the attenuation of superoxide and peroxynitrite formation have been described as a potential avenue for amelioration neuropathic pain (Kim *et al.* 2009c, Doyle *et al.* 2012, Janes *et al.* 2012). It is unclear whether such a rationale is contributing to the observations detailed in this thesis, nor do the BH_4/BH_2-nitrate correlations facilitate such a hypothesis. Notable caution is also required with the potential for interferences in pterin levels, primarily relating to comorbidities and pharmacological influences, which may be undiagnosed, unknown or not disclosed at the point of testing (Housby *et al.* 1999, Kraychete *et al.* 2009, Bendall *et al.* 2014, Firoz *et al.* 2015). Clearly, the impact of these factors may be pronounced and difficult to identify in small patient cohorts. Further analysis of the BH_4/BH_2 ratio in neuropathic pain, with particular focus on BH_2-mediated superoxide/peroxynitrite formation, is certainly worthy of further consideration.

6.3 Nitric oxide and CNP

Analysis of nitric oxide oxidation products in plasma was successfully conducted with consideration for the relatively short biological half-life of both nitric oxide and nitrite, which

necessitated enzymatic reduction of nitrate to nitrite. There was no significant difference in nitrite/nitrate when comparing healthy controls and CNP patients in both cohorts, although there was a notable trend towards elevated levels pertaining to those with CNBP in the discovery cohort. This observation may indeed be related to the clearly defined pain presentation within this cohort. However, the value of nitrite/nitrate as a biomarker is somewhat complicated both by the relatively wide range of typical plasma nitrate values in healthy humans, of 15-70 μM (Tsikas *et al.* 1998). In addition, the potential for endogenous contributors (e.g. comorbidities) and exogenous factors (e.g. medication and diet) to circulating nitrite/nitrate levels should be considered. These may complicate analysis seeking to attribute nitrite/nitrate to specific pathophysiological changes, particularly if concentrations change as a consequence of CNP, but frequently remain within a normal range. Aside from the exogenous factors previously described, other confounded factors may include variable expression of the NOS isoforms (including contributions from genetic variation) (Aminuddin *et al.* 2013), BH_4 availability (and related expression of pathway enzymes) and substrate (arginine) and cofactor (NADPH) availability (Luiking *et al.* 2010). Indeed, only one patient within the samples analysed from both the discovery, and discovery/validation cohorts, exhibited plasma nitrate exceeding the aforementioned normal plasma nitrate parameters, which infers that circulating nitrite/nitrate lacks feasibility as a stand-alone CNP biomarker.

Further analysis was conducted to elucidate correlations between nitrite/nitrate and other biomarker candidates, with noteworthy outcomes. Whilst nitrate and neopterin strongly correlated in the discovery cohort, it is pertinent that the degree of correlation was notably greater in those with pain, that healthy controls, suggesting an overall elevation in GTPCH activity. Indeed, trends towards upregulation of *GCH1* and downregulation of *PTS* were observed in patents in the same cohort, which may lead to increased neopterin production alongside probable simultaneous elevations in BH_4, thereby promoting nitric oxide production. A similar pattern of correlation between nitrate and neopterin was also present in the discovery/validation cohort, although this was notably weaker, which may be related to the mixed neuropathic aetiologies. Correlations between *GCH1* and nitrate in the discovery/validation cohort were however, comparatively strong, with notably greater correlation in those with an S-LANSS score suggestive of pain of predominantly neuropathic origin. Such correlations point to an underlying processes contributing to heightened activity within BH_4 synthesis pathways, with a degree of specificity towards CNP. The mechanisms and rationale underpinning elevated nitrite/nitrate in the plasma of patients with CNP remains to be confirmed and further validation would be necessary, particularly considering

previous observations that serum nitrite/nitrate did not vary after CCI in rats (Naik *et al.* 2006). Therefore, in the absence of further experimentation with larger cohorts comprising of neuropathic pain of specific aetiologies demonstrating notable separation of nitrite/nitrate levels between CNP and controls, current data may be suggestive of underlying pathophysiological processes pertaining to BH_4 and its role as a cofactor, but does not support its current use as a biomarker of CNP.

6.4 Transcriptional regulation and BH_4 synthesis

Differential regulation of genes pertaining to BH_4 production have been well documented in literature pertaining to rodent models of neuropathic pain, which largely relates to *Gch1*, *Spr* and *Qdpr* (Costigan *et al.* 2002, Tegeder *et al.* 2006, Latremoliere *et al.* 2015b). Analysis of gene expression changes in the rat DH was achieved using qRT-PCR, with some noteworthy outcomes. Although data did not always correspond between the different rat strains, *Gch1* displayed the greatest fold change (although not statistically significant), which is perhaps unsurprising considering the weight of evidence demonstrating *Gch1* upregulation during injury and inflammation (Latremoliere *et al.* 2011). However, other than downregulation of *Gchfr* in Sprague Dawley DH, there was general lack of remarkable results. The rationale for the mixed observations may be founded upon the previously discussed potential variabilities associated with the surgical procedures and dissection, although with limited evidence for chronic *Gch1* upregulation following nerve injury, it is possible that changes in gene expression were relatively diminished at 35 days post SNL. Indeed, the seminal article by Tegeder *et al* showed upregulation of *Gch1*, *Spr* and *Qdpr* in the DRG, peaking at 3-7 days after SNI, with a subsequent decline in the magnitude of upregulation towards the final data point at 21 days, at which point upregulation of *Qdpr* was no longer statistically significant (Tegeder *et al.* 2006). Although this suggests a downward trend, it cannot be surmised that *Gch1* and *Spr* expression continued on this trajectory towards a return to basal expression. In addition to changes in gene expression occurring over time, the use of the DH also represents a notable source of contrast when compared to the injured nerve or DRG, particularly as few genes are differentially regulated in both the DRG and DH after nerve injury (Zhang *et al.* 2005). Although a previous study highlighted upregulation of GTPCH in the DH after CCI (Meng *et al.* 2013), time-course gene expression analysis of transcripts pertaining to BH_4 pathways in the sciatic nerve, DRG and DH after SNI highlighted that whilst multiple genes were differentially regulated in the injured nerve, only *Gch1* was regulated in the DRG with no clear change in the expression of any gene pertaining to BH_4 in the DH (Latremoliere *et al.* 2015b).

Whilst this study did not consider *Gchfr*, upregulation of *Gch1* sciatic nerve and DRG peaked at one day after injury and subsequently declined and demonstrated a degree of stabilisation of upregulation after 14-21 days, thereby adding further uncertainty to the chronicity of *Gch1* upregulation in animal models of neuropathic pain (Latremoliere *et al.* 2015b).

Similarly, analysis of gene expression changes pertaining to BH_4 in the blood of CNP patients showed mixed results, although given the novelty of this approach, there is a distinct lack of literature available to allow for comparison with observations from previous studies. There was a general absence of concordance between both cohorts, which as previously suggested, may be a consequence of different neuropathic aetiologies. Of note, a general trend towards downregulation of *Gchfr*, which achieved statistical significance when analysed by qRT-PCR in the discovery/validation cohort, was observed. Similar observations of *GCHFR* downregulation in response to inflammatory mediators have been observed both *in vitro* (Gesierich *et al.* 2003) and *in vivo* (Werner *et al.* 2002, Ishii *et al.* 2005). Although attributions to elevated BH_4 in CNP are largely associated with upregulation of GTPCH, downregulation of GFRP may also facilitate BH_4 production, particularly alongside GTPCH upregulation, due to anticipated changes in the stoichiometry and reduced BH_4/GFRP-mediated inhibition of GTPCH.

6.5 Pain protective haplotype, nitric oxide and neopterin synthesis

Determination of genotype in relation to the pain protective haplotype, which is thought to decrease *GCH1* transcription and/or implicate RNA/protein stability (Tegeder *et al.* 2006, Lötsch *et al.* 2007), was enabled by means of PCR-RFLP methodology. Current evidence associating the pain protective haplotype to changes in sensitivity and/or susceptibility to painful conditions shows variable outcomes, which is unsurprising considering the wide variety of research themes (Table 3.1). Indeed, since the original observations by Tegeder *et al* (Tegeder *et al.* 2006), relatively few studies have considered patients with similar disease characteristics, with multiple studies focusing on experimentally induced pain sensitivity (Tegeder *et al.* 2008, Campbell *et al.* 2009), coital and labour pain (Dabo *et al.* 2010, Heddini *et al.* 2012, Pettersson *et al.* 2016), dental procedures (Kim *et al.* 2007, Lee *et al.* 2011) and pancreatitis (Lazarev *et al.* 2008). However, despite studies considering patients with likely neuropathic involvement, such as those with HIV-associated sensory neuropathy (Hendry *et al.* 2013), and those with similar disease characteristics to those analysed by Tegeder *et al* (Tegeder *et al.* 2006, Kim *et al.* 2010, Hegarty *et al.* 2012), there remains

a distinct lack of consensus amongst these studies with regards to the influence of the pain protective haplotype.

