AN EXPLORATORY STUDY TO INVESTIGATE POTENTIAL SENSORY BIOMARKERS OF CHEMOTHERAPY-INDUCED AND DIABETIC PERIPHERAL NEUROPATHY

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Medicine (Research), Imperial College, London

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2011
Abstract

Aims: Identification of neurophysiological or skin innervation biomarkers which can be used to assess and monitor progression of diabetic sensory polyneuropathy (DPN) and chemotherapy-induced neuropathy (CIPN). Sensitive and robust measures are needed to detect changes in the relatively short duration of clinical trials aimed to modify progression of neuropathy.

Methods: 40 patients with DPN were studied longitudinally over 1 year, and 33 patients with CIPN in a cross-sectional study. Clinical assessments, questionnaires, quantitative sensory testing, histamine-induced skin flare, nerve conduction studies and contact heat evoked potentials were measured. Repeat skin biopsies were performed at a 6 month interval to quantify intra- (IENF) and sub-epidermal (SENF) nerve fibres immunoreactive for PGP 9.5 (pan-neuronal marker), TRPV1 (heat and capsaicin receptor) and GAP-43 (marker of regenerating fibres) in the DPN group, and at baseline in the CIPN group.

Results: There was no change in symptoms and sensory tests in the DPN group. However, there was a significant reduction in IENF and SENF for both PGP 9.5 and TRPV1 fibres in the second DPN skin biopsy (n = 29 had repeat biopsy). GAP-43 fibres were present in the dermis and remained unchanged. Patients in the CIPN group had less painful neuropathy, but similar abnormalities on examination and sensory tests. Despite this, a preserved number of IENF and SENF were seen in the CIPN group, with abnormal morphology. This has not been reported previously.
Conclusion: PGP 9.5 and TRPV1-immunoreactive nerve fibres in sequential skin biopsies provide objective markers of progression of neuropathy, while the preserved GAP 43-immunoreactive fibres may detect enhanced regeneration. Novel findings in the CIPN group suggest prevention of degeneration and restoration of function should be the treatment strategy, rather than enhancing regeneration.
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Acknowledgements

My greatest thanks are to the participants of this study who kindly gave up their time. I was inspired by many who displayed tremendous resilience in the face of significantly painful peripheral neuropathy.

Many thanks go to my supervisor Professor Praveen Anand, Professor of Clinical Neurology at Imperial College, London. Also, thanks go to the all the staff in the diabetes and oncology clinics at Charing Cross and Hammersmith Hospitals where I recruited for the study and to my colleagues in the Peripheral Neuropathy Unit at Hammersmith Hospital; Mr Paul Facer, Dr Yiagos Yiangou, Dr Ravikiran Shenoy and Dr Katie Roberts.

This study was sponsored by JOHNSON & JOHNSON PHARMACEUTICAL RESEARCH & DEVELOPMENT (J&JPRD), a division of Janssen Pharmaceutica NV, Turnhoutseweg 30, B-2340, Beerse, Belgium.

Thanks go to GlaxoSmithKline, Harlow, UK for providing the TRPV1 antibody.
Declaration

The work presented herein was carried out at the Peripheral Neuropathy Unit, Hammersmith Hospital, Imperial College Healthcare NHS Trust, between September 2007 and August 2009 under the supervision of Professor Praveen Anand.

The study protocol was written by Professor Praveen Anand in agreement with J&J PRD who funded the study. I wrote an amendment to the protocol and the Ethics application for the study and amendment.

I recruited all patients from diabetes and oncology out-patient clinics at Charing Cross Hospital and Hammersmith Hospital. Patients were also referred directly to Professor Anand’s neurology clinic at Hammersmith Hospital.

I carried out all assessments in the Peripheral Neuropathy Unit. I had the help of two of my colleagues (Dr Ravikiran Shenoy and Dr Katie Roberts) in recording contact heat evoked potentials. Immunohistochemistry was performed by Mr Paul Facer and Dr Yiangos Yiangou.

Statistical analysis was carried out by myself and supervised by Professor Praveen Anand. Statistical review was performed by Mr Geert Byttebier, who was sponsored by J&J PRD.
## Abbreviations

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<td>AGE</td>
<td>Advanced glycation endproducts</td>
</tr>
<tr>
<td>ART</td>
<td>Artemin</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived growth factor</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin-gene related peptide</td>
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<tr>
<td>CHEPS</td>
<td>Contact Heat Evoked Potential Stimulator</td>
</tr>
<tr>
<td>CIPN</td>
<td>Chemotherapy-induced peripheral neuropathy</td>
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<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
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<td>DPN</td>
<td>Diabetic polyneuropathy</td>
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<td>DRG</td>
<td>Dorsal root ganglion</td>
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<td>EEG</td>
<td>Electroencephalogram</td>
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<tr>
<td>EVN</td>
<td>Enovin</td>
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<tr>
<td>GAP-43</td>
<td>Growth associated protein-43</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial-cell derived neurotrophic factor</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency virus</td>
</tr>
<tr>
<td>IENF</td>
<td>Intra-epidermal nerve fibre</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoproteins</td>
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<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
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<tr>
<td>MPQ</td>
<td>McGill pain questionnaire</td>
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<tr>
<td>NCIC-CTC</td>
<td>National Cancer Institute- Common Toxicity Criteria</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NIS</td>
<td>Neuropathy Impairment Score</td>
</tr>
<tr>
<td>NIS-LL</td>
<td>The Neuropathy Impairment Score Lower Limbs</td>
</tr>
<tr>
<td>NRS</td>
<td>Numerical Rating Scale</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>NT3</td>
<td>Neurotrophin 3</td>
</tr>
<tr>
<td>NTN</td>
<td>Neuturin</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>Protein gene product 9.5</td>
</tr>
<tr>
<td>PSP</td>
<td>Persephin</td>
</tr>
<tr>
<td>QST</td>
<td>Quantitative Sensory Testing</td>
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<tr>
<td>rhBDNF</td>
<td>Recombinant human brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>rhNGF</td>
<td>Recombinant human nerve growth factor</td>
</tr>
<tr>
<td>r-metHuNT3</td>
<td>Recombinant neurotrophin 3</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SENF</td>
<td>Sub-epidermal nerve fibre</td>
</tr>
<tr>
<td>SF-MPQ</td>
<td>The short-form McGill pain questionnaire</td>
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<tr>
<td>SOD3</td>
<td>Superoxide dismutases</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
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<tr>
<td>TNS</td>
<td>Total Neuropathy Score</td>
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<tr>
<td>Trk</td>
<td>Receptor for Tyrosine-kinase (Trk) gene products produce</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential receptors</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential cation channel, subfamily V, member 1</td>
</tr>
<tr>
<td>TRPM8</td>
<td>Transient receptor potential cation channel, subfamily M, member 8</td>
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<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
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<tr>
<td>VAS</td>
<td>Visual Analogue Scale</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>WHO</td>
<td>The World Health Organization</td>
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1.0 INTRODUCTION

1.1 Overview of Diabetes Mellitus

1.1.1 Definition

The World Health Organisation (WHO) has published guidelines on the definition, diagnosis and classification of diabetes mellitus since 1965. Major reviews of these guidelines, in collaboration with other organisations were undertaken and published in 1985, 1998 and 2006 (2006). WHO defines diabetes mellitus as a “metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both” (Alberti et al. 1998).

1.1.2 Diagnosis

Diabetes mellitus is considered when classical symptoms of polyuria, thirst, fatigue and weight loss are present. The name ‘diabetes’ is derived from the ancient Greek meaning ‘to run through’ and ‘mellitus’ was later added from the Latin word for ‘honey’, referring to the excess glucose passed in the urine giving it a sweet taste.

The main biochemical marker is hyperglycaemia which usually results in elevated glycosylated haemoglobin (HbA1c) levels. There has been a trend to lower the cut off at
which a diagnosis of diabetes is made, for example in 1985 a fasting glucose of ≥8.0 mmol/l was used and currently a fasting level of ≥7.0 mmol/l. WHO guidelines for the diagnosis of diabetes mellitus are shown in Table 1.1. In 2011 a WHO Consultation recommended using a HbA1c level of 48 mmol/mol (6.5%) as the cut point for diagnosing diabetes. Although, it should be noted that a value of less than this does not exclude diabetes diagnosed using glucose tests.

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<td>Fasting plasma glucose</td>
<td>≥7.0mmol/l (126mg/dl)</td>
<td>&lt;7.0mmol/l (126mg/dl)</td>
<td>6.1 to 6.9mmol/l (110mg/dl to 125mg/dl)</td>
</tr>
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<td>2–h plasma glucose*</td>
<td>≥11.1mmol/l (200mg/dl)</td>
<td>≥7.8 and &lt;11.1mmol/l (140mg/dl and 200mg/dl)</td>
<td>&lt;7.8mmol/l (140mg/dl)</td>
</tr>
</tbody>
</table>

* Venous plasma glucose 2–h after ingestion of 75g oral glucose load
* If 2–h plasma glucose is not measured, status is uncertain as diabetes or IGT cannot be excluded

**Table 1.1** World Health Organisation guidelines for the diagnosis of diabetes mellitus.

### 1.1.3 Epidemiology

The incidence and prevalence of diabetes mellitus is increasing at an accelerating rate. The estimated prevalence of total diabetes for all persons in England was 4.41% in 2001, equating to 2,168,000 persons. Of these, type 2 diabetes was estimated to affect 2,002,000 persons (92.3%), and type 1 diabetes 166,000 persons (7.7%) (Forouhi et al. 2006). By 2025 it is estimated that over four million people will have diabetes in the United Kingdom according to key statistics published by the organisation Diabetes UK in March 2010. Global estimates indicate there were 171 million people in the world with diabetes in the year 2000, and this is projected to increase to 366 million by 2030 (Wild et al. 2004), however already current
estimates by WHO published in their factsheet No. 312, August 2011 show a global prevalence of 312 million people.

1.1.4 Classification

The classification of the American Diabetes Association Expert Committee (1997) is used widely. This is based on aetiology and pathological mechanisms. The main previously used classification was based on pharmacological treatment used for management e.g. insulin-dependent or non insulin-dependent diabetes mellitus. The classification is shown in Table 1.2.

<table>
<thead>
<tr>
<th></th>
<th>Type 1 diabetes</th>
<th>Type 2 diabetes</th>
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<tbody>
<tr>
<td>I</td>
<td>A. Immune-mediated</td>
<td></td>
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<tr>
<td></td>
<td>B. Idiopathic</td>
<td></td>
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<tr>
<td>II</td>
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</tr>
<tr>
<td>III</td>
<td>Others</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. Genetic defects of β-cell function</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. Genetic defects in insulin action</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. Diseases of the exogenic pancreas</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F. Endocrinopathies</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G. Drug- or chemical-induced</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. Infections</td>
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</tr>
<tr>
<td></td>
<td>I. Uncommon forms of immune-mediated diabetes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>J. Other genetic syndromes</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Gestational diabetes</td>
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</tr>
</tbody>
</table>

Table 1.2 Classification of diabetes mellitus.

1.1.5 Complications

Diabetes mellitus gives rise to a number of acute and chronic complications. These are associated with significant morbidity and reduced life expectancy. Complications can be divided into two main types: those resulting from microvascular damage (retinopathy,
nephropathy and neuropathy), and macrovascular complications (ischaemic heart disease, stroke and peripheral vascular disease).

Microvascular damage or microangiopathy is thought to be related to hyperglycaemia, because incidence of this complication rises with poor glycaemic control and improves with lowering HbA1c to some extent (1993; Amthor et al. 1994; Ohkubo et al. 1995; 1998; Alberti et al. 1998).

1.1.6 Treatment of Diabetes Mellitus

The primary goal of treatment is to reduce hyperglycaemia and HbA1c is used as a marker. Comprehensive guidance is given by the National Institute for Health and Clinical Excellence and the principles outlines below. There is usually relief of symptoms such as excessive thirst and lethargy, and in the longer term reduction of complications.

Dietary advice is given and physical activity is encouraged, with view to weight loss if needed. Pharmacological treatment is vital in type 1 diabetes where insulin injections are required. Those with type 2 diabetes usually need more treatment over time, and often progress from no pharmacological measures to oral medication, and addition of insulin therapy. Recently two new classes of treatment (DPP4 inhibitors and GLP-1 analogues) have delayed the use of insulin in this group. Pancreas transplant and pancreas stem cell transplant are now available for the treatment of type 1 diabetes (2004; 2008; 2009).
1.2 Overview of the structure and function of the Peripheral Nervous System

The somatic nervous system is divided into motor and sensory components. This study will focus on the somatosensory components, which involve the sensations of light touch, vibration, temperature and pain (nociception).

1.2.1 Peripheral nerve fibre types

There are a number of nerve fibre types which together form a peripheral nerve. These are classified using the ABC classification, according to their function, size and myelination (Table 1.3). With regard to size, the largest diameter fibres are classified A. The smallest fibres are classified as C fibres and are unmyelinated. The A group is sub-divided into four groups; α, β, δ and γ. A secondary classification system is also used, based on conduction velocity I-IV in descending order of velocity.

Nerve fibres in skin originate in the spinal cord and traverse through dorsal root ganglia which house the cell bodies, into peripheral nerves. After entering the skin they course in nerve bundles to the superficial dermis, where they make up the sub-epidermal nerve plexus. Individual epidermal nerve fibres emerge from the bundles and shed their collagen collar and Schwann cell sheath as they pierce the dermal-epidermal basement membrane. They penetrate through the epidermis to the stratum corneum, usually vertically. Changes to these intra-epidermal (IENF) and sub-epidermal (SENF) nerve fibres are valuable in quantifying neuropathy.
Table 1.3 Nerve fibre types and classification.

1.2.2 Somatosensory receptors

There are three main groups: mechanoreceptors, thermoreceptors and nociceptors.

**Mechanoreceptors**

Mechanoreceptors contain ion channels that respond to stretching or changes in tension of the surrounding membrane. They mediate the sensations of light touch, pressure, vibration,
flutter, limb position and movement (kinesthesia) through various mechanisms that are based on punctuate fields and speed of adaptation. Mechanoreceptors are present in the dermis of hairy (hair receptors around hair shaft and free nerve endings) and glabrous skin (Meissner’s corpuscles, Merkel’s discs and free nerve endings). They are also found in the subcutaneous region (Pacinian corpuscles and Ruffini’s endings).

**Thermoreceptors**

There are separate thermoreceptors for warm or cold stimuli. They are mostly bare nerve endings. They have punctate receptive fields and temperature is related to the discharge rate. Transient receptor potential (TRP) receptors are a family of temperature sensitive ion channels that mediate thermosensitivity in primary sensory neurones. A number of different receptors have been found to be present on different sub-sets of neurones. TRPV1 is activated by noxious heat (≥42°C) and capsaicin (the noxious component from chilli peppers). TRPV2 is activated by heat (>43°C-52°C). TRPA1 mediates cold sensation (<17°C) and is activated by cinnamaldehyde. TRPM8 mediates cool sensation (<27°C-28°C) and is also activated by menthol compounds. TRPV3 and TRPV4 mediate warm sensation (>27°C-38°C) (Facer et al. 2007; Roberts et al. 2011).

Paradoxical cold sensation occurs when a hot stimulus of 45°C is selectively applied to a cold fibre field, for example where there has been selective destruction of nerve fibres. The stimulus is then perceived as cold instead of hot which would have been the case if the stimulus had been applied diffusely to the skin.
**Nociceptors**

Noxious stimuli are those that can cause tissue damage. Nociceptors are bare nerve endings and are selective for different types of noxious stimuli, for example thermal (heat or cold), mechanical, chemical and polymodal. Pain is the resultant unpleasant sensation which is triggered, along with sensations of pricking, burning, aching and stinging sensations. There is a high degree of subjectivity in the experience of pain such that stimulation of nociceptors does not always lead to pain transmission.

Damage to skin results in an initial sensation, which is conveyed by fast Aδ fibres. The more painful lasting sensation is transmitted by slower C fibres. Allodynia is the perception of pain sensation, without a noxious stimulus.

Two sub-groups of C fibres have been described. One group expresses the adenosine triphosphate (ATP)-gated P2X3 receptor, the IB4-lectin binding site and receptors for glial-cell derived neurotrophic factor (GDNF). The other group expresses TrkA (receptor for nerve growth factor, NGF) and produces neuropeptides important in nociception, substance P and calcitonin-gene related peptide (CGRP) (Hunt et al. 2001).

Three sub-groups of A fibre nociceptors have been described. Polymodal receptors, Type I - sensitive to high threshold temperature, low threshold mechanical and chemical stimuli; Type II – sensitive to lower threshold temperature, high threshold mechanical stimuli, and chemical stimuli. The third group are unresponsive to heat and are high threshold mechanoreceptors (Caterina 2005).
Some groups of Aδ and C fibres, known as ‘silent nociceptors’, may be insensitive to
mechanical or chemical stimuli, but become sensitized following inflammation and then can
be activated by mechanical stimuli. This may be the underlying mechanism for hyperalgesia
(Xu et al. 2000). Silent C fibres are thought to be important in inflammatory pain conditions
and central sensitization (Weidner et al. 1999), and are also responsible for the axon reflex-
mediated flare (Schmelz et al. 2000).

1.2.3 Transmission to the spinal cord

Each sensory neurone has a cell body in the dorsal root ganglia of the spinal cord. These
bipolar neurones have a long peripheral axon branch and a central axonal projection which
can be of varying length, for example those in the dorsal column-medial lemniscal pathway
have an extensive central projection. The cell bodies supply nutrients via axonal transport in
order to maintain neuronal function. The effect of diabetes on the dorsal root ganglion
microenvironment may explain why the sensory neurones are vulnerable to damage. There
have been a number of studies showing a reduction in spinal cross-sectional area (Eaton et al.
2001; Selvarajah et al. 2006).

There is segmental organisation of inputs into the spinal cord as a result of embryonic
development. Therefore an area of skin called a dermatome is supplied by axons from a
single dorsal root ganglion (DRG). In addition to the central projections there are local
connections within the spinal cord. The synaptic connections in the spinal cord are at
different layers, for example Aδ fibres synapse primarily in laminae I and V, C fibres connect
mainly in laminae II and I and Aβ fibre input goes to lamina V neurones.
Transduction of information is mediated by ion channels (Na\(^+\) and Ca\(^{2+}\)) with different properties conveyed according the distribution of different ion channels and receptors for nerve growth factors. Activation of these ion channels induces currents that depolarise the cell-membrane and open voltage-gated ion channels (such as Na\(_v\)1.8 and Na\(_v\)1.9), subsequently leading to initiation of an action potential. The intensity of the stimulus determines the level of activation and is reflected by the duration and frequency of the action potential (Snider et al. 1998).

**1.2.4 Pain**

Following injury, pain is a protective response preventing further damage to an affected area. For example injury to the sole of the foot leading to pain would cause the bearer to avoid putting further weight on the lesion until it was healed.

Initially, following damage to the somatic nervous system, there may be a partial or complete loss of sensory function (negative symptoms), and the development of symptoms such as pain, paraesthesia, dysesthesia, hypersensitivity (hyperalgesia - increased pain in response to a normally noxious stimulus, and allodynia - pain in response to a normally non-noxious stimulus). Neuropathic pain does not require any receptor stimulation and can be severe, chronic and intractable (Urch 2006). Several mechanisms are proposed.

Peripheral mechanisms include altered transmission; local cytokines induce changes in neuron receptor expression, altering thresholds and kinetics. Accumulation of neurotransmitters is also thought to be important in the altered properties of the injured neurone and dorsal root ganglion; electrical ‘cross-talk’ - ephaptic cross talk between axons.
of all calibres may occur in damaged nerves; phenotypic switch – following injury, there is changed expression of large numbers of genes (Devor 2006).

Central mechanisms include structural changes following lack of retrograde transport of nerve growth factors vital for trophic support; central sensitisation (“wind-up”), a form of activity-dependent plasticity in the spinal cord; disinhibition, following the depression of spinal inhibitory mechanisms (Devor 2006).

### 1.2.5 Vascular supply

The spinal roots and peripheral nerves have a good vascular supply composed of two integrated systems. These are the intrinsic and extrinsic circulations (Petterson et al. 1989). The extrinsic system is derived from local large arteries and veins, as well as those supplying surrounding muscle and periosteum. These are arranged segmentally and follow the surface along the length of the nerve. They form a plexus within the epineural layers of the nerve sheath. The intrinsic circulation, the vasa nervorum, consist of vessels within the perineurium that connect with the endoneural vascular bed. Terminal arterioles from the perineurium penetrate the nerve fascicles and form the endoneurial capillary bed which is a network of intrafascicular capillaries that run longitudinally throughout the length of the nerve.

The dorsal root ganglia have greater blood flow, perhaps reflecting increased metabolic rate. This blood flow is seen to be reduced in those with diabetic peripheral neuropathy (Cameron et al. 2001).
1.2.6 Neurotrophic factors and receptors

Neurotrophic factors play key roles in the development, maintenance and regeneration of the nervous system through retrograde transport of target-derived growth factors. Gene expression and protein synthesis in the cell-body are influenced. During foetal development or following nerve injury a variety of growth factors are important for axonal growth (Levi-Montalcini 1987). There is evidence to show that nerve growth factor (NGF) regulates nociception (Anand 1995).

Neurotrophins effect action through various receptors. Tyrosine-kinase (Trk) gene products produce the high-affinity receptors Trk A-C, co-expressed with the low-affinity neurotrophin receptor (P_
75
). Subpopulations of primary afferent fibres have differential expressions of the Trks corresponding to their dependence on different neurotrophic factors.

Many neurotrophic factors have now been discovered. Those relevant to this study are listed in Table 1.3 (please see section 1.2.1 for table), along with the nerve fibres they are associated with.

The nerve growth factor (NGF) superfamily includes NGF, which is trophic to sympathetic and small unmyelinated (including nociceptive) neurones (receptor TrkA, P_
75
); Neurotrophin NT-3, which is trophic to large and small unmyelinated (proprioceptive, mechanosensitive) neurones (receptor Trk C); Brain-derived growth factor (BDNF), which is trophic to medium and large sensory fibres including hair follicle afferents (receptor TrkB), and Neurotrophins NT-4 and NT-5, which are trophic to medium fibres (receptor TrkB). BDNF and NT-3 are thought to be important for myelination of nerves (Mohiuddin et al. 1995).
Another superfamily of growth factors is the transforming growth factor β family (TGF-β). Glial cell-derived neurotrophic factor (GDNF) has a multi-component receptor (consisting of a cell-surface protein, GDNFR-alpha and a tyrosine kinase receptor), and is important for dopaminergic mid-brain neurones and motor neurones in the periphery. Both NGF and GDNF are produced by basal keratinocytes in the skin (Lindsay et al. 1989; Meakin et al. 1992). Other members of this superfamily are Neuturin (NTN), which is important for survival of sympathetic fibres; Persephin (PSP), which is similar in effect to GDNF; Artemin (ART), which is a survival factor for sensory and sympathetic neurones, and Enovin (EVN), which seems to have an important role in development, maintenance, neuroprotection and regeneration of peripheral neurones (Masure et al. 1999).

Primary sensory neurons have the ability to regenerate if cut or crushed. Following nerve crush injury or transaction, there is physical loss of contact between the cell body in the dorsal root ganglion and the target tissues. Loss of target-derived NGF leads to induction of NGF synthesis in Schwann cells at the site of the lesion and in the distal stump. The amount of NGF available at the DRG is insufficient to maintain substance P and CGRP levels, affecting microvascular blood flow and vascular permeability (Lindsay et al. 1989). Atrophy and withdrawal of C fibres from outer laminae II of the dorsal horn of the spinal cord is observed over a period of weeks (Tandrup et al. 2000). Regeneration is favored in the periphery, where neurotrophic support and a favorable environment for axonal growth is present. The regenerative capacity of peripheral sensory nerves are associated with expression of growth associated protein-43 (GAP-43) and ongoing presence of NGF (Donnerer 2003). NT-3 can increase the level of nerve regeneration and up-regulate GAP-43 (Mohiuddin et al. 1995). GAP-43 is a major component of the axonal growth cone and its
synthesis correlates with an increased propensity of neurones to regenerate (Tetzlaff et al. 1989).

Vascular endothelial growth factor (VEGF) is a promoter of angiogenesis, induces Schwann cell proliferation and migration, stimulates growth and is important in survival of neurones, Schwann cells and DRGs (Sondell et al. 1999).
1.3 Assessment of Peripheral Neuropathy

A number of assessments are available for the diagnosis of peripheral neuropathy and to assess its severity and progression. Symptoms including pain are measured with the use of validated scales and questionnaires. Quantitative sensory tests (QST) are psychophysical, and by their nature are subjective; graded stimuli are often used with the threshold at which there is perception of the stimulus recorded. Few objective tests are available and these are discussed. Composite scores of symptoms, pain, clinical examination findings and sensory tests are often used.

1.3.1 Pain scores and scales

The Likert pain score is a numerical 11-point intensity rating scale of pain; zero is no pain and ten is worst pain imagined, or maximum pain. Other options for recording pain are the Numerical Rating Scale (NRS), and the Visual Analogue Scale (VAS), which is represented by a 10cm line, with one end representing no pain and the other representing worst pain; the patient is asked to mark a point on this line that represents their pain level, and this line is then measured to arrive at a numerical measurement (Melzack 2006).

1.3.2 Questionnaires

McGill Pain Questionnaire (MPQ)

The McGill pain questionnaire is one of the most widely used questionnaires for recording neuropathic pain. It was designed by Ronald Melzack in 1975 and takes into account a large number of descriptors of pain such as; ‘flickering’, ‘shooting’, ‘nauseating’ or
‘torturing’ (Melzack 1975). The short-form McGill pain questionnaire (SF-MPQ) was introduced in 1980 in recognition of the fact that the original questionnaire was too long to carry out in clinical trials (Melzack 1987). The most commonly used sensory and affective descriptors, along with a visual analogue scale for pain intensity are included. The SF-MPQ was found to correlate highly with the original questionnaire and has been used in several clinical trials (Melzack 2005). Please see Appendix A for illustration of the SF-MPQ described here.

*Total Neuropathy Score (TNS)*

The total neuropathy score was designed as a composite measure of peripheral nerve function, combining information from grading of symptoms, signs, nerve conduction studies, and quantitative sensory tests (Cornblath et al. 1999; Cavaletti et al. 2003). It was developed mainly in trials measuring chemotherapy-induced neuropathy (Chaudhry et al. 1994; Chaudhry et al. 1996).

*Neuropathy Impairment Score (NIS)*

The Neuropathy Impairment Score Lower Limbs (NIS-LL) is a widely used summed score of muscle power, reflex loss and sensation loss in the lower limbs (Dyck et al. 1997; Bril 1999). The various components are scored to take into account gender and age which is advantageous. Please see Appendix B for illustration of the Score described here.

1.3.3 Bedside examination

Neurological examination involves assessment of muscle tone and power, tendon reflexes and sensory examination. Usually sensory abnormalities are mapped out using cotton wool
for tactile sensation (Aβ fibres), pin-prick sensation with a small pin or cocktail-stick (Aδ fibres), gross temperature sensation with warm or cool objects (warm – C-fibres, cool-Aδ fibres) and vibration sense with a tuning fork (128Hz) (Aβ fibres) (Cruccu et al. 2004). The limitations with these tests are that they often vary according to examiner and are often not sensitive enough to show longitudinal change, hence the development of more standardized and validated quantitative sensory tests.

1.3.4 Quantitative Sensory Testing

Quantitative sensory testing (QST) is the term that is applied to tests where the intensity and characteristics of the test stimulus is well controlled and reproducible, and the detection threshold is determined in parametric units that can be compared to established normal values. Several instruments and accepted procedures for quantitative evaluation of neuropathy have been established, ranging from simple instruments such as monofilaments to complex computer-aided systems such as the computer aided sensory examination (CASE) IV system (Valk et al. 1997; Dyck 2003). Please see Appendix C for illustration of the equipment described here.

Monofilaments were originally made from horsehair and Von Frey used these in the early 19th century to study touch recognition. More commonly Semmes-Weinstein hairs are used to test touch perception (Aβ fibres). These nylon hairs test a broad range of forces and are uniform in length, but have different diameters. The 10g (5.07) monofilament has been associated with ulcer formation in patients with diabetes, and is now used for screening of ulcer risk in patients with diabetes (Birke et al. 1986).
Measurement of vibration perception thresholds is a reliable method of examining the function of large myelinated fibres (Aβ fibres), particularly when vibrotactile equipment such as a biothesiometer or neurothesiometer is used instead of a tuning fork (128Hz). A biothesiometer is a vibrating probe in which the amplitude of vibration increases as the voltage is increased. It has been validated in a number of different settings (Dyck et al. 2000; Duke et al. 2007).

Assessment of thermal thresholds; cool, warm, heat-pain and cold-pain detection thresholds using a probe that can change temperature is a test of small nerve fibre function, usually thinly myelinated Aδ fibres and unmyelinated C-fibres (Cruccu et al. 2004). Measurement of thermal thresholds has been validated in a number of different settings (Dyck et al. 2000; Shukla et al. 2005). The usefulness of QST is demonstrated in one study which showed abnormal thresholds in 25 patients with normal nerve conduction studies and normal vibration sense (Jamal et al. 1987).

It should be borne in mind that abnormalities of any of these sensory tests may mean pathology at any point in the sensory pathway; in the transduction of the sensory signal, transmission of the signal in the peripheral nerve, transmission in the spinal cord and correct functioning of the sensory cortex. Quantitative tests are dependent on patient concentration, a standardized environment and test regime being maintained for comparison (Dyck et al. 1999).

1.3.5 Histamine-induced axon reflex- mediated vasodilatation

Sir Thomas Lewis first described the triple response to injury of the skin in 1927. Local reddening (vasodilatation) of the skin at the site of injury is provoked by a number of stimuli;
mechanical, thermal (Krishnan et al. 2004), electrical as well as chemical agents including histamine. The histamine-induced axon reflex-mediated flare response has been demonstrated to be directly related to nociceptive C-fibre function and can be diminished in those with peripheral neuropathy (Hutchison et al. 1974; Schmelz et al. 2000; Caselli et al. 2003). The spreading flare is thought to be mediated by unmyelinated C-fibres via release of neuropeptides such as CGRP and substance P (Anand et al. 1983; Kuesgen et al. 2002). This test is one of the few objective measures of neuropathy available. Please see Appendix C for illustration of the triple response to injury and flare response described here.

1.3.6 Sudomotor function

Autonomic nerve fibres may be affected in patients with peripheral neuropathy and can present with excessive coldness, discolouration or sometimes erythromelalgia of the feet. Hyper- and hypohidrosis may be present. A number of methods are available to test autonomic nerve fibre function in the skin, and range from direct measurement of sweat evaporation from the skin using an evaporimeter (or sudorometer), to detection of nicotine-induced axon-reflex sweating or the quantitative sudomotor axon reflex test (QSART), which uses acetylcholine iontophoresis and is mediated by postganglionic sympathetic sudomotor C fibres (Walmsley et al. 1990; Facer et al. 1998; Low et al. 2006). Please see Appendix C for illustration of the equipment described here.