Although the size of the discovery cohort, and the number of samples available for *GCH1*, neopterin and nitrite/nitrate, was insufficient for robust analysis, such analysis was more feasible with the discovery/validation cohort. Although significantly higher *GCH1* levels were observed in healthy controls whom carried the haplotype, the remaining data pertaining to nitrite/nitrate and neopterin was generally unremarkable. Rationale for the lack of associations may be multifactorial. In reference to previous observations using cultured white blood cells, basal levels of *GCH1* mRNA and protein, in addition to biopterin, were not differentiable when categorising subjects according to genotype, yet administration of forskolin, which promotes *GCH1* transcription via cAMP in the proximal promoter, increases in GTPCH and biopterin were noted, which decreased in magnitude amongst heterozygous carriers, and further in homozygous carriers of the haplotype (Tegeder *et al.* 2006). Therefore, with consideration for previous observations of similar nitrite/nitrate and neopterin levels between controls and CNP, coupled with the previously discussed endogenous and exogenous influences, it is perhaps unsurprising that a lack of influence for the pain protective haplotype was observed amongst control subjects, given the probable absence of ongoing inflammatory processes. However, given a similar absence of significant variation amongst those with CNP, it is apparent that the pain protective haplotype has either no effect, or an indistinguishable effect, on circulating nitrite/nitrate and neopterin in this cohort. The two prominent rationale for such observations may be associated with the different pain phenotypes within this cohort; underpinned by variable pathophysiological changes in association with different anatomical features. A clear distinction should be made between the mechanisms and magnitude of effect obtained by *in vitro* forskolin challenge, which itself produced a relative modest fold change in *GCH1* and biopterin (Tegeder *et al.* 2006), and the complex and variable mechanisms in man, which may not be similarly or consistently observable in blood as is seen in the injured nerve.

6.6 Pain protective haplotype and GCH1 regulation

In order to improve our understanding of how the pain protective haplotype may confer reduced sensitivity or propensity to develop chronic pain, multiple methods were considered, including reporter gene assays, bioinformatics and EMSAs. Initially luciferase assays were used to help decipher whether a single base change, corresponding to a specific SNP within the haplotype, may

lead to changes in reporter gene expression. Assays using HEK293 and SH-SY5Y cells demonstrated that constructs representing the minor allele of the *GCH1* 5' SNP (rs8007267) showed significant reductions of relative luminescence, inferring that this allele may be at least somewhat involved in the regulation of *GCH1* transcription. Similar results were also observed after cloning of over 10 kb of the *GCH1* 5' region, incorporating the polymorphic locus, followed by transfection into HEK293 cells. However, considering previous observations that cell stimulation is required to demonstrate differential *GCH1* transcription according genotype (Tegeder *et al.* 2006), transfection of HUVECs was achieved alongside exposure to TNF-α and IFN-γ. A significant reduction in relative luminescence was observed only under the influence of cytokines, thereby affirming a potential regulatory role for the allele. Further *in-silico* analysis highlighted a potential role for the transcription factor ARNT, complexed with either AhR (as predicted by MatInspector), or potentially with HIF-1α. The associations between these pathways and inflammation/neuropathic pain were not immediately clear. However, research has demonstrated that hypoxia may result from persistent vascular dysfunction after peripheral nerve injury (Lim *et al.* 2015) with potential implications on sensitisation of TRPA1 (So *et al.* 2016), which is incidentally considered (alongside TRPV1) a downstream pro-nociceptive mediator of elevated BH_4 via nitric oxide synthesis (Miyamoto *et al.* 2009). Similarly, the previously hypothesised role of the AhR is further intrigued by evidence suggesting significant cross-talk between AhR/ARNT and inflammatory mechanisms (Nguyen *et al.* 2013, Vogel *et al.* 2014). Clearly confirmatory work is necessary to determine whether a role exists for the tryptophan degradation pathway in the regulation of BH_4 *de novo* synthesis, either by increased production of the SPR inhibitor xanthurenic acid (Haruki *et al.* 2015) or by genotype-dependent differential regulation of *GCH1* by means of L-kynurenine mediated AhR nuclear translocation.

6.7 GCH1 regulation

In order to build upon previous reporter gene assays illustrating an integral role for the first intron of *GCH1* in upregulation of gene expression in response to both LPS and IFN-γ/TNF-α (Liang *et al.* 2013), luciferase reporter assays were conducted using cytokine stimulated HUVECs. This incorporated a significantly larger region of the *GCH1* promoter and intron (28.6 kb). The rationale for the general reduction in relative luminescence observed over the majority of vector constructs remains unclear, but may relate to widespread transcriptomic changes after cytokine stimulation, potentially upregulating transcriptional repressors. However, the implication of such changes may clearly impact transcription of an episomal firefly luciferase construct differently to that of *GCH1*,

particularly considering the influence of chromatin structure/histone modifications. Similar observations were also made with both vector constructs pertaining to the *GCH1* 5' SNP (rs8007267) and to a less degree, the intronic SNP (rs3783641). In relation to the *GCH1* intron stimulation analysis, it was particularly noteworthy that the only construct to demonstrate a trend towards an increase in normalised firefly luminescence contained the loci for the reputed enhancer elements previously suggested to be at least partially responsible for upregulation of *GCH1* in response to TNF-α and IFN-γ (Liang *et al.* 2013). Future work in this regard should seek to assess the effect of incubation time post-transfection, further consideration for the incorporation of another cell line (such as RAW264.7) and a wider range of stimulatory factors, such as LPS and forskolin. After which, further cloning and mutations analysis may then facilitate the identification of enhancer regions and potentially, SNPs involved in *GCH1* regulation.

6.8 Transcriptional and translational biomarkers of CNP

Analysis of transcriptomics by microarray initially highlighted multiple differentially regulated genes in the discovery cohort, which comprised exclusively of patients with CNBP. After refinement of microarray data using modified statistical criteria, correlation analysis with previous microarray data and scrutiny of current literature, a total of 15 genes were proposed as potential CNBP biomarkers for further consideration. Similar methodology was used with the discovery/validation cohort, with the exception of correlation analysis, leading to the identification of 12 genes subject to further scrutiny. However, the discovery/validation cohort incorporated patients with variable pain aetiologies as collection rates meant there was insufficient data points to allow for categorisation by a particular diagnosis. Several genes displayed a degree of validation between the two cohorts, in particular *ARHGAP11B*, *CASP5* and *TLR5*. However, failure to validate between these cohorts does not discredit the potential of a candidate biomarker due to the aforementioned variation in cohort characteristics. Multiple differentially regulated genes, and their respective literature evidencing a role in mechanisms associated with pain, have been described in detail. Of the genes exhibiting strong associations, *CASP5, CX3CR1*, *MC1R*, *TIMP1*, and *TLR4*/*TLR5*, are perhaps of greatest interest.

Of the differentially regulated genes in the discovery cohort present after data refinement, *TIMP1* exhibited considerable supportive evidence. Correlation analysis initially highlighted that the expression of *TIMP1*, in conjunction with other genes, deviated from the norm generally observed in previous unrelated microarray studies. Considering the abundance of literature evidence

pertaining to TIMP1 in painful conditions (Table 4.1), analysis of plasma TIMP1 was sought. Relatively strong correlations were observed between plasma TIMP1 and blood *TIMP1* expression, which also highlighted differentiation between patients with CIBP and CNBP. This demonstrates that ELISAs, which are likely to be preferential as routine diagnostic methodology, may provide accurate representation of *TIMP1* expression, thereby possessing similar value as a CNBP biomarker. Clearly further validation is necessary in order to determine specificity and selectivity to CNBP, particularly considering that replication in the discovery/validation cohort was not observed, and to clarify whether upregulation of TIMP1 remains robust in spite of relatively common co-morbidities previously associated with TIMP1 (Wang *et al.* 2006, Kapsoritakis *et al.* 2008, Thorsen *et al.* 2013). Additional regard for the potential of MMP-dependent and -independent roles (Moore *et al.* 2012) of TIMP1 in the pathophysiology of CNP should also be given, particularly considering upregulation of *Timp1* in the DH after SNL and the reputed benefit of MMP-9 inhibition in the treatment of CNP (Gillon *et al.* 2013).

In addition to *TIMP1*, the accrued data suggests that *CASP5*, which was upregulated in both cohorts, is worthy of further scrutiny as a CNP biomarker, and may be indicative of spinal injury. Whilst CASP5 functions as a constituent of the inflammasome complex and triggers maturation of IL-1β, there is clear convergence of molecular pathways and functions with the TLRs. Activation of TLRs (of which *TLR4* and *TLR5* are upregulated in this study) leads to upregulation of inflammatory cytokines, including IL-1β, in addition to integral components of inflammasome complexes, such as NLRP3 (Bauernfeind *et al.* 2009) and *CASP5* (Lin *et al.* 2000). Indeed, both TLR/NF-κB-mediated increases in *IL-1β* and increased maturation (via the inflammasome complex) will lead to significant elevations in IL-1β. IL-1β has been shown to be integral to the development of neuropathic pain and related hyperalgesia and allodynia (Watkins *et al.* 1994, Wolf *et al.* 2006, Pineau *et al.* 2007), whilst also upregulating MMPs (Choi *et al.* 2010) and *TIMP1* (Bugno *et al.* 1999). In support of this, retrospective analysis showed strong correlation ($r = 0.72$, $p = 0.168$) of microarray probe intensities between *TLR5* and *CASP5* in CNBP patients when compared to controls ($r = -0.11$, $p = 0.862$) with similar observations in the discovery/validation cohort, although correlation remained remarkable between CNP patients ($r = 0.58$, $p = 0.003$) and controls ($r = 0.50$, $p = 0.013$). It is also worthy of note that other genes integral to inflammasome function were highlighted within this thesis, including *NAIP* and *NLRC4*. Similarly to *TIMP1*, further scrutiny is therefore necessary in order to determine whether *CASP5* and the TLRs function as reliable CNP markers, particularly considering previous observations of association with closely

related (Assassi *et al.* 2011, Lukkahatai *et al.* 2013) and unrelated conditions (Hosomi *et al.* 2003, Babas *et al.* 2010).

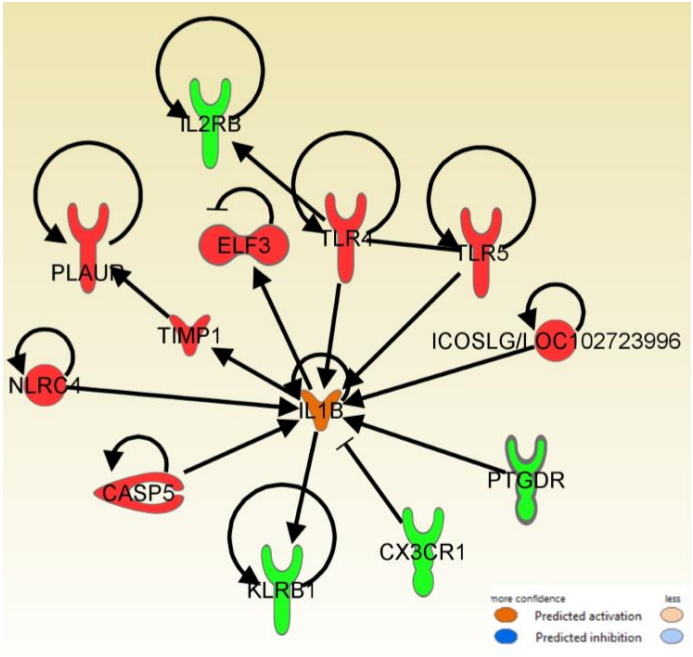

Figure 6.1: Molecular network of interactions between differentially expressed molecules and IL-1β

All differentially expressed genes present after data refinement (Table 4.1 and Table 4.2) were uploaded to IPA and molecular networks formed with the inclusion of IL-1β using the connect tool. The molecule activity prediction tool was then used by overlaying the level of regulation observed by microarray analysis. Molecules highlighted red and green were upregulated and downregulated, respectively. Only molecules which have downstream implications on IL-1β were selected to impact on the predicted effect on IL-1β. The IL-1β molecule is represented by IPA as deep orange, which illustrates the regulation of these genes strongly increases IL-1β activity.

Upregulation of *MC1R* was also observed in the discovery cohort. Literature evidence is suggestive of complex and diverse mechanisms by which MC1R mediates pain susceptibility and sensitivity and have detailed gender-specific influences (Mogil *et al.* 2003, Liem *et al.* 2005, Delaney *et al.* 2010). However, evidence exists which suggests that the MC1R agonist, α-MSH, is capable of exerting anti-inflammatory activity by mediating TLR4 signalling, which has been attributed to several mechanisms, including suppression of CD14 expression and inhibition of NF-κB activation (Sarkar *et al.* 2003, Yoon *et al.* 2003, Li *et al.* 2008). The anti-inflammatory role of MC1R is perhaps further evidenced by the attenuation and augmentation of inflammatory process by endogenous MC1R agonists and antagonists, respectively (Star *et al.* 1995, Ollmann *et al.* 1998, Lipton *et al.* 1999, Luger *et al.* 2007). Further intrigue in to the role of MC1R also provided two

additional observations. Firstly, that MC1R agonists have been shown to attenuate TNF-α-induced upregulation of vascular cell adhesion molecule (*VCAM1*), and intercellular adhesion molecule (*ICAM1*), thereby reducing the migration of circulating immune cells and their subsequent contributions to CNP (Brzoska 1999). Secondly, that central administration of MC1R agonists may function to reduce peripheral inflammation by neurogenic mechanisms (Lipton *et al.* 1991, Macaluso *et al.* 1994). Further analysis would be necessary in order to elucidate the value of MC1R as a CNBP biomarker, and whether observations of elevated MC1R in the CNS may function as compensatory mechanisms to reduce peripheral inflammation after nerve injury. Such investigation will likely necessitate the use of animal models and manipulation of *Mc1r*, which of particular note, was upregulated in the DH of both rat strains in this study.

Amongst the differentially expressed genes in the discovery/validation cohort, significant downregulation of the chemokine receptor, *CX3CR1*, was observed. The role of CX3CR1, and its ligand, CX3CL1, is heavily supported by literature evidence in the field of neuropathic pain (Milligan *et al.* 2004). The use of animal model has added considerable insight into the mechanism by which CX3CR1/CX3CL1 contribute to CNP (Milligan *et al.* 2004, Lindia *et al.* 2005, Staniland *et al.* 2010). Indeed, a recent study using transgenic mice concluded that microglia and monocytes synergistically facilitate the transition towards chronic pain after peripheral nerve injury, with observations that ablation of the $CX3CR1^{+}$ cell population had no implications on acute pain, but prevented the development of neuropathic pain after SNT (Peng *et al.* 2016). In addition to the previous association of caspase and TLRs in the regulation of IL-1β activity, IL-1β has also been shown to upregulate *CX3CL1* expression and increase its rate of release, which was notably reduced by broad-spectrum MMP inhibition (O'Sullivan *et al.* 2016). This reaffirms the previously documented role of MMP-2 and MMP-9 in CX3CL1 cleavage (Bourd-Boittin *et al.* 2009) (Gao *et al.* 2010), and infers potential downstream implications from TLR and caspase activity, and suggests a potential role for TIMP1 in attenuating CX3CR1 signalling. It has also been hypothesised, within this thesis, that downregulation of CX3CR1 in the blood of CNP patients may be reflection of high CX3CR1 expressing cells migrating towards the CNS. Considering the role described influencing of CX3CR1, it is perhaps pertinent that *CX3CR1* levels in the blood anti-correlated with pain severity measures.

Further work to be undertaken in light of the observations made in this thesis are numerous, and have been discussed in detail within the relevant chapters. To summarise, primary consideration for future studies should be given to increasing cohort size and the inclusion of distinct neuropathic

phenotypes, particularly considering the heterogeneity of its associated aetiologies (Beniczky *et al.* 2005). This is currently ongoing, though only ~25% of the expected total patient recruitment for the discovery/validation cohort could be incorporated within this thesis due to patient recruitment rates. Such increases in patient recruitment may clearly increase the likelihood of novel biomarker discovery pertaining to specific neuropathic aetiologies, which are not currently decipherable. Incorporation of longitudinal aspect to the study, inclusion of appropriate disease controls and analysis of exosomes present, all present valuable considerations for future work in human cohorts.

Subsequent analysis of the genes of particular interest derived from human studies, in the rat DH after SNL, highlighted several differentially regulated genes, including those which appeared to translate between clinical cases and animal models. The use of ddPCR undoubtedly facilitated such analysis, particularly in relation to lowly expressed genes. The incorporation of animal models of neuropathic pain in future studies presents numerous opportunities for expansion of study dynamics to enhance the assessment of a putative biomarker. This may involve a more comprehensive analysis, including the use of more than one model of neuropathic pain, in addition to transcriptomic analysis in the injured nerve and DRG. The assessment of gene expression changes over a longer period of time after nerve injury would clearly aid in the separation of translational biomarkers which may be representative of pain chronicity and those which are present within the immediate and early phase after nerve injury. Perhaps the most pertinent consideration for future planned studies is the inclusion of the rat blood transcriptomic profile, which will enable the assessment of nervous system-blood transcriptomic correlates.

6.9 Concluding remarks

This thesis has sought to assess the potential of blood to herald readily accessible biomarkers of CNP, with particular focus on transcriptomic changes and the BH_4 synthesis pathway. Efforts to elucidate the influence of the pain protective haplotype have demonstrated a potential role of the haplotype using *in vitro* methods, with particular reference to the *GCH1* 5' SNP. Clearly, further experimentation, potentially involving CRISPR-Cas9 or other methodology such as ChIP-seq, is necessary in order to elucidate transcription factor binding and to assess whether such interactions result in differential regulation of *GCH1*. Multiple candidate biomarkers are also presented within this study, of which several have strong literature evidence supporting their role in CNP. Several genes also exhibited differential regulation in the DH after SNL, and may therefore represent

potential translational biomarkers and investigational targets for amelioration of CNP. Clearly the challenges associated with biomarker discovery in CNP are considerable, though it is hoped this thesis provides multiple avenues for further investigation as larger, well defined studies incorporating multiple pain phenotypes, are commenced. Indeed, to coin a pertinent phrase by Bäckryd in relation to biomarkers of pain in the blood, ‘the task is immense and so is the need’ (Bäckryd 2015).