1.3.7 Contact heat-evoked potentials

The Contact Heat Evoked Potential Stimulator (CHEPS) is a useful objective measure of small nerve fibre function. Rapidly delivered heat pulses with adjustable peak temperatures
can be used to stimulate the differential warm/cold thresholds of receptors expressed by Aδ and C-fibres. The resulting evoked potentials are recorded and measured by electroencephalography (EEG). In volunteer models, reliable and quantifiable evoked potentials were produced with consistent Aδ peak latencies and amplitudes, particularly in the vertex Cz component of the EEG (Le Pera et al. 2002). Evoked potentials from supra and infra orbital electrodes are also recorded for artefact control and subsequently subtracted from the evoked potentials which are then averaged. A similar method using laser evoked potentials has also been described; however CHEPS is thought to be easier to use and avoids the risks of burns and need to use eye protection (Atherton et al. 2007). Please see Appendix C for illustration of the equipment described here.

Previous unpublished tests carried out in the Peripheral Neuropathy Unit showed a sensitivity of 96.3% and specificity 35.7% when comparing CHEPS measured from lateral calf compared with PGP 9.5 counts. Comparing Histamine-induced axon reflex-mediated vasodilatation instead of PGP 9.5, the sensitivity was 96.8% and the specificity 50%.

1.3.8 Nerve conduction studies

Nerve conduction studies are traditionally used as the gold standard non-invasive, objective measure of neuropathy. They are limited in scope however to measuring abnormalities of large diameter sensory and motor fibres. Three parameters are commonly tested; nerve conduction amplitude, velocity and presence of F-waves. Reduction of amplitude is usually associated with nerve fibre loss, whereas reduced velocity relates to demyelination in simple terms (Kimura 1984; Bril 1994; Dyck et al. 1996). F-waves are so called because they were first recorded in the foot. They are recurrent discharges of anti-dromically activated motor
neurons. A supramaximal stimulus applied to a nerve elicits a F-wave if intact and F-wave latency has been found to be one of the most sensitive measures for detection of nerve pathology (Andersen et al. 1997).

1.3.9 Nerve biopsy

Nerve biopsy is now performed infrequently. Sural nerve biopsy, typically posterior to the lateral malleolus, can be used to investigate nerve pathology, but has been superseded by skin biopsy particularly for investigation of small fibre neuropathy (Thomas 1997).

1.3.10 Skin biopsy

Skin biopsy for the assessment of neuropathy became established in the early 1980s after the discovery of an antibody against protein gene product 9.5 (PGP 9.5) which is present in almost all axons. Immunohistochemical use of this antibody allowed direct quantitative studies of intra-epidermal nerve fibres (which comprise of only Aδ and C-fibres), and allowed early detection of neuropathy in those with a ‘dying-back’ type of neuropathy, such as in diabetes. Over the years the method of nerve fibre assessment using this technique has been validated, standardized and guidelines established, such as those produced by the European Federation of Neurological Societies (EFNS) (McCarthy et al. 1995; McArthur et al. 1998; Lauria et al. 1999; Cruccu et al. 2004; Goransson et al. 2004; Lauria et al. 2005). The technique is particularly useful given that it is objective and minimally invasive. A limitation however is that it is only available in certain academic centres.
The utility of skin biopsy has been greatly enhanced by the discovery of other neuronal markers which allow mapping and quantification of sub-sets of clinically relevant neurones such as antibodies to transient receptor potential cation channel, subfamily V, member 1 (TRPV1), which is the heat and capsaicin receptor, and antibodies to growth associated protein-43 (GAP-43), a marker of nerve regeneration (Galkowska et al. 2006; Facer et al. 2007).

1.3.11 Confocal microscopy

A recently reported non-invasive technique for assessment of small-fibre neuropathy is the use of the corneal confocal microscope (Malik et al. 2003; Quattrini et al. 2007). The eye being examined is anaesthetised with anaesthetic eye drops. A large drop of liquid gel artificial tears is placed on the confocal microscope and advanced until the gel is in contact with the cornea. The microscope is then focused and scanning performed of the depth of the cornea. Several measures are then recorded; nerve fibre density, nerve fibre length and nerve branch density. These measures correlated with PGP 9.5 immunostaining from skin punch biopsies in the lower limb. Although very useful for repeat assessment in patients because of the non-invasive nature of the test, the usefulness of a surrogate marker at a totally different anatomical site, and testing with just one neuronal marker, are limitations which must be considered.
1.4 Diabetic Neuropathy

Diabetic neuropathy is a heterogeneous disorder that encompasses a wide range of abnormalities affecting sensory, motor and autonomic nerve fibres to varying degrees. These manifest as a number of clinical syndromes that can be focal or diffuse, and may be present with or without symptoms. For these reasons, estimating incidence and prevalence is difficult. Overall, the annual incidence in the United Kingdom Prospective Diabetes Study (UKPDS) and the Diabetes Control and Complications Trial (DCCT) was approximately 2% (1993; 1998). One study showed that approximately 7% of patients had neuropathy on diagnosis, and the prevalence approached 50% after 25 years of diabetes (Pirart 1977). The criteria and methods used to define neuropathy are also very important. In patients attending a diabetes clinic, 25% reported symptoms, 50% were found to have neuropathy on clinical examination (absent ankle jerk or reduced vibration perception), but almost 90% had neuropathy on sophisticated tests of autonomic function or sensation (Vinik 1999).

Neurological complications are thought to occur equally in the different forms of diabetes, although patterns of neuropathy may vary; symptomatic autonomic neuropathy where present is almost always seen in those with Type 1 diabetes, and reversible mononeuropathies are often seen in older men with type 2 diabetes (Dyck et al. 1993). Recently, metabolic factors other than hyperglycaemia have been shown to be associated with neuropathy, indicating that the initial disease process may be different in type 1 and type 2 diabetes (Green et al. 2010). The EURODIAB study showed that the presence of neuropathy was significantly correlated with age, duration of diabetes, quality of metabolic control, height, presence of retinopathy, smoking, high-density lipoprotein level, presence of cardiovascular disease, elevated blood pressure, elevated fasting triglyceride level and presence of microalbuminuria (Tesfaye et al. 1996).
The clinical spectrum can range from asymptomatic or mild in the majority to a severe, painful and disabling condition. Major morbidity results from neuropathic pain and increased risk of foot ulceration leading to gangrene and limb loss (Armstrong et al. 1998). The latter is responsible for significant health care costs. The American Diabetes Association reported the total annual economic cost of diabetes in 2007 to be $174 billion, and up to 27% of the direct medical cost of diabetes may be attributed to managing diabetic neuropathy and its complications (Gordois et al. 2003).

1.4.1 Diagnosis and staging

Given the heterogeneity of the forms of neuropathy, criteria for diagnosis and staging of severity are needed. A simple definition of diabetic neuropathy is “the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes” (Boulton et al. 1998). Neuropathy attributed to aetiology other than diabetes can be found in up to 10% of patients with diabetes (Dyck et al. 1993). Historically, absent ankle reflexes and elevated vibration thresholds in the feet were the main criteria, but these tests are not sensitive enough, and need to be age, sex and height matched. Dyck proposed that at least two abnormalities be present from among: symptoms, clinical deficits (signs), nerve conduction studies (NCS), quantitative sensory tests (QST) and quantitative autonomic tests (Dyck 2003). More recently this has been amended to include a validated measure of small-fibre neuropathy (with class 1 evidence) if NCS is normal (Tesfaye et al. 2010). Measures suggested include nerve biopsy (rarely performed now), skin biopsy, sudomotor innervation, corneal confocal microscopy and skin axon-reflex or flare response.
Dyck also proposed more comprehensive criteria for diagnosis and assessment of diabetic neuropathy (Dyck et al. 1993). A composite score was calculated as the Neuropathy Impairment Score (Lower Limbs), plus scores from up to seven tests (peroneal motor nerve conduction velocity, peroneal compound muscle action potential, peroneal motor distal latency, sural sensory nerve action potential and tibial motor distal latency, heart-pulse decrease with breathing, and vibratory detection threshold (using CASE IV). Given the complexity of measurement this composite score is not used routinely. Interestingly, further analysis of the Rochester Diabetic Neuropathy Study Cohort in which this score was validated, showed that changes in sensory tests were present before the minimum criteria for diagnosis of diabetic polyneuropathy was achieved (Dyck et al. 1993).

An international group of experts held a consensus meeting in 1998 and agreed the staging classification in Table 1.4 (Boulton et al. 1998).

| - No Neuropathy                  |
| - Clinical neuropathy           |
|     - Chronic painful           |
|     - Acute painful             |
| - Painless with complete/partial sensory loss |
| - Late complications           |

**Table 1.4** Staging of diabetic neuropathy (1998).
Using this staging method, progression from one stage to the other is not automatically implied. The staging of severity in diabetic polyneuropathy in Table 1.5, proposed by Dyck, is practical (Dyck et al. 1993).

**Table 1.5** Staging of diabetic neuropathy (1993).

Positive symptoms are hypersensitivity, prickling, burning pain, lancinating pain, deep throbbing and aching. Negative symptoms are decreased perception of tactile, thermal or painful stimuli.

**1.4.2 Classification**

The San Antonio Convention (1998) proposed three groups, Table 1.6.

1. Subclinical neuropathy – determined by abnormalities in electrodiagnostic and quantitative sensory testing.
2. Diffuse clinical neuropathy – distal symmetric sensorimotor and autonomic neuropathies.
3. Focal syndromes

**Table 1.6** The San Antonio Convention (1998) classification of diabetic neuropathy.
Several other classifications have been proposed which are based on presumed aetiology, pathological features and clinical manifestations, or combinations of these (Boulton et al. 1986; Thomas 1997; Said 2001). The classification proposed by Thomas (Thomas 1997), and adapted (Boulton et al. 2004) is shown in Table 1.7.

|---------------------------------------------|

<table>
<thead>
<tr>
<th>Rapidly reversible</th>
<th>Hyperglycaemic neuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generalised symmetrical polyneuropathies</td>
<td>-Sensorimotor (chronic)</td>
</tr>
<tr>
<td>-Acute sensory</td>
<td></td>
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<tr>
<td>-Autonomic</td>
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<tr>
<td>Focal and multifocal neuropathies</td>
<td>-Cranial</td>
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<tr>
<td>-Thoracolumbar radiculoneuropathy</td>
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<tr>
<td>-Focal limb (including entrapment and compression neuropathies)</td>
<td></td>
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<tr>
<td>-Proximal motor (amyotrophy)</td>
<td></td>
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<tr>
<td>Superimposed chronic inflammatory demyelinating polyneuropathy</td>
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</tbody>
</table>

**Table 1.7**

### 1.4.3 Sensorimotor polyneuropathy

Sensorimotor polyneuropathy, also known as distal symmetric polyneuropathy (DPN), is the most frequent form of diabetic neuropathy. It is usually insidious in onset, and more common with increasing age and duration of diabetes. Initially asymptomatic, patients may develop numbness, tingling and pain over time. Symptoms may be intermittent, and patients can experience severe burning type-pain, often worse at night. It is characterized by length-related, distally pronounced distribution of sensory symptoms and signs. It may be accompanied by autonomic neuropathy. A mild distal motor neuropathy may also co-exist.
The distal extremities of the lower limbs are usually affected, followed by upper limb involvement.

*Neurophysiological tests*

Vibration, thermal and pain thresholds have proven valuable in the detection of sub-clinical neuropathy, tracking large cohorts and predicting patients who are at risk of foot ulceration (Boulton et al. 2004).

Measurement of the axon reflex-mediated flare using Laser Doppler imaging in the foot showed reduced flare area in those with type 2 diabetes, with and even those without neuropathy (Krishnan et al. 2004). Contact heat evoked potentials have been shown to be reduced in patients with diabetes (Atherton et al. 2007; Chao et al. 2010).

Changes in nerve conduction studies (NCS) have been shown to correlate with duration of diabetes and severity of hyperglycaemia in some studies and not in others (Gregersen 1967; Fedele et al. 1980). The most common abnormality is reduction in amplitude of sensory nerves in the legs, mild reduction in motor NCS and prolongation of F-waves (Braddom et al. 1977).

1.4.4 **Histopathology**

DPN is characterized by a loss of both large and small myelinated nerve fibres, for example in the sural nerve where moderate to severe loss of fibres can occur with considerable variation between fascicles. Nerve fibre loss is pronounced distally, but is also seen in spinal roots, particularly the dorsal roots. There is a pronounced loss of unmyelinated nerve fibres
in the skin (Chopra et al. 1969; Dyck et al. 1986; Llewelyn et al. 1988; Thomas 1993). Axonal swellings of intra-epidermal nerve fibres are occasionally seen in patients with diabetes and are thought to result from damage to the cytoskeleton preceding axonal degeneration and loss. The ‘swelling ratio’ predicted IENF loss in painful diabetic neuropathy (Lauria et al. 2003). Degeneration and proliferative changes of Schwann cells in sural nerve samples is also observed (Kalichman et al. 1998). Damage to small and large sensory and autonomic fibres also occurs (Said et al. 1983).

A variety of fibre abnormalities including axonal degeneration, regeneration and segmental demyelination is reported, with axonal degeneration thought to be the primary event (Dyck et al. 1986). In one study, demyelination was found to precede axonal loss (Valls-Canals et al. 2002) in a proportion of patients.

Malik et al. performed repeat sural nerve biopsy and QST in 12 patients with minimal neuropathy in order to define early pathological changes. Repeat biopsy was performed 8.7±0.6 years later. No evidence of myelinated fibre loss was found. Un-myelinated nerve fibres showed increased numbers of un-associated Schwann cell profiles and axonal sprouts suggestive of concomitant degeneration with regeneration. QST was normal at baseline, with no significant change in vibration sense over this time period. A significant increase in thermal thresholds was found. A decline in median and peroneal nerve conduction velocity, with no significant change in sural nerve conduction velocity was found on repeat testing (Malik et al. 2005).

There is loss of PGP 9.5 immunostained intra-epidermal nerve fibres (IENF) in patients with diabetic neuropathy. The extent of loss is in keeping with the duration of neuropathy
Moreover, IENF density was found to be reduced even in those with DPN who have normal NCS. This suggests early damage to small fibres (Loseth et al. 2008). In the majority of patients complete loss of these fibres is seen by the time symptoms are established. Fibres may be detected in the papillary and reticular layer of the skin. TRPV1 expressing intra-and sub-epidermal fibres are decreased in diabetic skin (Facer et al. 2007). GAP-43 positive fibres are very rarely seen in the epidermis, but have been consistently seen in the dermal layer of patients with diabetes (Galkowska et al. 2006).

IENF reduction in patients with diabetes has been correlated with cold and heat thresholds (Shun et al. 2004). Inverse correlation with neurological disability score (Quattrini et al. 2007) has been found, and IENF density was found to be lower in patients with painful neuropathy (Sorensen et al. 2006). No difference in IENF density between patients with type 1 and type 2 diabetes has been found (Sumner et al. 2003). IENF density was inversely correlated with duration of type 2 diabetes and also correlated with elevation of warm thresholds (Shun et al. 2004).

Thickening of the basement membrane of endoneurial capillaries with reduced luminal area is seen and a number of other abnormalities in endoneurial microvessels have been observed (Powell et al. 1985; Timperley et al. 1985; Malik et al. 2005). There are also changes to connective tissue, such as fibrosis and extensive deposition of endoneurial collagen (Bradley et al. 2000; Malik et al. 2005).
1.4.5 Pathogenesis

The pathogenesis of diabetic neuropathy is not fully understood. It is accepted that it is a multifactorial process which depends on factors which are both genetic and acquired. Important contributing biochemical mechanisms are discussed briefly.

**Glycaemia**

The process by which hyperglycaemia exerts its effects are still not entirely clear, but three effects are thought to play a role at the cellular level; activation of the polyol pathway, non-enzymatic glycation, and generation of reactive oxygen species (ROS).

**Polyol pathway**

Increased levels of intracellular glucose in nerves leads to saturation of the normal glycolytic pathway. Extra glucose is shunted into the polyol pathway and converted to sorbitol and fructose by the enzymes aldose reductase and sorbitol dehydrogenase (Greene et al. 1999). Accumulation of sorbitol and fructose lead to reduced nerve myoinositol, decreased membrane Na⁺/K⁺ -ATPase activity, impaired axonal transport, and structural breakdown of nerves, causing abnormal action potential propagation.