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Appendices

Appendix 1

Details of study participants in the discovery cohort.

Table A: Control and CNP patient demographic information for the discovery cohort

Code	Sex	Age	Location	Duration	Severity*	Comorbidities/Medical history	Medication
Control							
NP6	M	70	-	-	-	Migraine	Dipyrone
NP16	M	69	-	-	-	-	-
NP7	M	71	-	-	-	-	-
NP17	F	76	-	-	-	-	-
NP8	F	67	-	-	-	Cystitis	Antibiotics
NP18	M	80	-	-	-	Hypertension	Captopril
NP9	F	80	-	-	-	-	-
NP19	M	74	-	-	-	Cataract	Aspirin
NP10	M	61	-	-	-	-	-
NP20	F	36	-	-	-	-	-
CNBP							
NP1	M	72	Lower back	>6 months	Grade 2/3	Hypertension	Duloxetine, tramadol
NP11	F	71	Lower-mid back	>6 months	Grade 2/3	Migraine, depression	Dipyrone
NP2	M	68	Lower-mid back	>6 months	Grade 3	Haemorrhoid	Tramadol
NP12	M	80	Lower back	>6 months	Grade 3	Ciliary arrhythmia	Tramadol
NP3	M	75	Lower back	>6 months	Grade 3	Anxiety	Tramadol
NP13	F	73	Lower back	>6 months	Grade 2/3	Depression	Dipyrone
NP4	F	75	Lower-mid back	>6 months	Grade 2/3	Cataract	Reboxetine
NP14	F	75	Lower back	>6 months	Grade 3	-	Tramadol
NP5	F	78	Lower back	>6 months	Grade 2/3	Chronic gastritis	Morphine
NP15	F	81	Lower-mid back	>6 months	Grade 2/3	-	Dipyrone

CIBP							
CIBP1	F	27	Lower back	>6 months	Grade 2	Previous hip dislocation and osteotomy	Paracetamol, diclofenac
CIBP2	M	62	Lower back	>6 months	Grade 1	Degenerative disc disease	Diclofenac
CIBP3	F	40	Lower back	>6 months	Grade 3	Degenerative disc disease	Diclofenac
CIBP4	F	54	Lower back	>6 months	Grade 2	Osteoarthritis	Diclofenac, Ibuprofen
CIBP5	M	74	Lower back	>6 months	Grade 1	Degenerative disc disease	Methylprednisolone
CIBP6	F	72	Lower back	>6 months	Grade 4	Inflammatory back	Tramadol, paracetamol
CIBP7	F	57	Lower back	>6 months	Grade 1	Degenerative disc disease	Lidocaine patch
CIBP8	F	69	Lower back	>6 months	Grade 4	Degenerative disc disease	Diclofenac, paracetamol, capsaicin, tapentadol
CIBP9	F	50	Lower-mid back	>6 months	Grade 4	Degenerative disc disease	Codeine, paracetamol, tramadol, oxycodone, diazepam
CIBP10	F	88	Lower back	>6 months	Grade 3	Osteoarthritis, degenerative disc disease, sacroiliac joint disease	Paracetamol, steroid injections
CIBP11	F	85	Lower back	>6 months	Grade 2	Degenerative disc disease	Paracetamol
CIBP12	F	63	Lower-mid back	>6 months	Grade 4	Scoliosis, osteomyelitis	Tramadol, paracetamol

*Measures of pain severity are not directly comparable between the CNBP and CIBP cohorts. Those pertaining to CNBP patients are based on a simple numerical rating scale are converted to a pain grade (0-3). The pain grading system used with CIBP patients involved the Chronic Pain Grade Questionnaire (0 = no pain, 1 = low disability-low intensity pain, 2 = low disability-high intensity pain, 3 = high disability-moderately limiting and 4 = high disability-severely limiting). Controls and patients in red font were subject to gene expression analysis by microarray.

Appendix 2

Details of study participants in the discovery/validation cohort.

Table A: Control and CNP patient demographic information for the discovery/validation cohort

Code	Sex	Age	Location	Duration Post-Diagnosis (months)	Modified Pain Grade Score (5 measures; max. 50)*	Severity**	S-LANSS Score (max. 24)	Medication
Control								
CH001	F	45	-	-	-	-	-	-
CH002	F	53	-	-	-	-	-	-
CH003	F	22	-	-	-	-	-	-
CH004	F	61	-	-	-	-	-	-
CH005	F	27	-	-	-	-	-	-
CH006	M	53	-	-	-	-	-	-
CH007	M	44	-	-	-	-	-	-
CH008	F	49	-	-	-	-	-	-
CH009	F	22	-	-	-	-	-	-
CH010	M	24	-	-	-	-	-	-
CH011	F	62	-	-	-	-	-	-
CH012	F	23	-	-	-	-	-	-
CH013	M	25	-	-	-	-	-	Propranolol
CH014	M	46	-	-	-	-	-	Levothyroxine, Lisinopril, ezetimibe
CH015	F	21	-	-	-	-	-	-
CH016	F	30	-	-	-	-	-	-
CH017	F	21	-	-	-	-	-	Citalopram
CH018	F	26	-	-	-	-	-	-
CH019	F	51	-	-	-	-	-	-
CH020	M	24	-	-	-	-	-	-
CH021	M	49	-	-	-	-	-	-

CH022	M	21	-	-	-	-	-	-
CH023	M	18	-	-	-	-	-	-
CH024	F	51	-	-	-	-	-	-
Neuropathic pain								
NPL001	M	68	Abdomen	24	28	Grade 2	0	Tramadol, ramipril
NPL002	M	64	Scrotum	36	37	Grade 2	17	Pregabalin, amlodipine, formoterol, ciclesonide
NPL003	F	40	Back	84	41	Grade 2	16	Aciclovir, frovatriptan, naproxen
NPL004	F	38	Lower back, right leg	36	43	Grade 4	23	Fentanyl, morphine, paracetamol ibuprofen
NPL005	F	48	Leg	108	46	Grade 4	18	Estradiol, fentanyl, morphine, paracetamol, dihydrocodeine
NPL006	M	39	Leg	60	35	Grade 2	17	Oxycodone, duloxetine, gabapentin, naproxen, paracetamol
NPL007	M	21	Thoracic	18	34	Grade 3	13	Pregabalin, tramadol
NPL008	F	68	Shoulder (PHN)	120	24	Grade 3	19	Buprenorphine, pregabalin, amitriptyline
NPL009	M	56	Right leg	96	16	Grade 1	5	Amitriptyline, gabapentin, latanoprost
NPL010	F	40	Leg (bilateral)	38	40	Grade 4	22	Citalopram, codeine, paracetamol, lansoprazole, fentanyl, ferrous sulphate, calcium, colecalciferol, Creon (amylase, lipase, protease), cyclizine
NPL011	M	60	Leg (bilateral)	224	20	Grade 3	10	Duloxetine
NPL012	F	44	Lower back, leg	24	35	Grade 4	17	Gabapentin, diclofenac, paracetamol, amitriptyline
NPL013	F	42	Back, right leg	24	34	Grade 4	6	Tramadol, paracetamol, imipramine
NPL014	F	49	Back, right leg	120	42	Grade 4	4	Tramadol, omeprazole, trazodone, ranitidine

NPL015	F	60	Back, leg	132	36	Grade 4	19	Eletriptan, estradiol, fluoxetine, tramadol, simvastatin, paracetamol, omeprazole, morphine, topiramate
NPL016	M	40	Right arm (crush injury)	168	40	Grade 4	24	Paroxetine, amitriptyline, tramadol, simvastatin, amlodipine, bisoprolol, ramipril, lansoprazole, paracetamol
NPL017	M	74	Back, leg	588	42	Grade 4	0	Fenyanyl, losartan, bisoprolol, amlodipine, simvastatin, omeprazole, warfarin
NPL018	F	40	Back, leg	192	45	Grade 4	23	Pregabalin, buprenorphine, amitriptyline, omeprazole
NPL019	F	36	Back, leg (CRPS)	84	39	Grade 4	24	Tramadol, citalopram, lansoprazole, pregabalin
NPL020	M	37	Left leg	42	34	Grade 4	18	Gabapentin, tramadol, paracetamol, naproxen, morphine
NPL021	M	41	Leg	24	38	Grade 3	7	Gabapentin, paracetamol, codeine, amitriptyline
NPL022	M	48	Back, leg	18	42	Grade 4	24	Gabapentin, diclofenac, omeprazole
NPL023	F	43	Abdomen, pelvis	36	40	Grade 4	9	Levonorgestrel, ethinylestradiol
NPL024	F	50	Right leg	13	14	Grade 1	5	Ibuprofen

*This relates to the accumulative score of the first 5 measure of the modified Chronic Pain Grade tool. Higher scores are an indicator of persistent severe pain. Chronic Pain Grade Questionnaire (0 = no pain, 1 = low disability-low intensity pain, 2 = low disability-high intensity pain, 3 = high disability-moderately limiting and 4 = high disability-severely limiting).