**Advanced glycation end products**

The non-enzymatic reaction of excess glucose with proteins, nucleotides, and lipids results in advanced glycation end products (AGE) (Ryle et al. 1995). These products undergo irreversible cross-linking and cause a change in their structure and function, for example glycated low-density lipoprotein (LDL) is more atherogenic than non-glycated LDL. Basement membranes may become resistant to degredation leading to an increase in
thickness and stiffness, and disruption of neuronal integrity and axonal transport. Nitric Oxide may be used up by AGE products, causing the release of adverse cytokines affecting nerve cell metabolism. Resulting ischaemia causes increased activity of the diacylglycerol-protein kinase C pathway, further affecting endothelial cell permeability, vascular contractility and prostaglandin metabolism (Xia et al. 1994). In addition, activation of the polyol pathway leads to the accumulation of sorbitol which ultimately contributes to further to the formation of advanced glycation products.

**Oxidative stress**

Hyperglycaemia and AGE are able to induce the formation of reactive oxygen species (ROS) in vascular cells by a number of postulated mechanisms. The formation of ROS is thought to be the central, initiating step for the transformation of endothelial cells into an active, pro-thrombotic state (Figueroa-Romero et al. 2008). There is emerging evidence that polymorphisms of the genes for mitochondrial (SOD2) and extra-cellular (SOD3) superoxide dismutases may confer increased risk (Zotova et al. 2003).

**Neurotrophic factors**

Studies of nerve growth factors in the pathogenesis of neuropathy have been performed in animal models. There is reduction of NGF in target tissues causing hypoalgesia and retrograde axonal transport of NGF is impaired in diabetic animal models (Hellweg et al. 1990; Fernyhough et al. 1994; Ordonez et al. 1994). It was postulated that exogenous administration of neurotrophic factors may provide new treatments (Lewin et al. 1993; Brewster et al. 1994). Exogenous NGF prevented some of the pathological changes (Apfel et al. 1994). Substance P and CGRP were found to be reduced in diabetic rat sciatic nerve and dorsal root ganglia, but administration of NGF prevented this loss (Diemel et al. 1994).
Anand et al. showed that diabetic neuropathy in humans is associated with deficiency of NGF. Early length-dependent dysfunction of small sensory fibres, prior to dysfunction of sympathetic fibres, with depletion of NGF and substance P in the skin was found. NGF depletion was significantly correlated with skin axon-reflex vasodilatation. Immunostaining of diabetic skin showed reduced NGF in keratinocytes (Anand et al. 1991; Anand et al. 1994; Anand et al. 1996). Reduction of NGF in skin may relate in part to reduced keratinocyte turnover (Yajima et al. 1991) and a combination of the effects of other mechanisms relating to hyperglycaemia. Exogenous NGF reversed hypoalgesia (Petty et al. 1994).

Systemic administration of NGF was thought to bypass the need for retrograde axonal transport and allow the protein to reach the DRG directly thereby promoting survival of remaining neurones and promoting protein synthesis, repair and sensory nerve sprouting (Kessler et al. 1980; Tiercy et al. 1986; Diamond et al. 1992). This is the rationale behind clinical trials of nerve growth factor discussed later. In excess, NGF may produce hyperalgesia (Anand 1995).

Interestingly increased NGF messenger RNA (mRNA) was found in skin biopsy from the lateral calf of patients with diabetes (Diemel et al. 1999). An increase in NT-3 in diabetic skin was found in another study (Kennedy et al. 1998). This may indicate a compensatory mechanism. NT-3 has been shown to have a role in preventing neurotoxic effects of cisplatin and diabetes in animal models (Chaudhry et al. 2000).

BDNF has been shown to reverse deficits in GAP-43 mRNA in rats, and increased levels of BDNF mRNA was found in diabetic rat skeletal muscle (BDNF is also trophic to
motorneurones) (Fernyhough et al. 1994). Mice lacking BDNF or Trk B receptors showed a 30%-50% reduction in DRG cells with apparent preservation of unmyelinated nerve fibres (Snider 1994). Reduced BDNF mRNA was found in subclinical human diabetic neuropathy (Wellmer et al. 2001). Mutations of Trk A have been found in hereditary sensory and autonomic neuropathy type IV (Indo et al. 1996).

Epidermal VEGF expression was shown to be decreased in human diabetic skin and this was related to severity of neuropathy (Quattrini et al. 2008).

A number of factors combine to cause impaired peripheral nerve regeneration in diabetes: microangiopathy, inadequate metabolic support for repair, defects in the entry and actions of inflammatory cells to the site of injury, less robust support of axons by their Schwann cells, and a lack of a full repertoire of trophic factors (Kennedy et al. 2005). A defect in turnover or axonal transport in GAP-43 has been implicated in impaired peripheral nerve regeneration in diabetes (Pekiner et al. 1996). Disturbed nerve regeneration or sprouting may occur in the nerve trunk, but also in the dermis and around DRGs thereby being implicated in pain sensation. (Bradley et al. 1995).

1.4.6 Natural History

Given the complexities of diagnosis, classification and stage of neuropathy, there is still only limited information available on the natural history of DPN. In general a worsening of quantitative measures of sensory function over time has been shown.
Progression of neuropathy is related to glycaemic control and duration of diabetes in both type 1 and type 2 diabetes (1993; 1998) and markers of macrovascular disease such as hypertension, hyperlipidaemia and smoking (Tefaye et al. 2005).

Longitudinal studies have been performed to look at changes in large nerve-fibre sensory tests in patients with DPN. After diagnosis of Type 2 diabetes, slowing of peroneal nerve conduction velocity can be seen of approximately 1 m/s/year (Ziegler et al. 1988). Another study showed worsening of median nerve conduction velocity, but not peroneal conduction velocity over an average of 4.7 years in those with established diabetes (mean duration of diabetes 11 years) (Boulton et al. 1983). Electrophysiologic abnormalities in the lower limbs increased from 8% at baseline to 42% after 10 years (Partanen et al. 1995).

Changes in vibration sense have been looked at with no significant change at 1 year found in one study (Apfel et al. 1998), and 80% of patients retained a normal ‘VPT’ score (age-matched) after 12 years, with worsening in the remaining (Coppini et al. 2001). Worsening of vibration thresholds was noted over 5 years in another study, and each 1 mmol rise in fasting glucose was equated with a worsening in vibration threshold to the equivalent of adding 5 years of age in another study (Hillson et al. 1984).

Sympathetic thermoregulation and sudomotor function was studied after 4 years, but no significant change over this time was found (Sun et al. 2008).

Small fibre function was assessed in one study after an average of 3.6 years and significant worsening of heat-pain threshold and weighted pinprick threshold was seen (Benbow et al.)
The Rochester Diabetic Neuropathy Study Cohort showed a worsening of composite score (NIS, NCS and QST) over 2 years (Dyck et al. 1993).

Correction of hyperglycaemia following pancreas transplant usually shows a prevention of worsening and in some cases a small improvement in nerve conduction studies (Kennedy et al. 1990; Solders et al. 1992; Muller-Felber et al. 1993; Allen et al. 1997; Navarro et al. 1997). The improvement tended to be more pronounced when mild dysfunction was present initially (Kennedy et al. 1990), however pancreas transplant is most often performed in patients with advanced secondary complications, including diabetic neuropathy (Kennedy et al. 1995). Pancreas transplant has been shown to have an independent effect (Martinenghi et al. 1997) over and above the effect of improvement in urea level following simultaneous kidney-pancreas transplant. The longest duration of follow up was 10 years (Navarro et al. 1997), but one should bear in mind that reversal of diabetic nephropathy has been seen after 10 years (Fioretto et al. 1998), so longer follow up is needed to fully evaluate the effects of normoglycaemia where it is achieved.

Placebo groups in clinical trials of pain-relieving drugs have tended to focus on symptoms but not QST. Tesfaye discusses results from a placebo arm of a trial investigating the protein kinase inhibitor (ruboxistaurin). Interestingly the placebo group showed an improvement in symptoms (likely placebo effect), but also improvement in vibration detection threshold which has not been explained, but could be a test learning effect. NCS and heart-rate deep breathing did show worsening of function over time (Tesfaye et al. 2007). Placebo arms in intervention trials are discussed in the next section. In general patients in placebo arms of trials have shown equivocal changes or slight worsening of neuropathy.
There have only been a few studies that have investigated repeat skin biopsy as a clinical marker of progression of neuropathy or treatment response. These are discussed here:

Lauria et al. showed improvement in epidermal innervation after 18 months in one patient with diabetic truncal neuropathy after symptoms had resolved (Lauria et al. 1998). Nodera et al. published a case report in 2003 detailing the case of a 47 year old man with pain in the feet, trunk and shoulder (no evidence of diabetes on OGTT) who had a similar improvement in intra-epidermal nerve fibre density (IENF) stained with PGP 9.5 in a skin biopsy taken from the right ankle after 1 year following treatment with prednisolone. The improvement in IENF correlated with improvement in symptoms (Nodera et al. 2003).

Lauria et al. performed repeat skin punch biopsy (at a proximal and distal site) in the leg in 15 patients with painful neuropathy in their feet. Neuropathy was related to diabetes in 6 patients, one patient had HIV-associated neuropathy, one Taxol-induced neuropathy and the rest had idiopathic neuropathy. Intra-epidermal nerve fibres were stained with PGP 9.5 and IENF density was measured. The first biopsy showed reduced IENF density in patients compared with controls at the distal leg, but not thigh. Repeat biopsy an average of 19.2 months later, in the distal leg showed a reduction of IENF 8.4±4.6/mm to 4.9±3.1/mm and a reduction in thigh IENF density was seen compared with controls at this time point. There was a worsening in symptoms and sural nerve amplitude during this time (Lauria et al. 2003).

Polydefkis performed repeat skin biopsy in 19 patients with diabetes after administration of capsaicin. Neuropathy was present in 8 patients. Regeneration rates were calculated. Epidermal nerve fibre densities were reduced in the diabetic neuropathy group compared with
healthy volunteers at baseline. Repeat biopsies in patients with DPN or controls, without capsaicin administration were not performed (Polydefkis et al. 2004).

Smith et al. performed repeat skin biopsy with measurement of IENFD in 32 patients with impaired glucose tolerance (IGT) after 1 year, at distal and proximal sites in the leg. Diet and exercise advice was given as an intervention. After one-year there was a 0.3±1.1 fibre/mm improvement distally and 1.4±2.3 fibre/mm improvement proximally. The proximal change correlated with improvement in pain and sural sensory amplitude (Smith et al. 2006).

Corneal confocal microscopy was used to quantify small nerve fibre pathology in 15 patients with type 1 diabetes under-going simultaneous pancreas-kidney transplants, prior to surgery and after six months. A significant improvement in nerve fibre density was found at this time (Mehra et al. 2007).

Repeat skin biopsy was performed in 18 patients with type 1 diabetes, at baseline and after 21-40 (median 29) months post simultaneous pancreas/kidney transplant. Although baseline IENF density was reduced compared with controls without diabetes, no significant change in IENF density was seen on repeat biopsy (Boucek et al. 2008). The difference in results from the corneal confocal microscopy group was attributed to the different site of testing and perhaps patients with more severe neuropathy at baseline in this group.

In patients without diabetes there have been two studies of repeat skin biopsy. McArthur et al. performed repeat skin biopsy with recombinant nerve growth factor (rhNGF) in HIV associated sensory neuropathy. A significant reduction in pain and improved pin sensibility was found. No change in IENF density from baseline to repeat biopsy at 18 weeks was seen.
in the control group (HIV associated neuropathy) or the treatment group (McArthur et al. 2000). Gibbons et al. performed repeat skin biopsy in patients with idiopathic small fibre neuropathy (OGTT normal), at three sites; in the distal leg, distal thigh and proximal thigh, and repeated these biopsies between 11-22 months later to assess progression of neuropathy (Gibbons et al. 2006). Significant reduction in IENF was seen at each site for a sub-set of patients who were noted to have large axonal swellings.

In order for comparison, skin punch biopsy performed in controls is discussed here. Lauria et al. performed skin punch biopsy in controls and found a consistent gradient in IENF from proximal to distal sites in all subjects, but minimal effect of age was noted, until above 75 years of age (Lauria et al. 1999). No significant effect of race, sex, height or weight was noted in another study, but did show significantly higher intra-epidermal fibre density in those aged 10-19 years of age (McArthur et al. 1998). Other studies have shown a mild inverse, gender-adjusted relationship with IENF density and age with a decrease of 0.6-1.8/mm per decade (Umapathi et al. 2006) but this change was not seen in skin biopsy from a more proximal site such as the thigh (Goransson et al. 2004).

1.4.7 Treatment

Please note only agents which have been tested in clinical trials in humans are presented here.
Treatment aimed at pathogenetic mechanisms

Normoglycaemia

Several studies have shown an association with hyperglycaemia and neuropathy (Partanen et al. 1995; Dyck et al. 1999). Neuropathy is milder in patients with impaired glucose tolerance, before the development of overt diabetes (Sumner et al. 2003) and progression has been shown to slow with improved glycaemic control (1993). Intensive glycaemic control in type 1 diabetes has been associated with improvement of NCS with insulin treatment (1995) and with pancreas transplant (Navarro et al. 1997). Improvement in neuropathy symptoms in patients with type 1 diabetes was also associated with improved glycaemic control (Reichard et al. 1996).

The UK Prospective Diabetes Study (UKPDS) showed that there was a significant reduction in microvascular endpoints with improved glycaemic control in type 2 diabetes. This would imply improvement in neuropathy, but one should bear in mind that composite end-points were assessed. Actual measures were of percentage of patients with vibration score above 25V which showed improvement in the intervention arm at 15 years, however only 217 patients were available for examination compared to 3836 at baseline (1998). Other measures of rates of absent knee or ankle jerks and heart rate response to deep breathing did not differ between groups. Improvement in NCS was seen in a Japanese group of patients with type 2 diabetes, studied over 6 years (Ohkubo et al. 1995). More recently this effect has not been seen in patients with type 2 diabetes (Azad et al. 1999), but one study did show improvement in autonomic neuropathy (Gaede et al. 2003). A diet and exercise programme over one year, for patients with evidence of small-fibre neuropathy and impaired glucose tolerance led to increase in IENF, which correlated with improvement in pain and improvement in sural nerve amplitude (Smith et al. 2006).
Polyol pathway

The antioxidant A-lipoic acid (ALA) has been shown to be beneficial in alleviating symptoms (Ametov et al. 2003). The SYDNEY 2 trial showed a significant improvement in symptom score and NIS (Ziegler et al. 2006) over 5 weeks. Longer term data has just been published; the NATHAN 1 Trial. This showed that treatment over 4 years did not significantly change a composite score of NIS-LL plus 7 other neurophysiological tests, but did prevent progression of the NIS-LL score, in particular the motor component of this. There was minimal change in the placebo group over 4 years. The lack of progression of NCS over this period may relate to a statistically significant improvement in HbA1c of 0.6%. There was a significant worsening of NIS in the placebo group however (Ziegler et al. 2011). γ-LA (GLA) treatment for one year showed an improvement in electrophysiological tests (Keen et al. 1993).

A number of Aldose reductase inhibitor (ARI) trials have been performed. Minimal benefit has been seen, however the use of a potent agent (Zenarestat) which achieved >80% suppression of nerve sorbitol content appeared to improve nerve conduction velocity slowing and small myelinated nerve fiber loss in sural nerve biopsy samples, however a significant increase in serum creatinine was noted (Greene et al. 1999).