Appendix 3

The following tables relate to PCRs undertaken in order to facilitate reporter gene assays. The table directly beneath describes the SNP or locus implicated. The *GCH1* intron 1 cloning procedure has been graphically represented (Figure 3.17). The second table details the specific reaction components and PCR cycling parameters. The primer sequences and PCR condition across both tables are linked by the reaction number in the leftmost column.

Table A: Primer sequences relating to PCRs undertaken for assessment of *GCH1* regulation in reporter gene assays

No.	SNP/Gene	Amplicon (bp)	Forward Primer	Reverse Primer
1	rs10483639	155	TGTGGTACCGCTGCCGGTGAGCATTGG	AAGCTCGAGGGAAAAGGAGGAAGAATA
2	rs3783641	161	TTTGGTACCTCTCTGCTATTTGCTTTGTCCA	CCTCTCGAGGTGAGTTTTAACACTGCA
3	rs8007267	161	CCTGGTACCTTTAAAATATCCTCAGAA	GGTCTCGAGGATACAGTTTAATTAGAAG
4	rs8007267	387	GGAGGTACCTTCCCCAGCCTGGCCACA	CCTCTCGAGCTCAGCCTCCCAAAGTGC
5	rs8007267	10166	TCCGGTACCTCTATGATCAGTTGGATGCCA	GTTCTCGAGCAGCGAGCTCAGGATGGA
6	GCH1 promoter 3.4 kb	3387	AGAGGTACCCTCCCAGACTGGGTCCAAGC	GCACTCGAGCCGAATCCCGCGCTGG
7	GCH1 Intron 1 region A	4275	CTGCTCGAGCAAAGTTGCCCACGATAAGGATC	GGCCTCGAGTTCTGCAGTCAGACTCTCTGG
8	GCH1 Intron 1 region B	4417	CCACTCGAGGGCAACTGAGGCAGAGAGG	CCTCTCGAGGTGAGTTTTAACACTGCA
9	GCH1 Intron 1 region C	3863	ATTCTCGAGCTGACTCATTTGCCAGTGA	AGACTCGAGTCACGGCCAATTTCCTC
10	GCH1 Intron 1 region D	4019	CTGCTCGAGTTGCCTCCTAGCTTGTCC	GGTCTCGAGCTCCAGTGGCAGTGTAC
11	GCH1 Intron 1 region E	4270	TCGCTCGAGCAAAGTGCGGGGATTAC	GCACTCGAGATCCCTCCACCCTGATGC
12	GCH1 Intron 1 region F	1445	TGACTCGAGTGCTGGGTAGCTTGGGAC	CCTCTCGAGAGAGCTTCCGACACTCACAG

13	GCH1 Intron 1 region G	2342	TCTCTCGAGCCGCCATTGTGTCAGTCTG	ATACTCGAGTTAGCCGGATGTGGTGCCA
14	GCH1 Intron 1 region H	2118	TGGCTCGAGCTCTACATATCCTTCTGCTACAC	TCTCTCGAGACAGGGTCTCCTC
15	GCH1 Intron 1 region I	756	CACCTCGAGTTGTATTGTGGGCCTGTTAC	TGTCTCGAGAGTGGAAGGAGGAGCTATGAAC

Table B: PCR conditions relating to reactions undertaken for assessment of *GCH1* regulation in reporter gene assays

No.	PCR Enzyme	PCR Constituents (per 10 µL Reaction)	Cycling Conditions	Additional Information
1	Phire Hot Start II DNA Polymerase	Water 5.6 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, (20 ng) gDNA 1 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 60ºC 5 sec, 72ºC 5 sec [x 37 cycles]), 72ºC 1 min	
2	Phire Hot Start II DNA Polymerase	Water 5.6 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, (20 ng) gDNA 1 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 60ºC 5 sec, 72ºC 5 sec [x 37 cycles]), 72ºC 1 min	
3	Phire Hot Start II DNA Polymerase	Water 6.1 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, PCR product 0.5 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 45ºC 5 sec, 72ºC 5 sec [x 37 cycles]), 72ºC 1 min	
4	Phire Hot Start II DNA Polymerase	Water 5.6 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, (20 ng) gDNA 1 µL, (25 mM) Mg^{2+} 1 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 72ºC 5 sec, 72ºC 5 sec [x 37 cycles]), 72ºC 1 min	PCR product used as template for reaction No.3.
5	KOD Extreme Hot Start DNA Polymerase	Water 2 µL, 2x buffer 5 µL, (2 mM) dNTPs 2 µL, (10 µM) F-primer 0.3 µL, (10 µM) R-primer 0.3 µL, (17 ng) gDNA 0.2 µL, polymerase 0.2 µL.	94ºC 2 min, (98ºC 10 sec, 63.8ºC 30 sec, 68ºC 10 min [x 40 cycles])	
6	KOD Extreme Hot Start DNA Polymerase	Water 2 µL, 2x buffer 5 µL, (2 mM) dNTPs 2 µL, (10 µM) F-primer 0.3 µL, (10 µM) R-primer 0.3 µL, (17 ng) gDNA 0.2 µL, polymerase 0.2 µL.	94ºC 2 min, (98ºC 10 sec, 68ºC 30 sec, 68ºC 3.5 min [x 38 cycles])	
7	Phire Hot Start II DNA Polymerase	Water 6.2 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, (15 ng) gDNA 0.4 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 72ºC 5 sec, 72ºC 45 sec [x 35 cycles]), 72ºC 1 min	
8	Ranger DNA Polymerase (BIOLINE, UK)	Water , 5x buffer (with dNTPs) 2 µL, (10 µM) F-primer 0.4 µL, (10 µM) R-primer 0.4 µL, (17 ng) gDNA 0.2 µL polymerase 0.2 µL.	95ºC 1 min, (98ºC 10 sec, 64.5ºC 4.5 min [x 30 cycles])	For TA cloning into pCR2.1 prior to sub-cloning.

9	Phire Hot Start II DNA Polymerase	Water 6.2 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, (15 ng) gDNA 0.4 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 72ºC 5 sec, 72ºC 75 sec [x 35 cycles]), 72ºC 1 min	
10	Phire Hot Start II DNA Polymerase	Water 6.2 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, (15 ng) gDNA 0.4 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 72ºC 5 sec, 72ºC 75 sec [x 35 cycles]), 72ºC 1 min	
11	Phire Hot Start II DNA Polymerase	Water 6.2 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, (15 ng) gDNA 0.4 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 69.4ºC 5 sec, 72ºC 75 sec [x 35 cycles]), 72ºC 1 min	
12	Ranger DNA Polymerase (BIOLINE, UK)	Water , 5x buffer (with dNTPs) 2 µL, (10 µM) F-primer 0.4 µL, (10 µM) R-primer 0.4 µL, (17 ng) gDNA 0.2 µL polymerase 0.2 µL.	95ºC 1 min, (98ºC 10 sec, 64ºC 4.5 min [x 30 cycles])	For TA cloning into pCR2.1 prior to sub-cloning.
13	Ranger DNA Polymerase (BIOLINE, UK)	Water , 5x buffer (with dNTPs) 2 µL, (10 µM) F-primer 0.4 µL, (10 µM) R-primer 0.4 µL, (17 ng) gDNA 0.2 µL polymerase 0.2 µL.	95ºC 1 min, (98ºC 10 sec, 64ºC 4.5 min [x 30 cycles])	For TA cloning into pCR2.1 prior to sub-cloning.
14	Phire Hot Start II DNA Polymerase	Water 6.2 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, (15 ng) gDNA 0.4 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 69.4ºC 5 sec, 72ºC 40 sec [x 35 cycles]), 72ºC 1 min	
15	Phire Hot Start II DNA Polymerase	Water 6.2 µL, 5x buffer 2 µL, (10 mM) dNTPs 0.2 µL, (10 µM) F-primer 0.5 µL, (10 µM) R-primer 0.5 µL, (15 ng) gDNA 0.4 µL, polymerase 0.2 µL.	98ºC 30 sec, (98ºC 5 sec, 72ºC 5 sec, 72ºC 10 sec [x 35 cycles]), 72ºC 1 min	

Appendix 4

The following tables relate to qRT-PCRs undertaken in order to determine changes in gene expression after *in vitro* experiments, in humans with CNP and in the dorsal horn after SNL.