Advanced glycation products

Long-chain fatty acid metabolism accumulation was targeted with a trial of Acetyl – L carnitine, which was associated with significant improvement in pain scores in one trial, but not in another (Sima et al. 2005). Aminoguanidine inhibits the formation of AGE products, but clinical trials have focused on nephropathy.
Nerve blood flow

Significant improvement in vibration sense and sural nerve velocity was found in a study of 46 patients with type 1 diabetes who were treated with C-peptide (600 nmol/24 h, four doses s.c.) after 3 months. Proinsulin C-peptide stimulates enzymes known to be important for nerve function, Na\(^+\) K\(^+\)-ATPase and endothelial nitric oxide synthase (Ekberg et al. 2003).

A trial of a Protein kinase C inhibitor (ruboxistaurin) over 1 year in patients with diabetes and evidence of neuropathy was carried out and showed a reduction in symptoms and improvement in vibration sense in a sub-group of those with less severe neuropathy, but overall there was no difference compared with placebo (Vinik et al. 2005).

Prostaglandin analogs may be effective in increasing endoneurial blood flow (Toyota et al. 1993). ACE inhibitor treatment has been shown to improve electrophysiological measures in diabetic patients with mild neuropathy (Malik et al. 1998).

Neurotrophic factor clinical trials

In 1998, a trial of recombinant human nerve growth factor (rhNGF) in 250 patients with diabetic polyneuropathy showed a significant improvement after 6 months when compared with controls, in clinical tests of sensation, two quantitative sensory tests (cool detection threshold and heat pain) and global symptom assessment (Apfel et al. 1998). A longer term study was performed to see if the usual progression of neuropathy could be delayed or reversed. In 2000, a randomized controlled trial of rhNGF was performed over 12 months in 1019 patients with diabetic polyneuropathy. This showed an improvement in global symptom assessment and pain in legs (patient benefit questionnaire), but failed to show any
significant change in neuropathy impairment score or quantitative sensory testing (Apfel et al. 1998; Apfel et al. 2000). A lack of clinical effect was disappointing given previous success in animal models and smaller clinical trials. Various explanations were postulated; robust placebo effect, different study population, inadequate dosage, changes in formulation of rhNGF. Placebo arms in both studies did not show any change in neuropathy over the time course of the studies, although there was improvement in NIS in the first study, although not to the same extent as the treatment group, and not significantly.

Recombinant human brain-derived neurotrophic factor (rhBDNF) was trialled (Wellmer et al. 2001) in 30 patients with diabetes over 3 months. A sub-set of patients who had abnormal, but measurable cool detection thresholds at baseline, showed a significant improvement of cool detection threshold. This is possibly related to the fact that cool sensation is mediated primarily by larger Aδ fibres for which BDNF is trophic, rather than unmyelinated fibres which mediate warm/heat pain sensation. Skin biopsy was performed at the end of the study and no significant morphological changes were seen apart from those in keeping with diabetic neuropathy. BDNF is trophic to hair follicle afferents, but hair follicles were too few to comment on any change in the skin biopsies. There was no significant change in the placebo group.

VEGF is an endothelial-specific growth factor with a prominent role in angiogenesis which was trialled in 39 patients with diabetes. Improvement in pain, symptoms and area of sensory loss were reported after 6 months, but no change in objective sensory tests was found. There were no significant changes in the placebo group. The drug was not well tolerated with a number of adverse events (Ropper et al. 2009).
Neurotrophic factors have also been used in other diseases and discussed here briefly for reference. McArthur et al. performed repeat skin biopsy with recombinant nerve growth factor (rhNGF) in HIV associated sensory neuropathy. A significant reduction in pain and improved pin sensibility was found. No change in IENF density from baseline to repeat biopsy at 18 weeks was seen in the control group (HIV associated neuropathy) or the treatment group (McArthur et al. 2000). Ciliary neurotrophic factor, brain-derived neurotrophic factor and insulin-like growth factor have been tested in clinical trials for the treatment of amotrophic lateral sclerosis, with variable results (Apfel 2001).

A tolerability study of recombinant NT-3 (r-metHuNT3) was trialled in humans (Chaudhry et al. 2000) and was shown to be safe and well-tolerated. A clinical trial studied eight patients with Charcot –Marie-Tooth Type 1A, who were given r-metHuNT3 over 24 months. An improvement in NIS and nerve regeneration was seen. Regeneration was measured by an increase in the number of thinly myelinated fibres in sural nerves from these patients which formed regeneration units, clustered with fibres containing thicker myelin. Another study successfully used r-metHuNT3 to treat chronic constipation (Parkman et al. 2003). The role of r-metHuNT3 in patients with diabetes is not clear given that NT-3 was found to be increased in diabetic skin (Kennedy et al. 1998).

Others

Actovegin is a deproteinized haemoderivative produced from calf blood. Ziegler showed a reduction in symptoms, vibration perception threshold and NIS–LL over 160 days, with an improvement of HbA1c in the treatment group. Vibration perception threshold and total symptom score also improved in the placebo group, although not significantly (Ziegler et al. 2009).
Treatment aimed at symptoms

Pharmacological agents

These are efficacious and work through variable mechanisms: antidepressant drugs (Amitriptyline, Duloxetine, Venlafaxine), calcium-channel blockers (Gabapentin, Pregabalin), Glycerol trinitrite spray, Non-steroidal anti-inflammatory drugs (NSAIDs), Opioids and Capsaicin cream (Ziegler 2008).

Non-pharmacological agents

Transcutaneous electrical nerve stimulation (TENS machine) has been used in a small number of patients with success (Ziegler 2008), spinal cord stimulation has been shown to be effective in patients with severe disabling pain (Tesfaye et al. 1996). Botulinum toxin injection in feet has been trialled (Yuan et al. 2009).
1.5 Chemotherapy-induced Neuropathy

Peripheral neuropathy can result from treatment with a number of chemotherapeutic agents. Taxane, platinum based chemotherapy agents, capecitabine and bortezomib are discussed.

Paclitaxel (in combination with carboplatin) (Clark et al. 2001; 2002) is used first-line in the treatment of ovarian cancer and oxaliplatin is commonly used first-line in the treatment of colorectal cancer (de Gramont et al. 2000; Hind et al. 2008); both agents are also often used in recurrent disease, exacerbating the neurotoxic side effects (Blackledge et al. 1989). The use of these agents is increasing because of their efficacy in treating cancer; however, as a result, an increasing number of patients are living with the neuropathic side-effects of these treatments, have dose reductions in their chemotherapy, or cannot complete their full course of treatment because of neurotoxicity (Kannarkat et al. 2007). There is no effective treatment or prevention of these neurodegenerative side effects available, apart from reducing the dose of chemotherapy given, which can lessen the neurotoxic effects to some degree (Chaudhry et al. 1994).

Symptoms can range from mild numbness in toes and/or fingertips, to tingling and pain, which can be severe. Paraesthesia, hyperaesthesia, hypoaesthesia, and dyasaesthesia have all been described to varying degrees depending on the type of chemotherapy agent used and dose. There is a length-dependent symmetrical distribution of symptoms which characterizes chemotherapy-induced neuropathy (CIPN), similar to other patients who have small-fibre neuropathy, for example those with diabetes. The symptoms tend to wax and wane between each chemotherapy dose. Overall symptoms may progress and eventually may become persistent between treatments, or by the end of the course of chemotherapy treatment.
Persisting symptoms may gradually lessen over months or years, or occasionally worsen after treatment has stopped, termed ‘coasting’, and is seen particularly after oxaliplatin treatment (Grunberg et al. 1989; Lipton et al. 1989; Rowinsky et al. 1993; Hilkens et al. 1997; Quasthoff et al. 2002).

Sensory findings include loss of proprioception, vibration, touch, two-point discrimination, sharp/dull discrimination, temperature sensation, and are typically diminished in the stocking-glove distribution in symptomatic patients.

1.5.1 Diagnosis and staging

Diagnosis of chemotherapy-induced neuropathy is made when there is a temporal association with a chemotherapeutic agent, and after the exclusion of other potential causes of neuropathy.

There are several factors which influence the development of CIPN. Dosing regimens and cumulative dose are one of the most important factors (Hilkens et al. 1997). Others include concurrent neurotoxic chemotherapy (Chaudhry et al. 1994), previous treatment with chemotherapy and pre-existing neuropathy, for example from diabetes, or excess alcohol intake (Wiernik et al. 1987; Lipton et al. 1989; Chaudhry et al. 1994; Chaudhry et al. 2003).

Symptoms, clinical examination findings and nerve conduction studies are traditionally used for assessment. Several toxicity grading scales are also used to provide accurate and reliable reporting. The most well known is the National Cancer Institute- Common Toxicity Criteria.
(NCIC-CTC), which was updated in 1999 (Oken et al. 1982). The neurotoxicity scale is outlined in Table 1.8

<table>
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<th>Neuropathy</th>
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<tbody>
<tr>
<td>Sensory</td>
<td>Normal</td>
<td>Loss of deep tendon reflexes or paresthesia, but not interfering with function</td>
<td>Objective sensory loss or paresthesia interfering with function, but not interfering with activities of daily living</td>
<td>Sensory loss or paresthesia interfering with activities of daily living</td>
<td>Permanent sensory loss that interferes with function</td>
</tr>
<tr>
<td>Motor</td>
<td>Normal</td>
<td>Subjective weakness, but no objective findings</td>
<td>Mild objective weakness interfering with function, but not interfering with activities of daily living</td>
<td>Objective weakness interfering with activities of daily living</td>
<td>Paralysis</td>
</tr>
</tbody>
</table>

Table 1.8 NCIC-CTC grading scale for chemotherapy-induced neuropathy

The chemotherapy-induced peripheral neuropathy outcome measure study (CI-PERINOMS) was proposed in 2009 as a prospective trial to assess and monitor CIPN (2009). A number of scales and questionnaires are being evaluated; Total neuropathy Score, visual analogue scale (VAS), 11-point pain intensity numerical scale (PI-NRS), calibrated overall disability sum score (C-ODSS) and quality of life questionnaire (QLQ-CIPN20). Nerve conduction studies seem to be the most widely performed objective test in assessing CIPN. In general, a reduction in sensory potential amplitude is seen (Argyriou et al. 2005). The potential role of clinical and electrophysiological tests in predicting the final outcome of CIPN in 46 patients receiving cisplatin and paclitaxel was studied (Argyriou et al. 2005). A reduction in sural nerve amplitude >50% of baseline was a predictor of worse neurological outcome.

Quantitative sensory testing (QST) is increasingly being used to further assess neuropathy in patients with cancer and those receiving chemotherapy. QST findings are discussed below in relation to different chemotherapeutic agents. Lipton et al. found abnormal vibration sense
present in 12% of patients with cancer, compared with 1.7% of healthy subjects, and abnormality was present even accounting for age, coexisting diabetes and/or renal disease, poor nutrition or chemotherapy treatment, and this should be borne in mind when considering chemotherapy-induced neuropathy (Lipton et al. 1987).

1.5.2 Clinical presentation and pathogenesis

The underlying mechanisms of CIPN have been partially described and relate to the effect of neurotoxic agents in the peripheral nervous system, given that these agents cannot cross the blood-brain barrier (Gregg et al. 1992). Although clinical presentation may vary slightly between the chemotherapeutic agents, mechanisms of pathogenesis are thought to be similar, with the exception of oxaliplatin, which will be considered separately.

Mechanisms postulated are disruption of axoplasmic microtubule mediated transport, distal axon (Wallerian) degeneration and direct damage to the sensory dorsal root ganglia (Masurovsky et al. 1983; Hilkens et al. 1997; Fazio et al. 1999; McKeage et al. 2001).

Taxanes

Taxanes are organic compounds derived from plants of the genus Taxus (yews). Taxanes include paclitaxel (Taxol®, Bristol Myers-Squibb) and docetaxel (Taxotere®, Sanofi-Aventis). Paclitaxel is a chemotherapeutic agent derived from rare Pacific Yew tree bark (Taxus brevifolia) (Wani et al. 1971). It was discovered in 1963 as part of an American National Cancer Institute program in which thousands of plants were screened for anticancer activity. It is an effective and commonly used anti-neoplastic drug for the treatment of ovarian, breast and lung carcinomas. Docetaxel is a newer semi-synthetic analogue that is
derived from more common European Yew tree needles (*Taxus baccata*) (Chevallier et al. 1995). Docetaxel is used as first line therapy for breast cancer and a number of other solid tumours (Pronk et al. 1995). Three main toxic side-effects were noted during initial trials of taxanes; hypersensitivity reactions, neutropenia and peripheral neurotoxicity (Rowinsky et al. 1993). Pre-medication and granulocyte colony-stimulating factor (GCSF) treatment effectively counteract the side-effects of hypersensitivity and neutropenia respectively, therefore in most patients, neurotoxicity is the significant dose-limiting side-effect (Rowinsky et al. 1993; Wasserheit et al. 1996).

Neurotoxic effects are progressive and usually start as a sensory neuropathy with dysasthesia (numbness and tingling) in a glove and stocking distribution, associated with neuropathic pain, which can be severe (Rowinsky et al. 1993). As neuropathy progresses, loss of vibration sense and axon reflexes are seen (Hilkens et al. 1997), even in asymptomatic patients, leading to the conclusion that taxanes affect all modalities, and can affect myelinated nerve fibres (New et al. 1996), even preferentially (Dougherty et al. 2004). Onset of symptoms is usually within 24-72 hours. Most cases resolve within months after stopping treatment, but can become chronic (Rowinsky et al. 1993; Chaudhry et al. 1994). Occasionally symptoms are seen to transiently worsen after treatment has been stopped (New et al. 1996).

Incidence of CIPN following paclitaxel standard monotherapy (250mg/m²) ranges between 22% - 100%, but varies with single dose intensity, duration of treatment, cumulative dose, prior or concurrent treatment with cisplatin, co-existing conditions such as diabetes and alcohol abuse, and pre-existing neuropathy (Wiernik et al. 1987; Lipton et al. 1989; Chaudhry...
et al. 1994). Cumulative doses >1000mg/m² are invariably associated with the development of neuropathy (Chaudhry et al. 1994).

Paclitaxel acts as an anti-cancer agent by causing mitotic cell arrest via polymerization of tubulin (Schiff et al. 1979). Microtubules formed from tubulin are vital, for the correct functioning of the neuronal growth cone. In animal models paclitaxel has been shown to inhibit neuronal growth and cause structural damage to neurons and Schwann cells (Yamada et al. 1971). Nerve fibre loss, axonal atrophy and secondary demyelination have been seen in human sural nerve biopsies, indicating a ganglionopathy rather than axonopathy or demyelination as the most likely pathogenesis of neuropathy (Sahenk et al. 1994). Following docetaxel, axonal neuropathy with a preferential loss of large myelinated-fibres was found in sural biopsy of one patient. There was also evidence of considerable nerve fibre regeneration (Fazio et al. 1999).

An acute effect of paclitaxel has also been demonstrated in-vitro and in-vivo. Paclitaxel induced an acute membrane depolarisation via an axonal membrane leak and a non-specific ion influx (Schilling et al. 1997) which may explain some of the acute effects of infusion.

At the time of peak pain severity, rats were found to show heat-hyperalgesia, mechano-alldynia, mechano-hyperalgesia and cold-alldynia, but with no effect on motor performance. No axonal degeneration was seen in this study, but endoneurial oedema was observed (Polomano et al. 2001). Similar findings in rats were seen in a study by Flatters and Bennett, but in addition to endoneurial oedema in C-fibres, a significant number of swollen and vacuolated mitochondria were also seen in these fibres. These changes resolved in a
comparable manner to the pain behaviour, which may indicate a causative role (Flatters et al. 2006).

Intra-or sub-epidermal nerve fibres have not been investigated in human CIPN. In one study intra-epidermal nerve fibres were assessed in the rat following administration of paclitaxel. At the time of peak pain severity rats showed a decrease of PGP 9.5 IENF in the hind paw glaborous skin, but an increase in Langerhans cells, which are known to be increased following nerve transaction and associated with inflammatory, immune-mediated responses. The relevance of this increase is unclear (Siau et al. 2006).

Cremophor is a polyoxyehylated castor oil vehicle (also in cyclosporine preparations) that is used to keep lipophlic paclitaxel in solution for i.v. administration. It was found to be neurotoxic in itself (causing axonal swelling, vesicular degeneration, and demyelination in vivo) and now cremophor-free taxol preparations are being trialled; however, these still show significant dose-limiting neurotoxicity, indicating effects from paclitaxel itself (Windebank et al. 1994; Gelderblom et al. 2001; Kim et al. 2004; Kim et al. 2011).

Platinum compounds
Platinum compounds were first associated with anti-mitotic features over 40 years ago (Rosenberg et al. 1965). Cisplatin and later carboplatin were developed and found to be very effective agents in a number of solid tumors (Windebank et al. 2008). Initially nephrotoxicity and gastro-intestinal disturbances were the main dose-limiting effects, however hydration schedules and careful monitoring of renal function have minimised these effects. Neurotoxicity is now the main dose-limiting feature (Windebank et al. 2008).
Reported incidence of CIPN for cisplatin ranges between 49%-100%, and for carboplatin from 13%-43% depending on dosing schedule and combination of other drugs administered (Gregg et al. 1992). Significant neurotoxicity is apparent at doses greater than 400-500 mg/m² (Thompson et al. 1984). A length-dependent sensory neuropathy with symptoms of paraesthesia and numbness is found in most patients one month after starting treatment with cisplatin or carboplatin, with diminished vibration perception and proprioception, leading to ataxia in advanced cases. L’hermitte’s sign is also described and thought to be due to demyelination of the dorsal roots and columns (Siegal et al. 1990; LoMonaco et al. 1992).

There is evidence of worsening of neuropathy 2.5-5.5 months after cessation of cisplatin (Siegal et al. 1990), termed coasting, and may relate to accumulation of the drug in the dorsal root ganglion, but is still not fully understood (Grunberg et al. 1989).

Anti-tumor effects of platinum compounds arise as a result of binding to DNA (DNA alkylating agents), forming intra- and inter-strand crosslinks which cause apoptotic cell death in rapidly dividing cell lines and cancer cells (Huang et al. 1995). Platinum agents have been found to have high affinity for the peripheral nervous system with accumulation in DRGs, up to 10-fold higher concentrations than found in the brain (Thompson et al. 1984). This accumulation in DRGs and sensory nerves causes shrinking of the nuclear and cytoplasmic compartments, likely causing neuropathy via disturbance of cellular metabolism and axonal transport (Gregg et al. 1992). Platinum-induced neuropathy is thought to be a primary neuronopathy given the accumulation in the dorsa root ganglia, rather than an axonopathy. Nerve conduction studies have shown amplitude reduction and slowing of the sural nerve action potential, indicating axonal loss and demyelination (Walsh et al. 1982).
Acutely, cisplatin has been found to disturb electrolyte homeostatic mechanisms in cultured dorsal root ganglion neurones, causing a reduction in voltage activated potassium currents by 50% and calcium currents by 60% (Scott et al. 1995). Sural nerve biopsy has shown degeneration of large myelinated axons with signs of segmental demyelination and remyelination (Roelofs et al. 1984; Thompson et al. 1984).

Cavaletti et al. found a highly significant correlation between a decrease in circulating NGF levels and severity of CIPN, as judged by the total neuropathy score (TNS) in 129 patients treated with cisplatin and paclitaxel chemotherapy in combination (Cavaletti et al. 2004). Circulating NGF levels were not predictive of the final neurological outcome however. This study shows a link between NGF and CIPN which may be important in pathogenesis.

**Oxaliplatin**

Oxaliplatin is a third generation platinum based chemotherapy agent and is mainly used in the treatment of colorectal cancer, but also pancreatic and gastric malignancies (Becouarn et al. 2001). It works in a similar way to cisplatin, causing cross-binding of DNA, but has less nephrotoxic and haematological side effects than cisplatin (Extra et al. 1998; Wiseman et al. 1999). Neurotoxicity is more frequent and can produce neurotoxicity with a concentration in nervous tissue far lower than those of other platinum preparations (Screnci et al. 2000). Platinum accumulation in the DRG is associated with axonal loss with selective atrophy of DRG cells, indicating a primary neuronopathy (Cavaletti et al. 2001).

Infusion time and cumulative dose are important, with almost every patient who receives >540mg/m² developing neuropathy. Sensory symptoms, particularly mechanical and heat and cold allodynia, can become apparent from 30-60 minutes after oxaliplatin infusion (Extra
et al. 1998; Cersosimo 2005; Joseph et al. 2008). Other symptoms include muscle cramps and fasciculation, indicating motor nerves are affected in the acute syndrome (Cersosimo 2005). These acute symptoms tend to disappear within a few days and reappear after each subsequent dose of chemotherapy. A sensory length-dependent neuropathy can subsequently develop (Argyriou et al. 2007). In one study 20% of patients were left with residual symptoms following cessation of chemotherapy. After 6 to 8 months neuropathy had completely resolved in 40% (Extra et al. 1998). Another study showed that a dose-reduction because of neuropathic side-effects was needed in 40% of patients undergoing treatment with oxaliplatin for treatment of colorectal cancer (Park et al. 2009).

Patients receiving oxaliplatin chemotherapy had QST measured at baseline, and after 3, 6 and 9 cycles of chemotherapy. Hyperalgesia to cold in the upper limb was an early marker of oxaliplatin neurotoxicity. There was a significant worsening of vibration perception threshold over the four visits and heat pain threshold was lowered, whereas cold pain threshold increased (Attal et al. 2009). Nerve conduction studies have shown reduced sural nerve amplitudes (Krishnan et al. 2005).

The mechanism of the acute neuropathy seems to be interference of axonal ion conductance, altering neuronal excitability. One study showed that these acute effects of oxaliplatin were not prevented when voltage-gated potassium channels were blocked, indicating that it is likely voltage-gated sodium channels are likely to be the ones affected (Adelsberger et al. 2000). Another study showed that this effect on voltage-gated sodium channels involves calcium ions (Grolleau et al. 2001). Joseph et al. showed that oxaliplatin acts on IB4-positive, GDNF-dependent nociceptors which have their own repertoire of voltage-gated sodium channels (Joseph et al. 2008). Given the propensity of oxaliplatin to produce cold
hyperalgesia, it is postulated that oxaliplatin modulates or binds to TRPM8 and/or TRPA1 receptors to cause sensitisation and pain, in a similar way to the menthol model of cold hyperalgesia (Wasner et al. 2004). It has been postulated that prolonged voltage-gated sodium channel activation may induce cellular stress, and along with the reduction in cellular metabolism disruption caused by accumulation of oxaliplatin in the DRG, may contribute to the development of the chronic form of sensory neuropathy (Grothey 2003).

Oxaliplatin-induced neuropathy is predicted by certain gene polymorphisms relating to glutathione s-transferase enzyme function (Lecomte et al. 2006).

**Capecitabine**

Capecitabine is an oral prodrug formulation of 5-fluorouracil, and is used to replace the intravenous formulation in colon, lung, pancreas, head and neck, and breast cancers. It is rarely thought to cause neuropathy, but has been associated in some cases. The mechanism of neuropathy is not clear (Saif et al. 2004; Videnovic et al. 2005).

**Bortezomib**

Bortezomib is a proteasome inhibitor and is used extensively for the treatment of multiple myeloma, which in itself may cause a peripheral neuropathy. In one study 83% of patients were found to have pre-existing neuropathy prior to Bortezomib treatment, with 35% of these patients having worse symptoms after treatment. Neuropathy was found to be reversible in most cases after dose-reduction or discontinuation of treatment (Richardson et al. 2006).
Pathological changes in rats have been studied. Vacuolisation of Schwann cells, found to be due to damaged mitochondria and primary degeneration of myelin sheaths have been observed (Cavaletti et al. 2007).

1.5.3 Treatment

*Treatment aimed at pathogenetic mechanisms*

Altering the way chemotherapy is given in terms of dose, frequency, duration and cumulative dose has an impact on the development of the neurotoxic side effects, and numerous studies have been conducted with varying regimes to find the best approach. The ‘Stop-and-Go’ method was proposed in order to try and maximize the cumulative chemotherapy dose that can be given before the neurotoxic threshold is reached. The aim is to stop chemotherapy when sensory neurotoxicity of a certain grade has developed or a predefined dose has been reached, and to restart chemotherapy when neurotoxicity has regressed or if there is concern about tumor progression (Grothey 2003; Petrioli et al. 2006).

The effect of oxaliplatin on sodium channels was shown to be antagonized by carbamazepine, an antiepileptic that blocks sodium-channels in an animal model (Adelsberger et al. 2000), but has shown mixed results in clinical trials (Eckel et al. 2002; Wilson et al. 2002). Other antiepileptic drugs have been trialled; gabapentin showed mixed results (Grothey 2003; Mitchell et al. 2006), but pregabalin did show an effect in a case report (Saif et al. 2008).

Calcium and magnesium infusions were shown to reduce the frequency and severity of acute symptoms and are now used in some centres when administering oxaliplatin (Grothey et al. 2011). This ability is thought to be due to their oxalate chelating abilities. Oxalate is a
metabolite of oxaliplatin that is thought to be responsible for the sodium-channel disturbance associated with oxaliplatin-induced neuropathy (Gamelin et al. 2004).

Amifostine is an organic thiophosphate pro-drug that has strong free radical scavenging and antioxidative abilities that are thought to counteract the production of free radicals, including those produced as a result of the action of oxaliplatin within the cell. Clinical trials in humans have shown some benefit in treating acute neuropathy (Penz et al. 2001; Lu et al. 2008). Glutathione is a nonessential amino acid that has antioxidant properties and has similarly been shown to be effective in reducing the severity and frequency of oxaliplatin induced neuropathy. After 12 cycles, grade 2 to 4 neurotoxicity was observed in three patients in those treated with glutathione and oxaliplatin and in eight patients treated with oxaliplatin alone (p =0.004). Sural nerve conduction amplitudes and velocity showed a statistically significant reduction in the oxaliplatin arm but not in the glutathione and oxaliplatin arm (Cascinu et al. 2002). Such improvements in electrophysiology were not seen in another study of glutathione and oxaliplatin, but significant symptomatic improvement was seen (Wang et al. 2007). Other anti-oxidants have also been tested and shown to have beneficial effects in terms of attenuating the acute neuropathy; alpha lipoic acid (Gedlicka et al. 2002) and vitamin E (Pace et al. 2003). Acetyl-L-carnitine has been shown to have neuroprotective effects in animal models and small clinical trials (Bianchi et al. 2005; Flatters et al. 2006). It has been postulated that these supplemental antioxidants might interfere with the oxidative breakdown of cellular DNA and cell membranes necessary for the cytotoxic chemotherapy agent to work, and therefore need to be evaluated further before routine use is recommended (Wolf et al. 2008).
Xaliproden is a nonpeptide, 5-HT$_{1A}$ receptor agonist that has been shown to mimic or enhance the effects of neurotrophins in animal models (Fournier et al. 1998; Labie et al. 1999). In clinical trials, this compound was found to lessen the frequency of severe neuropathy (Grade 3 neuropathy) when administered along with oxaliplatin, but Grade 1 or 2 neuropathy, number of cycles and cumulative dose tolerated did not alter (Gibson et al. 2006).

Several studies have been performed to investigate the role of neurotrophic factors in chemotherapy-induced neuropathy (Apfel et al. 1991). NGF, which is necessary for the development of the dorsal root ganglia and the maintenance of normal ganglion function in adults, has been shown to have a protective effect in paclitaxel- and cisplatin-induced neuropathy in animal models (Apfel et al. 1992; Windebank et al. 1994). Recombinant NGF was shown to be protective in rats treated with cisplatin (Tredici et al. 1999) and also restored depleted levels of CGRP in taxol-treated mice (Schmidt et al. 1995). Neurotrophins 3, 4 and 5 have also been studied and shown to prevent peripheral neuropathy and spiral ganglion neuron damage, which is important for hearing, during treatment with cisplatin (Gao et al. 1995; Zheng et al. 1995). Recombinant human glial growth factor 2 has also been studied and shown to have neuroprotective effects in the rat (ter Laak et al. 2000).

In a rat model, gene transfer of vasoactive endothelial growth factor (VEGF) resulted in improvement of electrophysiological measures of neuropathy in sciatic nerve. It was hypothesized that neuropathic side effects resulted from damage to the vasa vasorum and restoring VEGF would help (Kirchmair et al. 2005). A protective effect of herpes simplex virus-mediated nerve growth factor and neurotrophin-3 gene transfer in cisplatin-induced neuropathy was found in another animal model (Chattopadhyay et al. 2004). Leukaemia
inhibitory factor (LIF) restored the cisplatin-induced decrease in the number of migrating cells and length of axonal outgrowth in DRG cultures (Ozturk et al. 2005). However a trial of recombinant LIF did not prevent neuropathy in a clinical trial (Davis et al. 2005).

An adrenocorticotropic hormone (ACTH) analogue (Org 2766), prevented cisplatin-induced reduction of sensory nerve conduction velocities in rat models. A clinical study also showed a benefit (van der Hoop et al. 1990), but a later study did not (van Gerven et al. 1994).

Treatment with dihydroprogesterone and progesterone in a rat model protected against docetaxol-induced neuropathy, and Roglio et al. proposed further investigation of neuroactive steroids in this area (Roglio et al. 2009).

Minocycline, an inhibitor of macrophage activation, was used in rats to prevent paclitaxel-evoked allodynia and reduction of IENF loss, possibly by slowing Wallerian degeneration (Liu et al. 2010).

*Treatment aimed at symptoms*

A number of analgesic agents have been found to be efficacious; antidepressant drugs (Amitriptyline, Nortriptyline), anticonvulsants (Gabapentin, Lamotrogine) and opiates are used (Wolf et al. 2008).
2.0 Aims

The development of peripheral neuropathy leads to significant morbidity in patients with diabetic polyneuropathy (DPN) and patients with chemotherapy-induced neuropathy (CIPN). While a number of studies have investigated the risk factors associated with diabetic polyneuropathy and the underlying pathological changes, there are no interventions currently available to reverse or halt the development of the condition, apart from improving glycaemic control, which is effective to only a small degree. Clinical trials with neuroprotective agents including neurotrophic factors have failed to show meaningful efficacy, perhaps because more sensitive and robust objective markers are needed to detect changes produced by the relatively short duration of clinical trials. A number of factors may explain this, including lack of suitable biomarkers.

Peripheral neuropathy resulting from treatment with taxane and platinum based chemotherapy agents is increasingly more common because of their efficacy in treating cancer, however as a result an increasing number of patients are living with the neuropathic side-effects of these treatments, have dose reductions in their chemotherapy, or cannot complete their full course of treatment because of neurotoxicity. The symptoms are similar to other patients who have small-fibre neuropathy, for example those with diabetes.

Small unmyelinated nerve fibres in the skin can show the earliest signs of damage and regeneration in patients with diabetes and impaired glucose tolerance. These nerve fibres are therefore potentially good biomarker candidates, particularly as their regulatory neurotrophic growth factors are available for clinical trials.
The aim of this study in patients with DPN and CIPN was to assess and measure changes in novel sensory tests and markers in skin biopsies, in order to find a biomarker for use in future clinical trials.

**Objective**

The primary objective of this study was to examine changes in novel morphological and functional measures of peripheral sensory nerve function in 40 patients with diabetic polyneuropathy and 40 patients with chemotherapy-induced neuropathy, which could potentially serve as biomarkers of drug response in future clinical trials.
3.0 Methods

3.1 Ethical approval

Written informed consent was obtained from all patients and approval from the Charing Cross Research Ethics Committee, London, was gained (ref. 07/H0706/61).