Table A: Primer sequences relating to qRT-PCR for *in vitro* experiments and gene expression analysis in CNP patients

Accession ID	Gene Symbol	Forward primer	Reverse primer
NM_001039841	*ARHGAP11B*	CGCAGAAAAGAAGGGCGTG	AAGCCTTCCAGTGATGGAGT
NM_004347	*CASP5*	GGCTACACTGTGGTTGACGA	GTGCTGTCAGAGGACTTGTGC
NM_001831	*CLU*	GCGTGCAAAGACTCCAGAAT	GTCTTGCGCTCTTCGTTTGT
NM_001171174	*CX3CR1*	GAGGCGTTTAAGTTGGCAGA	AAAGACCACGATGTCCCCAAT
NM_000499	*CYP1A1***	CAAGGGGCGTTGTGTCTTTG	ACACCTTGTCGATAGCACCA
NM_000791	*DHFR*	GCTCCCTGTCCTGTGTGTG	TGTTTGGCATTCACTGTTCC
NM_005700	*DPP3*	CCCCGAGTGCTTCCTCAC	GGCGAGTGTTGGGCTGAA
NM_004433	*ELF3*	AAAAACAAAACAAAATGGAGATGAGTA	GACCTCCTAAATCTAAAGTAAAAGTTG
NR_026643	*FAM99A*	TGTGGCTGTTTTGTGATGCG	GAGTGAGGGGTGCAGTTAGG
NM_002046	*GAPDH*	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
NM_000161	*GCH1*	ACAAACAAAACCGCAACTCC	TGGGATGAATTTGAAGAGCA
NM_000161	*GCH1 (d/v)*	GCCATGCAGTTCTTCACCAA	ATGGAACCAAGTGATGCTCA
NM_005258	*GCHFR*	TCTGCCTTGCTCCTCTCTTC	CCCTCTCCCACTGCTTGAC
NM_015259	*ICOSLG*	TTCCAGGAGGTTTTGAGCGTT	GGGGTAGCCGTTTATGGATG
NM_000878	*IL2RB*	CACGTGGAGACCCACAGATG	CTTGACCCGCACCTGAAACT
NM_002258	*KLRB1*	ACTGGAAGTGGATAAACGGCT	TGGCAGATCCATCTGATTTCTG
NM_024674	*LIN28A*	GTATTGGGAGTGAGAGGCGG	TTCAGCGGACATGAGGCTAC
NM_002386	*MC1R*	Bio-Rad PrimePCR Assay	Bio-Rad PrimePCR Assay
NM_000615	*NCAM1*	GTTCATGTGCATTGCGGTCA	CGTTTCTGTCTCCTGGCACT
NM_021209	*NLRC4*	GGTCTGACTGACAGCTTGGG	CAGGTTTTTCAGGCCTTCAGC

NM_006056	*NMUR1*	GCAGCCAGGTCCAGATACAC	CAGATGCCAAACACCACGAC
NM_000608	*ORM2*	GACTGCTTGTGCATTCCCAG	TTCTCGTGCTGCTTCTCCAG
NM_000281	*PCBD1*	ACCACTCCCCTCCCAAGA	AGCCCCCAGGATGAAGAG
NM_002620	*PF4V1*	ACTGCCCAACTCATAGCCAC	GTTAGATTGAAAGTGCACACTTAGG
NM_002659	*PLAUR*	ACCCATGGATGCTCCTCTGA	CATGTTGGCACATTGAGGC
NM_000953	*PTGDR*	CTACGCTCAGAACCGGAGTC	GAAAGGTAGCGCGCAGAAAG
NM_000317	*PTS*	CTGTTTGGGAAATGCAACAA	CCGTAGCAGGGTCAATCTCT
NM_000320	*QDPR*	CACAGAAGGAAGGACGGAAC	AAACACAAGGCTGGACAAGG
NM_152267	*RNF185*	CTGTGCGGAGGCAGGATTT	CACCTGTCTGTTAGGTCTGGTC
NM_003124	*SPR*	GGCTCTCTTGGGGATGTGT	TTCAGGACGCTGGAAGTCA
NM_003124	*SPR (v/d)**	AAGGCTGCTCGTGATATGCT	CCAACTGCTGCATGTCTGTG
NM_003254	*TIMP1*	GCAATTCCGACCTCGTCATC	TCTTGATCTCATAACGCTGGTATAA
NM_138554	*TLR4*	CCAAGAACCTGGACCTGAGC	AGGCTCTGATATGCCCCATC
NM_003268	*TLR5*	TGACCATCCTCACAGTCACA	GTCCTTGAACACCAGTCTCTG
NM_001171623	*VEGFA*	CCTGGTGGACATCTTCCAG	TGGTGAGGTTTGATCCGC
NM_024911	*WLS*	CCTTACGCCCAGCATCTTCA	CCCTGTCGGATGTCACCAAA
NM_002995	*XCL1*	AAGAGCCCGATCCTCACTCT	CTCTCACCCATGTGGCTTG

(v/d) denotes that this primer pair was used in the discovery/validation cohort. Primer sequences relating the reference genes obtained through PrimerDesign were not available from the manufacturer. *Deviates from standard cycling conditions with 15 second extension time and T_a of 64.3^{o}C. ** Deviates from standard cycling conditions with a 15 second extension time.

Table B: Primer sequences relating to qRT-PCR and ddPCR for *in vitro* experiments and gene expression analysis in rat dorsal horn

Accession ID	Gene Symbol	Forward primer	Reverse primer
NM_001168524	*Arhgap11a*	TGTTGCTGTCGTGCCTTATG	CAAGATTGCTGCTGTCCATTT
NM_053736	*Casp4*	Bio-Rad PrimePCR Assay	Bio-Rad PrimePCR Assay
NM_053021	*Clu*	GGGCGATGACCAGTACCTT	TCAAACAGCTTCACCACCAC
NM_133534	*Cx3cr1**	GACACTTCTGTGCTGACCCA	GTGTCACATTGTCCACACGC
NM_130400	*Dhfr*	TGACAAGGATCATGCAGGAGT	GGATCTCAGAGAGGACGCC
NM_053748	*Dpp3*	GATCCGGTCAGTGGGCAAA	CCTGCAACCACATCCCCT
NM_001024768	*Elf3*	TCTTCGTTCAGAGGCTGTGG	CGTTCCAGGATCTCCCGTTT
NM_017008	*Gapdh*	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
NM_024356	*Gch1*	GCCATGCAGTTCTTCACCAA	CCTTCACAATCACCATCTCGT
NM_133595	*Gchfr*	ACGAGTCCTGGGAAACAACT	CCACCCCTGTCATGCTTAAC
XM_006256260	*Icoslg*	TCAGTACGCCTGTCATCAGC	ACGTCATACAGGCCCAACTC
NM_013195	*Il2rb*	Bio-Rad PrimePCR Assay	Bio-Rad PrimePCR Assay
NM_001085405	*Klrb1*	AGAGTGCCCAAGAGATTGGC	CAGCAAAGTGGCTCCTCTCA
NM_001109269	*Lin28a*	GTATTGGGAGTGAGCGACGG	ACAGTTGTAGCACCTGTCTCC
XM_006255795	*Mc1r*	Bio-Rad PrimePCR Assay	Bio-Rad PrimePCR Assay
NM_031521	*Ncam1**	AGACAAGGTACGCGGTACGA	GGAGGCGAGCGCTCTGTA
NM_001309432	*Nlrc4*	Bio-Rad PrimePCR Assay	Bio-Rad PrimePCR Assay
NM_023100	*Nmur1*	CAGGCCTCCCACAGAAGTAT	GGTCAGCATGGAATGGAACC
NM_053288	*Orm1*	Bio-Rad PrimePCR Assay	Bio-Rad PrimePCR Assay
NM_001007601	*Pcbd1*	CCCGAGTGGTTTAACGTGTAC	GTTCAGAAAGACCGGCACAT
NM_001007729	*Pf4*	TCTCAAACGCATCACCAGC	TTCAGCGTGGCTATGAGCT
NM_134352	*Plaur*	GCAGTGCCGGTATCCTACAG	GCCTTTGGTGTAGGGTTCGT
NM_001135164	*Ptgdr*	GTCCCCGAATCCTTTGGAG	GTCTTCCGAGTCTCCGTCAG
NM_017220	*Pts*	CTTGAAAGTGTTTGGGAAATGCA	AACCATTCCTGTAACCGGATC
NM_022390	*Qdpr*	CCCTTAGAGTTCCTGGTGGAG	CTGTCTTCCCGTCTGTGGTT
NM_001024271	*Rnf185*	TGGCTTTCAGATGTCTTTTGGA	CAAACAGGAAGAGGCGTGAC

NM_019181	*Spr*	TACTGTGCAGGGAAGGCTG	TCCTCAACTCTGGGTCCATG
NM_053819	*Timp1*	CCTGGCATAATCTGAGCCCT	TTTGCAAGGGATGGCTGAAC
NM_019178	*Tlr4*	Bio-Rad PrimePCR Assay	Bio-Rad PrimePCR Assay
NM_001145828	*Tlr5*	CTGTCTGACCTCAAGCGTGT	GGGCCACCTCAAATACTGCT
NM_199408	*Wls**	ATGAGCCCATGGTTCCAGTT	CTCTTTCGTGCGCCATTTCA
NM_134361	*Xcl1*	ACCTACACCATCAAGGAGGG	CCTGCCATCTACGGTTTTGA

Primer sequences relating the reference genes obtained through PrimerDesign were not available from the manufacturer. Primer sequences relating to the Bio-Rad PrimePCR Assays were also unavailable.