3.2 Assessment of neuropathy

A number of assessments were carried out at each study visit; over one year in the DPN group and over six months in the CIPN group; clinical history and questionnaires were undertaken, general and neurological examination was conducted, and sensory tests were performed in both lower limbs. The right lower limb values were used for the analyses presented. Please see Appendix A, B and C for illustrations of the questionnaires and equipment used in this study.

The following assessments and tests were carried out at each study visit unless otherwise stated:

Clinical symptoms and pain scores
Symptoms were recorded using the short-form McGill Pain Questionnaire (Melzack 1987) (maximum score 55, indicating severe symptoms) and by recording a numerical pain score (Likert pain score 0 = no pain and 10 = maximum pain).
Neurological deficits were recorded using the Neuropathy Impairment Score Lower Limbs (NIS-LL) which is a summed score of muscle power, reflex sensory loss (maximum score 88 indicating severe neuropathy) (Bril 1999).
**Quantitative Sensory Testing (QST)**

Thresholds for light touch were measured using Semmes-Weinstein hairs (made by A. Ainsworth, University College London, UK) No.1 (0.0174 g) to No.20 (263.0 g). The number of the hair with the lowest force reliably detected by the patient after 3 applications on the dorsum of the toe was recorded. Values >No. 3 monofilament (0.0479 g) were considered abnormal (Atherton et al. 2007).

Vibration perception thresholds were measured using a biothesiometer (Biomedical Instrument Co., Newbury, OH, USA) placed on the metatarsophalangeal joint of the big toe. Three ascending and three descending trials were carried out, and the mean value obtained. Values >12 V were considered abnormal based on previous work by Coppini et al. and Peripheral Neuropathy Unit internal controls (Coppini et al. 2001).

Thermal perception thresholds were performed as previously described (Anand et al. 1996; Wellmer et al. 2001) using the TSA II – NeuroSensory Analyzer (Medoc Ltd., Israel). A 30 × 30 mm thermode was used and thermal thresholds determined in the soles of the feet (under the instep) for warm perception, cool perception, heat pain and cold pain from a baseline temperature of 32°C, with a change in temperature of 1°C/sec. The mean of three consecutive tests for each modality was recorded. Values >6.4°C for warm sensation, >2.3°C for cool sensation and >10.4 °C for heat pain, were considered abnormal (Anand et al. 1996; Wellmer et al. 2001; Atherton et al. 2007).

**Histamine Skin Flare**

Intra-dermal injection of Histamine (0.03 ml of 1 mg/ml Martindale Pharmaceuticals, Romford, UK) was given into the lateral calf of patients as described previously (Anand et al.
1996), and the area of flare measured after 10 minutes by scanning laser Doppler imaging (Moor Instruments Ltd, Axminster UK) (Krishnan et al. 2004). Abnormal skin flare was taken to be \(<14.9 \text{ cm}^2\) based on unpublished Peripheral Neuropathy Unit control data.

**Sweat rate**

Sweat rates were measured as an indication of autonomic function on the sole of the foot using an Evaporimeter EPI (Servo Med AB, Sweden). Values \(<10 \text{ g/m}^2/\text{h}\) were considered abnormally low and \(>30 \text{ g/m}^2/\text{h}\) considered abnormally high, based on unpublished Peripheral Neuropathy Unit control data.

**Nerve Conduction Studies**

Nerve conduction studies (NCS) of the common peroneal (including F-wave studies) and sural nerves in the right leg were performed by the same experienced consultant neurophysiologist on a Medtronic Keypoint EMG machine. If the sural nerve was not found by this examiner it was presumed to be absent, rather than not found. NCS was performed once at the start of the study. Sural antidromic sensory action potential values of less than 5 \(\mu\text{V}\) amplitude and 40 m/s conduction velocity were considered abnormal, and common peroneal nerve (compound muscle action potential from extensor digitorum brevis) values less than 3 mV amplitude and 40 m/s conduction velocity were considered abnormal (Atherton et al. 2007). F–wave latency greater then 60 ms were considered abnormal in patients measuring \(<185 \text{ cm}\).

**Contact Heat Evoked Potentials**

A Contact Heat Evoked Potential Stimulator (Medoc Ltd, Ramat Yishai, Israel) was used as described previously (Atherton et al. 2007). The baseline temperature was 32°C, destination
temperature 51°C, and stimulus interval 7 seconds. Responses from 10 stimuli were recorded from each patient with the thermode placed over 3 sites (lateral aspect of calf, volar forearm and cheek). Evoked potentials were recorded from six midline electrodes (Fz, FCz, Cz, CPz, Pz, POz) (Granovsky et al. 2005; Atherton et al. 2007). Data was reported from the vertex (FCz) position for averaged Aδ potentials. Latency was measured from the first definitive negative peak (N2), and the amplitude measured peak-to-peak (N2 to P2). Please see Appendix C for illustration. N2 to P2 amplitude was considered abnormal if <14.7 µV at the leg, <18.1 µV at the arm and <23.4 µV at the face. When responses could not be recorded, patients were given a nominal value of 0.1µV for statistical analysis.

Calf skin biopsy and Immunohistology

Two 3 mm diameter skin punch biopsies were collected at first and third visits. Biopsies were taken under local anaesthesia from the lateral calf. Please see Appendix C for illustration of site used. Control calf skin biopsies (from healthy volunteers or patients undergoing elective sural nerve harvesting for upper limb nerve grafts) were studied with informed consent and Ethics approval, alongside the diabetic patient biopsies (n = 11 for PGP9.5, n=17 for GAP-43, n = 13 for TRPV1; controls mean age 43 years). The immunohistological methods and antibodies used here have been described previously (Facer et al. 1998; Facer et al. 2000; Gopinath et al. 2005; Ragé et al.). Briefly, one of the two skin biopsies was snap frozen and stored at -70°C for TRPV1, whilst the other biopsy was fixed (modified Zamboni's fluid – 2% w/v formalin; 0.01 M phosphate buffer; 15% saturated picric acid; pH 7.2) before snap freezing for PGP 9.5 and GAP-43. Frozen sections (15 µm for PGP9.5 and TRPV1, 30µm for GAP-43 to increase the sensitivity of this marker in the epidermis) were collected onto glass slides, and unfixed sections post-fixed in 4% w/v paraformaldehyde in 0.15M phosphate buffered saline (PBS) for 30 minutes. Endogenous
peroxidase was blocked by incubation in industrial methylated spirit containing 0.3% w/v hydrogen peroxide for 30 minutes. Sections were incubated overnight with primary antibodies to PGP 9.5 (1:40,000, Ultraclone Ltd, Isle of Wight, UK), TRPV1 (1:8,000; GlaxoSmithKline, Stevenage, Herts., UK) or GAP-43 (1:80,000, Sigma-Aldrich Co., Dorset, UK). Sites of primary antibody attachment were revealed using nickel-enhanced, avidin-biotin peroxidase (ABC - Vector Laboratories, Peterborough, UK). Omission of primary antibodies and sequential dilution of antibodies gave appropriate results for specificity. Tissue sections were counter-stained for nuclei in 0.1% w/v aqueous neutral red.

Intra-epidermal nerve fibres (IENF) were counted along the length of four non-consecutive sections. The length of epithelium in each counted section was measured using computerised microscopy software (Olympus ANALYSIS 5.0 Soft, Olympus UK Ltd., Southend, Essex, UK) and results expressed as fibres/mm length of section. Sub-epidermal fibres (SENF) were measured by image analysis where digital photomicrographs were captured via video link to an Olympus BX50 microscope. The grey-shade detection threshold was set at a constant level to allow detection of positive immunostaining and the area of highlighted immunoreactivity obtained as a percentage (% area) of the field scanned. Images were captured (x40 objective magnification) along the entire length of section and the mean values used for statistical analysis. Quantification was performed by two independent blinded observers and there was no significant difference between observers.

3.3 Patients

Patients were recruited from neurology, oncology and diabetes clinics at Charing Cross and Hammersmith Hospitals, Imperial College Healthcare NHS Trust. Male or female participants aged 18-80 years were eligible.
3.3.1 DPN group

In total, 43 patients were recruited, 3 of whom were subsequently excluded as quantitative tests showed that a cause other than diabetes was likely and one patient had no objective signs of neuropathy found in quantitative sensory tests or skin biopsy.

Patients (n=40) with either type 1 or type 2 diabetes mellitus and a history of symptomatic distal symmetric sensory polyneuropathy (affecting lower limbs) for at least 6 months, confirmed on clinical examination and/or sensory testing, were studied. Patient demographics and characteristics are shown in Table 3.1.

<table>
<thead>
<tr>
<th>DPN group patients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>40</td>
</tr>
<tr>
<td>Gender</td>
<td>26 male      14 female</td>
</tr>
<tr>
<td>Type of diabetes</td>
<td>11 Type 1   29 Type 2</td>
</tr>
<tr>
<td>Age</td>
<td>57 (36-76)</td>
</tr>
<tr>
<td>Duration of Diabetes</td>
<td>17.4 (0.5-56.0)</td>
</tr>
<tr>
<td>Duration of symptoms</td>
<td>4.0 (0.5-15.0)</td>
</tr>
<tr>
<td>HbA1c %/mmol/mol (at first visit)</td>
<td>7.9 (5.5-11.0)/63 (37-97)</td>
</tr>
<tr>
<td>HbA1c %/mmol/mol (at final visit)</td>
<td>8.0 (5.9-12.7)/64 (41-115)</td>
</tr>
</tbody>
</table>

Table 3.1 DPN group: patient demographics.

All patients fulfilled criteria for neuropathy outlined by Dyck (Dyck 2003). Study assessments were performed at 0, 3, 6 and 12 months. Skin punch biopsy was performed in the lateral calf, at the first and third study visits. Table 3.2 shows the number of patients who attended for each visit.
Out of the 40 patients who were recruited, all four visits were attended by 22 patients. Missed appointments and drop outs were due to intercurrent illness, patients reporting lack of time, holidays and a death (from cardiovascular disease). Analysis of data from the patients who withdrew from the study did not show a significant difference from the rest of the group. Three patients who attended the third visit declined to have a repeat skin biopsy because of dislike of the procedure. All patients had uncomplicated healing of the skin biopsy site.

**Clinical symptoms and pain scores**

Patients described a number of symptoms in their lower limbs, most commonly numbness, pins and needles, tingling and burning pain. Out of 40 patients, 34 reported pain in their feet, and 17 patients were taking treatment for neuropathic pain at the start of the study (Gabapentin, Pregabalin, Amitriptyline, Tramadol, Oxycontin or a combination of these). If required, patients were commenced on analgesia during the study as part of their care (n=7), and others had optimization of analgesia. There were no overall differences in test results in the group taking analgesia treatment. The mean ± SEM Likert pain score at the first visit was 5.3 ± 0.5. Out of 40 patients, 29 scored 4 or above, 4 scored 10 and 6 scored zero. The mean ± SEM McGill pain questionnaire score at the first visit was 17.1 ± 1.9. There was no significant change in pain scores over the year (Figure 3.1).

---

### Table 3.2 DPN group: number of patients tested at each visit.

<table>
<thead>
<tr>
<th>Visit</th>
<th>Number of Patients tested</th>
<th>Number of patients biopsied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit 1 (baseline)</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Visit 2 (3 months)</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Visit 3 (6 months)</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>Visit 4 (12 months)</td>
<td>29</td>
<td>-</td>
</tr>
</tbody>
</table>

---

82
Figure 3.1 DPN group: Mean ± SEM at each visit for a) Likert Pain Score, b) McGill Pain Score (maximum score 55). Results shown are of the 22 patients who attended all 4 visits.

Clinical Examination

Clinical examination and tests confirmed that patients had a predominantly sensory, length-dependent neuropathy. Distal weakness (lower limbs) was present in 15/40 patients at Medical Research Council (MRC) grade 4 out of 5 or less. Neurological deficits were recorded using the Neuropathy Impairment Score Lower Limbs (NIS-LL) (Bril 1999) which is a summed score of muscle power, reflex loss and sensation loss (maximum score 88, indicating severe neuropathy). The mean ± SEM Neuropathy Impairment Score (LL) at the first visit was 12.9 ± 1.6. There was no significant change in score over the year (Figure 3.2). All patients had palpable peripheral pulses at the first visit.
3.3.2 CIPN group

In total, 40 patients were recruited consisting of two groups of patients. One group of 33 patients had a diagnosis of chemotherapy-induced peripheral neuropathy (National Cancer Institute of Canada Common Toxicity Criteria, NCIC-CTC, grade 1–4) and the other group consisted of 7 patients who were chemotherapy naïve and had no evidence of neuropathy prior to the study. There was poor recruitment and significant loss of follow up in the chemotherapy-naïve group mainly because patients having started chemotherapy did not feel well enough or did not want to spend the time to attend the extra visits; therefore this data is not presented here.

Demographics of patients with CIPN are shown in Table 3.3. Out of 33, 2 patients had a significant prior alcohol history which might have contributed to their peripheral neuropathy. Type 2 diabetes was present at time of testing (mean HbA1c 6.8%) in 3 patients, with no prior history or signs of neuropathy as recorded by their Oncologists. The sensory testing and

Figure 3.2 DPN group: Mean ± SEM at each visit for Neuropathy Impairment Score (LL, maximum score 88). Results shown are of the 22 patients who attended all 4 visits.
intra-epidermal nerve fibre results from these three patients were in keeping with the others in
the CIPN group and therefore included in the analysis. Out of 33 patients, 15 had prior
Oxaliplatin, 10 had prior Carboplatin and Paclitaxel, 3 had prior Cisplatin, 2 had prior
Docetaxel, 1 had prior Capecitabine, 1 had prior Bortezomib and 1 had prior Cisplatin and
Carboplatin. The mean time since last dose of chemotherapy was 9.3 (range 0.25-91) months.

<table>
<thead>
<tr>
<th>CIPN group patients</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>6 male</td>
<td>27 female</td>
</tr>
<tr>
<td>Mean (range), years</td>
<td>62 (43-73)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.3** CIPN group: patient demographics.

Assessments were performed at 0, 3 and 6 months with repeat skin biopsy at the third visit or
end of chemotherapy treatment. Table 3.4 shows the number of patients who attended for
each visit. Again there were a large number of missed appointments and patients who
dropped out from the study because of fatigue, intercurrent illness, breaks for abdominal
surgery or radiotherapy and one death (progressive ovarian cancer). All three visits were
attended by only 10 patients, with repeat biopsies performed at the third visit.

<table>
<thead>
<tr>
<th>Number of Patients tested</th>
<th>Number of patients biopsied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit 1 (baseline)</td>
<td>33</td>
</tr>
<tr>
<td>Visit 2 (3 months)</td>
<td>18</td>
</tr>
<tr>
<td>Visit 3 (6 months)</td>
<td>18</td>
</tr>
</tbody>
</table>

**Table 3.4** CIPN group: number of patients tested at each visit.
Clinical symptoms and pain scores

Patients in the CIPN group described a number of symptoms in their toes and fingertips, most commonly numbness, pins and needles, tingling and pain. The symptoms were comparable to the DPN group except the latter described symptoms affecting the feet and lower limbs mainly, rarely the upper limbs. A comparison of symptoms as a percentage of the total number of patients was obtained (Figure 3.3). Symptoms developed in the CIPN group after commencement of chemotherapy with symptoms persisting throughout the course of chemotherapy treatment. Usually symptoms started to subside after chemotherapy was stopped, however 6 patients reported that their symptoms worsened after cessation of chemotherapy, so called ‘coasting’ phenomenon. Of these, 5 had treatment with Oxaliplatin and 1 patient had treatment with Cisplatin.

![Figure 3.3](image)

Figure 3.3 Incidence of symptoms in the DPN and CIPN groups. Patients with a Likert score ≥1 were counted as having pain.