Appendix 5

Oligonucleotide sequences and related reaction conditions for mutagenesis. Numbers in the left hand column correspond between Table A and Table B.

Table A: Oligonucleotide sequences used for mutagenesis

No.	SNP	Forward primer	Reverse primer
1	rs10483639	GTGTATGTACAACTTC***G***TCTCAGGCTATTG	GAAAATCAATAGCCTGAGA***C***GAAGTTGTACATAC
2	rs3783641	CCTGACTCATTTGCC***T***GTGATTTCTATATG	CCTCATATAGAAATCAC***A***GGCAAATGAG
3	rs8007267	GACTGAAGTTTGGCGT***A***TACTGTTCAAAC	CGTGTGTTTGAACAGTA***T***ACGCCAAACTTC
4	rs8007267	AGTTTGGCGT***A***TACTGTTCAAAC	TCAGTCATTCAAGTACCATC

Table B: Mutagenesis reaction constituents and PCR cycling conditions

No.	PCR enzyme	PCR constituents (per 10 µL reaction)	Cycling conditions	Additional information
1	Pwo DNA Polymerase (Roche, X)	Water 5.8 µL, 10x buffer 1 µL, 5x GC solution 2 µL, (10 mM) dNTPs 0.2 µL, (50 µM) F-primer or R-primer 0.5 µL, ~200 ng DNA 0.4 µL, polymerase 0.1 µL.	A) 95°C 30 sec, (95°C 30 sec, 55°C 1 min, 72°C 7 min [x 18 cycles]) B) 95°C 5 min, 90°C 1 min, 80°C 1 min, 70°C 30 sec, 60°C 30 sec, 50°C 30 sec, 40°C 30 sec, 37°C hold	Two reactions, each with F or R primer, which are combined after part A of thermal cycling
2	Pwo DNA Polymerase (Roche, X)	Water 5.8 µL, 10x buffer 1 µL, 5x GC solution 2 µL, (10 mM) dNTPs 0.2 µL, (50 µM) F-primer or R-primer 0.5 µL, ~200 ng DNA 0.4 µL, polymerase 0.1 µL.	A) 95°C 30 sec, (95°C 30 sec, 55°C 1 min, 72°C 7 min [x 18 cycles]) B) 95°C 5 min, 90°C 1 min, 80°C 1 min, 70°C 30 sec, 60°C 30 sec, 50°C 30 sec, 40°C 30 sec, 37°C hold	Two reactions, each with F or R primer, which are combined after part A of thermal cycling
3	Pwo DNA Polymerase (Roche, X)	Water 5.8 µL, 10x buffer 1 µL, 5x GC solution 2 µL, (10 mM) dNTPs 0.2 µL, (50 µM) F-primer or R-primer 0.5 µL, ~200 ng DNA 0.4 µL, polymerase 0.1 µL.	A) 95°C 30 sec, (95°C 30 sec, 55°C 1 min, 72°C 7 min [x 18 cycles]) B) 95°C 5 min, 90°C 1 min, 80°C 1 min, 70°C 30 sec, 60°C 30 sec, 50°C 30 sec, 40°C 30 sec, 37°C hold	Two reactions, each with F or R primer, which are combined after part A of thermal cycling
4	KOD Extreme Hot Start DNA Polymerase (Merck Milllipore, USA)	Water 2 µL, 2x buffer 5 µL, (2 mM) dNTPs 2 µL, (10 µM) F-primer 0.3 µL, (10 µM) R-primer 0.3 µL, (2.5ng) DNA 0.2 µL, polymerase 0.2 µL.	94°C 2 min, (98°C 10 sec, 54.5-64.6°C 30 sec, 68°C 12 min [x 24 cycles])	PCR product purified before kinase-ligase-DpnI reaction

Appendix 6

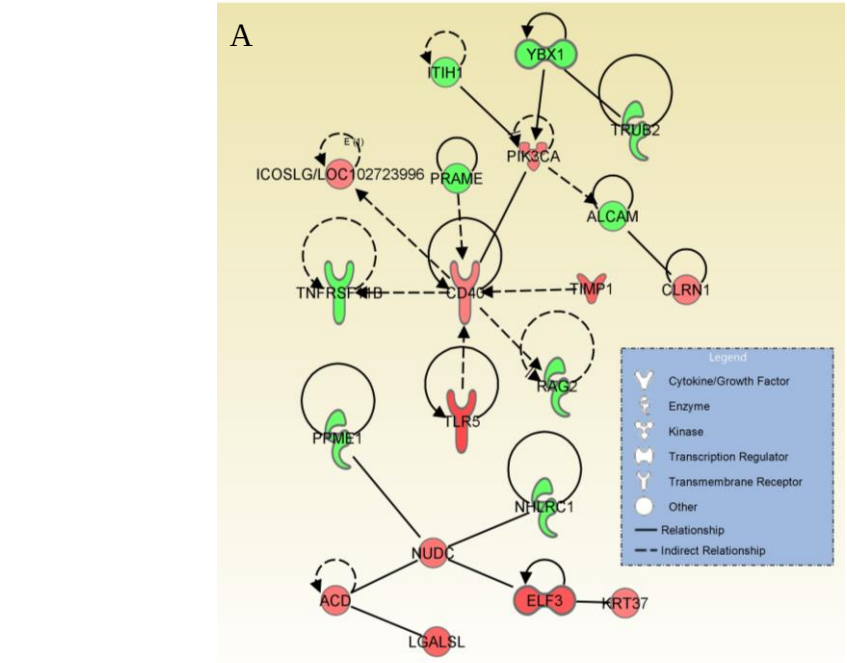
Oligonucleotide sequences for annealing and subsequent EMSAs.

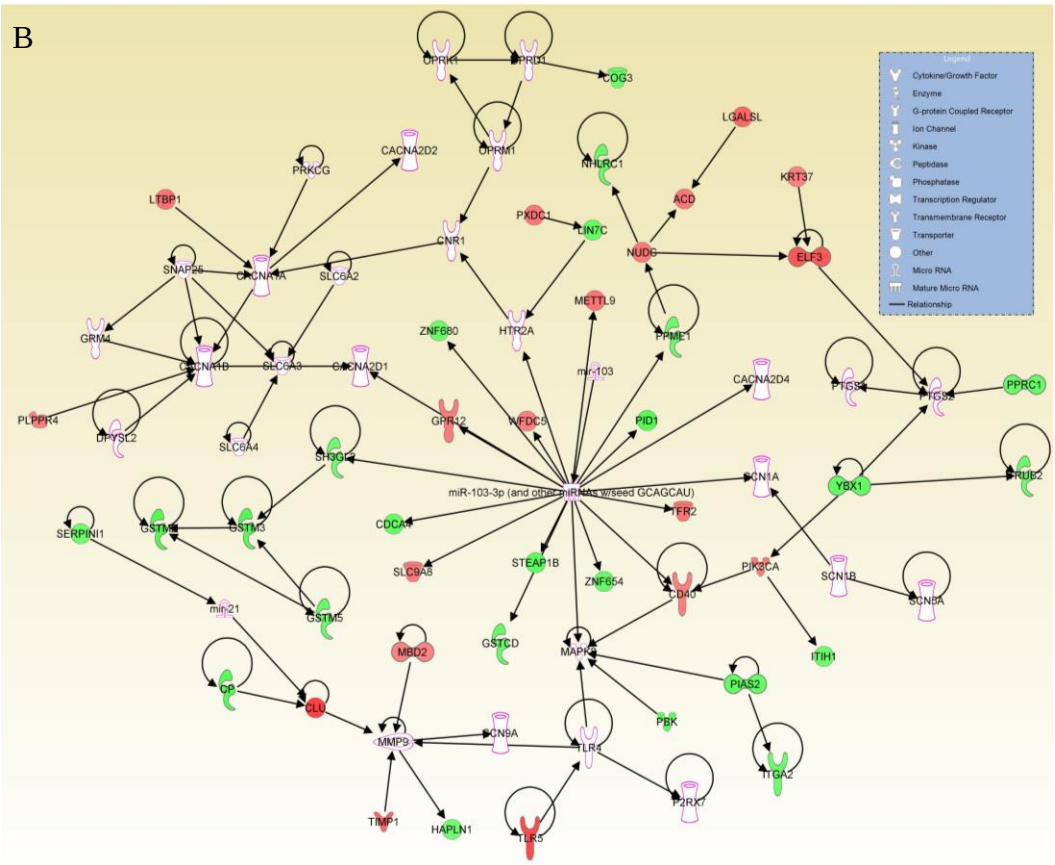
Table A: Labelled and unlabelled oligonucleotides used to conduct EMSAs

Probe	**Oligonucleotide sequence (5'-3')**
rs8007267 WT labelled	**Cy5**-GACTGAAGTTTGGCGT***G***TACTGTTCAAAC
rs8007267 MUT labelled	**Cy5**-GACTGAAGTTTGGCGT***A***TACTGTTCAAAC
rs8007267 WT	GACTGAAGTTTGGCGT***G***TACTGTTCAAAC
rs8007267 WT	GTTTGAACAGTA***C***ACGCCAAACTTCAGTC
rs8007267 MUT	GACTGAAGTTTGGCGT***A***TACTGTTCAAAC
rs8007267 MUT	GTTTGAACAGTA***T***ACGCCAAACTTCAGTC

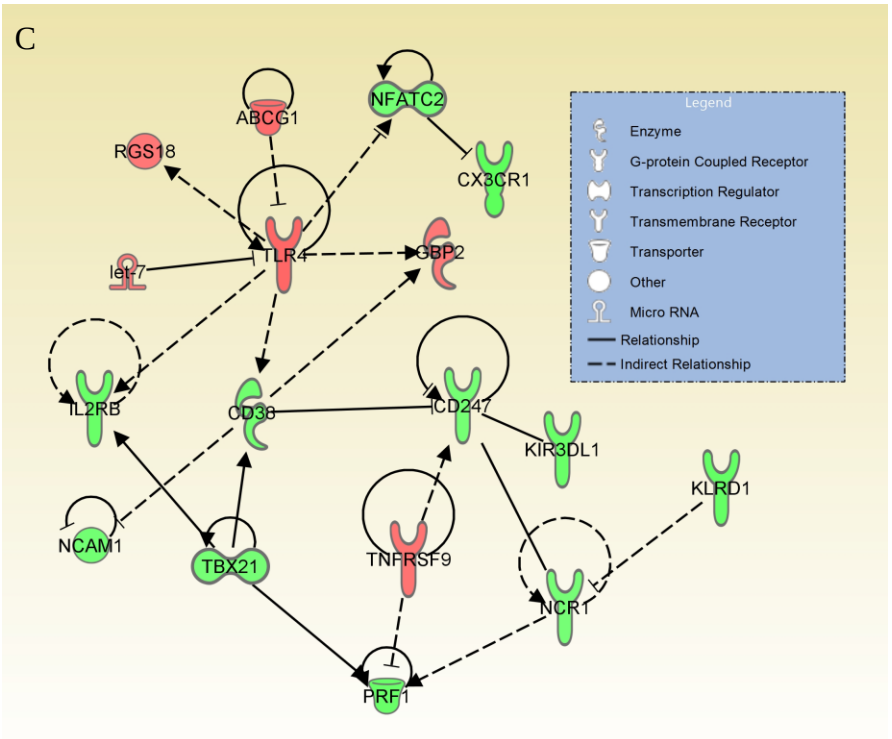
Appendix 7

Molecular interactions between regulated genes in the discovery (A+B) and discovery/validation cohort (C+D). Red and green molecules represent upregulated and downregulated, respectively. Molecules with no colour originate from the 'Neuropathic Pain' database in IPA software.

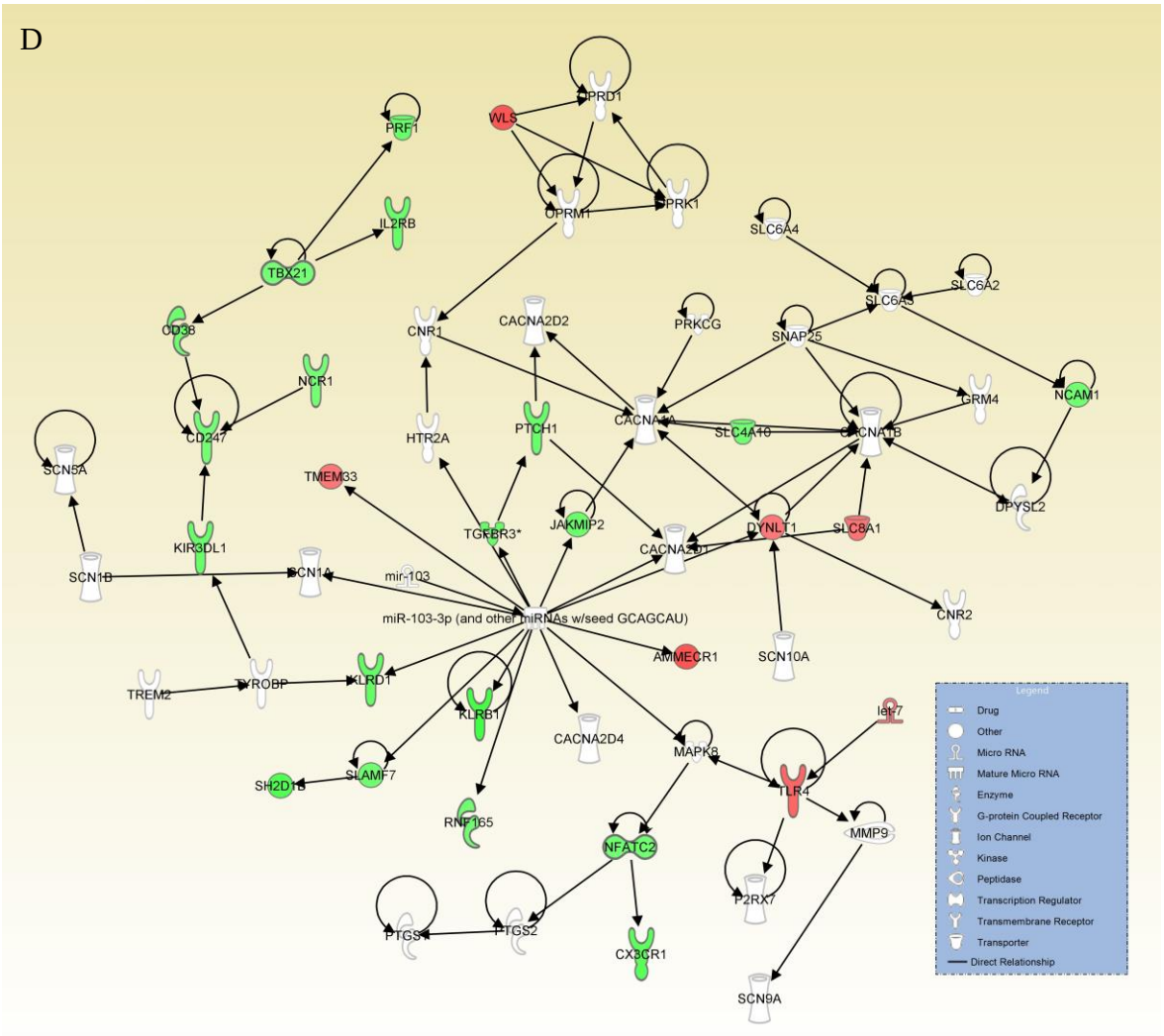




C


ABCG1
NFATC2
RGS18
CX3CR1
TLR4
GBP2
let-7
IL2RB
CD38
CD247
KIR3DL1
KLRD1
NCAM1
TBX21
TNFRSF9
NCR1
PRF1
Legend
Enzyme
G-protein Coupled Receptor
Transcription Regulator
Transmembrane Receptor
Transporter
Other
Micro RNA
Relationship
Indirect Relationship

D


PRF1
WLS
GPRD1
OPRM1
GPRK1
IL2RB
TBX21
SLC6A4
SLC6A3
SLC6A2
CD38
CNR1
CACNA2D2
PRKCG
SNAP25
NCR1
CD247
HTR2A
PTCH1
CACNA1A
SLC4A10
CACNA1B
GRM4
NCAM1
SCN8A
TMEM33
DPYSL2
TGFBR3*
JAKMIP2
DYNLT1
SLC8A1
KIR3DL1
CACNA2D1
SCN1B
SCN1A
mir-103
miR-103-3p (and other miRNAs w/seed GCAGCAU)
CNR2
AMMECR1
SCN10A
TREM2
TYROBP
KLRD1
KLRB1
CACNA2D4
MAPK8
let-7
SH2D1B
SLAMF7
TLR4
RNF165
MMP9
NFATC2
P2RX7
PTGS1
PTGS2
CX3CR1
SCN9A
Legend
Drug
Other
Micro RNA
Mature Micro RNA
Enzyme
G-protein Coupled Receptor
Ion Channel
Kinase
Peptidase
Transcription Regulator
Transmembrane Receptor
Transporter
Direct Relationship

Appendix 8

Publications relating to this thesis:

Buckley, D.A., Jennings, E.M., Burke, N.N. Roche, M., McInerney, V., Wren, J.D., Finn, D.P., McHugh, P.C. (2017) The Development of Translational Biomarkers as a Tool for Improving the Understanding, Diagnosis and Treatment of Chronic Neuropathic Pain, Molecular Neurobiology; https://doi.org/10.1007/s12035-017-0492-8

Buckley, D., Radford, H., Finn, D., Johnson, M., and McHugh, P.C. (2014) Improving the understanding and treatment of neuropathic pain. British Journal of Pain; Vol 8, p. 41.