Out of 33 patients with CIPN, 30 (91%) reported pain in their feet and 4 were taking analgesia for neuropathic pain (either amitriptyline, gabapentin, tramadol, diclofenac, morphine sulphate or a combination of these). The mean ± SEM Likert pain score was 2.6 ± 0.5 at the first visit. Out of 33, 12 patients scored 4 or above, 15 patients scored zero. There
was no significant change over the year (Figure 3.4). The mean pain score differed significantly from the DPN group, with the latter being more painful (p=0.0100).

The mean ± SEM McGill pain questionnaire score in the CIPN group was 6.6 ± 1.5 at the first visit. The maximum possible score is 55. There was no significant change over the year (Figure 3.4). The mean McGill pain questionnaire score differed significantly from the DPN group, with the latter having more symptoms (p=0.0099).

**Figure 3.4** CIPN group: Mean ± SEM at each visit for a) Likert Pain Score, b) McGill Pain Score (maximum score 55). Results shown are of the 10 patients who attended all 3 visits.

Clinical Examination

Distal muscle weakness (lower limbs) was present in 3/33 (9%) patients at MRC grade 4/5 in the CIPN group. Neuropathy Impairment Score Lower Limbs (NIS-LL) which is a summed score of muscle power, reflex loss and sensation loss was calculated (maximum score 88 indicating severe neuropathy). This was relatively low at 9.0 ± 1.0 in the CIPN group at the first visit. There was no significant change over the year (Figure 3.5). There was no
significant difference between patients in the CIPN and DPN groups (p=0.2284). All patients had palpable peripheral pulses in the lower limbs.

**Figure 3.5** CIPN group: Mean ± SEM at each visit for Neuropathy Impairment Score (LL, maximum score 88). Results shown are of the 10 patients who attended all 3 visits.

### 3.4 Statistical analysis

Data was analysed using GraphPad Prism version 5.0 for Windows (GraphPad Prism Software, San Diego, California, USA), p <0.05 indicated significance. Statistical tests calculated were: the two-sided Mann Whitney U test, Wilcoxon matched pairs test and Spearman correlation coefficients. A statistical advisor supervised the statistical analysis of the data and accounted for the multiple variables involved; the normality of the distributions was assessed by means of the Shapiro-Wilk test. In case the null-hypothesis of normality could not be rejected (p>0.05) then in principle a parametric test could be applied. In that situation the Folded F statistic was calculated first to test the equality of variances of the two study groups. In case the null-hypothesis of equality of variances could not be rejected (p>0.05) then the two-sided unpaired pooled Student t-test applied. If the assumption of equality of variances was not reasonable and had to be rejected (p<0.05) then the two-sided unpaired Satterthwaite Student t-test was used. In case the null-hypothesis of normality had
to be rejected (p<0.05) then the non-parametric Mann-Whitney U test, also known as the Wilcoxon Rank Sum test, was applied. The Spearman correlation coefficients between indicated variables were calculated. Statistically significant differences between the two study groups or correlation coefficients, which were statistically significantly different from zero were indicated by p<0.05.
4.0 Results

4.1 DPN group

4.1.1 DPN group: First Visit

*DPN group: Quantitative Sensory Testing*

Out of 40, 38 (95%) patients had an abnormal touch threshold [Monofilament No. >3, 0.0479 g; Semmes-Weinstein hairs No.1 (0.0174 g) to No.20 (263.0 g)], mean ± SEM (range) monofilament number at the first visit was 9 ± 1 (3-20) (Figure 4.1).

Out of 40, 34 (85%) patients had an abnormal vibration threshold (>12mV), mean (mV) ± SEM (range) at the first visit was 32.0 ± 2.4 (5-50) (Figure 4.1).

*Figure 4.1 DPN group: Touch perception (values >No. 3 monofilament (0.0479 g) were considered abnormal and Vibration threshold (values >12V were considered abnormal).*

Thermal thresholds performed on the sole of the right foot showed abnormal cool perception thresholds in 38/40 (95%) patients, mean ± SEM (range) at first visit was 13.3 ± 1.3(2.2-32.0) °C, abnormal warm perception thresholds in 38/40 (95%) patients, mean ± SEM (range) at
first visit was 12.7 ± 0.6 (4.2-18.0)°C and abnormal heat pain thresholds in 39/40 (98%) patients, mean ± SEM (range) at first visit was 17.0±0.3 (10.4-18.0)°C. Cold pain threshold, mean ± SEM (range) at first visit was 28.3 ± 1.1 (7.1-32.0)°C (Figure 4.2). No control value is available for cold pain threshold in this range.

![Temperature change from baseline](image)

**Figure 4.2** DPN group: Thermal thresholds for a) Cool, b) Warm, c) Heat pain and d) Cold pain. Values >6.4°C for warm sensation, >2.3°C for cool sensation and >10.4°C for heat pain, were considered abnormal.

**DPN group: Histamine Skin Flare**

Out of 35, 13 (37%) patients had an abnormally small histamine skin flare, mean ± SEM (range) at the fist visit was 16.8 ±1.0 (6.1-34.6) cm² (Figure 4.3).
DPN group: Sweat Rate

Out of 40, 3 (8%) patients had an abnormally low sweat rate and 5 had an abnormally high sweat rate (13%), mean ± SEM (range) at the first visit was 19.8 ± 1.3 (5.0-37.0) g/m²/h (Figure 4.3).

Figure 4.3 DPN group: Histamine flare area and sweat rate. Abnormal skin flare was taken to be <14.9 cm² and abnormal sweat rate values <10 g/m²/h were considered abnormally low and >30 g/m²/h considered abnormally high.

DPN group: Nerve Conduction Studies

Out of 38, 30 (79%) patients had at least one abnormality on nerve conduction study recorded (Figure 4.4).

Out of 37, 15 patients had reduced amplitude recorded of the common peroneal motor action potential and was absent in 5 patients. The mean ± SEM (range) peroneal motor action potential was 3.4 ± 0.5 (0.0 - 14.0) μV. Of the 32 patients in which an amplitude was recorded, 11 had reduced common peroneal conduction velocity recorded. The mean ± SEM (range) peroneal conduction velocity was 42.1 ±1.1 (31.3 – 54.7) m/s. F-wave latency was absent in 10 patients. Where recorded 6/27 were abnormally slow. The mean ± SEM (range) F-wave latency was 54.5 ± 1.6 (42.0-79.3) ms (all patients measured<185cm²).
Out of 38, 9 patients had reduced amplitude recorded of the sural sensory action potential and was absent in 16 patients. The mean ± SEM (range) sural sensory action potential was 3.8 ± 0.7 (0.0 - 14.0) μV. Of the 22 patients where an amplitude was recorded, 4 had reduced sural conduction velocity. The mean ± SEM (range) sural conduction velocity was 44.4 ± 1.3 (31.2 – 52.2) m/s.

**Figure 4.4** DPN group: Peroneal (upper panels) and sural (lower panels) nerve conduction amplitude (left panels) and velocity (right panels). Peroneal motor action potential values < 3 mV amplitude and < 40 m/s conduction velocity were considered abnormal and Sural sensory action potential values <5 μV amplitude and <40 m/s conduction velocity were considered abnormal.
A summary of results is shown in Table 4.1.

<table>
<thead>
<tr>
<th>Clinical Test</th>
<th>(n)</th>
<th>Mean ± SEM (range)</th>
<th>% patients in abnormal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Touch Threshold *</td>
<td>40</td>
<td>8.7 ± 0.9 (3-20)</td>
<td>95%</td>
</tr>
<tr>
<td>Vibration threshold (mV)</td>
<td>40</td>
<td>32.0 ± 2.4 (5-50)</td>
<td>85%</td>
</tr>
<tr>
<td>Cool perception threshold (˚C)**</td>
<td>40</td>
<td>13.3 ± 1.3(2.2-32.0)</td>
<td>95%</td>
</tr>
<tr>
<td>Warm perception threshold (˚C)**</td>
<td>40</td>
<td>12.7 ± 0.6 (4.2-18.0)</td>
<td>95%</td>
</tr>
<tr>
<td>Heat Pain Threshold (˚C)**</td>
<td>40</td>
<td>17.0±0.3 (10.4-18.0)</td>
<td>98%</td>
</tr>
<tr>
<td>Cold Pain Threshold (˚C)**</td>
<td>40</td>
<td>28.3 ± 1.1 (7.1-32.0)</td>
<td>-</td>
</tr>
<tr>
<td>Histamine skin flare (cm²)</td>
<td>31</td>
<td>16.8 ±1.0 (6.1-34.6)</td>
<td>37%</td>
</tr>
<tr>
<td>Sweat rate (g/m²/h)</td>
<td>40</td>
<td>19.8 ± 1.3 (5.0-37.0)</td>
<td>8%</td>
</tr>
<tr>
<td>Peroneal action potential (µV)</td>
<td>37</td>
<td>3.4 ± 0.5 (0.0 -14.0)</td>
<td>54%</td>
</tr>
<tr>
<td>Peroneal conduction velocity (m/s)</td>
<td>37</td>
<td>#42.1 ±1.1 (31.3 –54.7)</td>
<td>43%</td>
</tr>
<tr>
<td>F-wave latency (ms)</td>
<td>37</td>
<td>## 54.5 ± 1.6 (42.0-79.3)</td>
<td>43%</td>
</tr>
<tr>
<td>Sural action potential (µV)</td>
<td>38</td>
<td>3.8 ± 0.7 (0.0 -14.0)</td>
<td>66%</td>
</tr>
<tr>
<td>Sural conduction velocity (m/s)</td>
<td>38</td>
<td>###44.4 ± 1.3 (31.2 –52.2)</td>
<td>53%</td>
</tr>
</tbody>
</table>

Table 4.1  DPN group: A summary of findings from clinical tests performed. * Monofilament No.1 (0.0174 g) to No.20 (263.0 g) ** temperature change from baseline (˚C). # Mean from 33 patients where a value was recorded (absent in 5 patients). ##Mean from 27 patients where a value was recorded. ### Mean from 22 patients where value was recorded (absent in 16 patients).

DPN group: Contact Heat Evoked Potentials

Contact heat evoked potentials were measured from the leg (Figure 4.5). Out of 34 patients, responses were recorded in 7. No response could be recorded in the remaining 27 patients. These patients were given a nominal value of 0.1 µV for analysis. Mean ± SEM (range) leg amplitude 1.8 ± 0.7 (0.1-15.3) µV. Age-matched control data from other studies in the Peripheral Neuropathy Unit (n=6) had a mean ± SEM (range) leg amplitude 14.6 ± 7.8 (0.1-47.2) µV. CHEPS (Aδ) N2-P2 amplitudes were significantly reduced from the leg (p=0.0260) compared with control values at baseline.

Contact heat evoked potentials were measured from the arm (Figure 4.5). Out of 34 patients, responses were recorded in 17. No response could be recorded in the remaining 17 patients.
These patients were given a nominal value of 0.1 μV for analysis. Mean ± SEM (range) arm amplitude 4.2 ± 0.8 (0.1-15.5) μV. Age-matched control data from other studies in the Unit (n=8) had a mean ± SEM (range) arm amplitude 18.3 ± 2.7 (4.5-27.0) μV. CHEPS (Aδ) N2-P2 amplitudes were significantly reduced from the arm (p<0.0001) compared with control values at baseline.

Contact heat evoked potentials were measured from the face (Figure 4.5). Out of 21 patients, responses were recorded in 14. No response could be recorded in the remaining 7 patients. These patients were given a nominal value of 0.1 μV for analysis. Mean ± SEM (range) leg amplitude 9.2 ± 1.7 (0.1-20.7) μV. Age-matched control data from other studies in the Unit (n=6) had a mean ± SEM (range) face amplitude 18.0 ± 3.6 (9.5-32.0) μV. CHEPS (Aδ) N2-P2 amplitudes were significantly reduced from the face (p=0.0353) compared with control values at baseline.
Figure 4.5 DPN group: Contact Heat Evoked Potential (Aδ) N2-P2 amplitudes were significantly reduced for a) Leg (n=34, p=0.0260), b) Arm (n=34, p<0.0001) and c) Face (n=21 p=0.0353). Examples of recordings from a control subject and diabetic patient are given in d) and e) respectively.
**DPN group: Immunohistology**

Intra- and sub-epidermal fibres for PGP 9.5 and TRPV1 were significantly decreased in the DPN group, in comparison with controls (Figure 4.6a - d). GAP-43 IENF were of very fine calibre in control skin (Figure 4.6e), but absent or reduced in the DPN group (Figure 4.8f). GAP-43 SENF were often close to the dermo-epidermal junction (Figure 4.6e, f), and were relatively preserved in deep dermis in diabetic skin (Figure 4.7). Strongly positive GAP-43-immunoreactive nerve fibres were found in deep dermal nerve fascicles in the diabetic patients, even in those with marked loss or absence of IENF (Figure 4.7).

![Figure 4.6](image.png)

**Figure 4.6.** Intra- and sub-epidermal nerve fibres immunoreactive for PGP 9.5 (a, b), TRPV1 (c, d) GAP-43 (e, f) and in control subjects (left panels) and diabetic patients (right panels). Magnification x40.
Figure 4.7 DPN group: GAP-43- immunoreactive nerve fibres within deep dermal nerve fascicles in a diabetic patient. Magnification x 10 (top panel); x40 (bottom panels).

**DPN group: Intra-epidermal nerve fibre (IENF) counts**

PGP 9.5 (15μm section thickness, Figure 4.8)

Intra-epidermal nerve fibres for PGP 9.5 were significantly decreased in the DPN group in comparison with controls at 15μm section thickness (p= 0.0003).

Patient (n=40) PGP 9.5 mean ± SEM IENF/mm (range) was 1.7 ± 0.4 (0.0-9.5). Out of 40, 25 patients had PGP 9.5 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values PGP 9.5 mean ± SEM IENF/mm (range) was 4.6 ± 0.6 (1.7-9.5).

Control (n=11) PGP 9.5 mean ± SEM IENF/mm was 6.1 ± 0.9 (1.6-11.3).
TRPV1 (15μm section thickness, Figure 4.8)

Intra-epidermal nerve fibres for TRPV1 were significantly decreased in the DPN group in comparison with controls (p<0.0001).

Patient (n=40) TRPV1 mean ± SEM IENF/mm (range) was 1.2 ± 0.3 (0.0-9.1). Out of 40, 25 patients had TRPV1 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values TRPV1 mean ± SEM IENF/mm (range) was 3.1 ± 0.6 (0.3-9.1).

Control (n=13) TRPV1 mean ± SEM IENF/mm was 5.9 ± 0.5 (2.0-8.7).

**Figure 4.8** DPN group: Intra-epidermal nerve fibres per mm length of tissue section (15μm thickness) vs. control. PGP 9.5 (***p = 0.0003) and TRPV1 (***p < 0.0001) fibres are significantly reduced.

PGP 9.5 (30μm section thickness, Figure 4.9)

Intra-epidermal nerve fibres for PGP 9.5 were significantly decreased in the DPN group in comparison with controls at 30μm section thickness (p<0.0001).
Patient (n=39) PGP 9.5 mean ± SEM IENF/mm (range) was 2.4 ± 0.5 (0.0-10.5). Out of 39, 21 patients had PGP 9.5 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values PGP 9.5 mean ± SEM IENF/mm (range) was 5.2 ± 0.7 (0.5-10.5).

Control (n=16) PGP 9.5 mean ± SEM IENF/mm was 7.6 ± 0.9 (0.0-12.8), excluding one zero value control (n=15) PGP 9.5 mean ± SEM IENF/mm was 8.1 ± 0.8 (2.3-12.8).

GAP-43 (30μm section thickness Figure 4.9)

Intra-epidermal nerve fibres for GAP-43 were significantly decreased in the DPN group in comparison with controls (p=0.0032).

Patient (n=40) GAP-43 mean ± SEM IENF/mm (range) was 0.1 ± 0.1 (0.0 – 1.8). Out of 40, 37 patients had GAP-43 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values GAP-43 mean SEM IENF/mm (range) was 1.2 ± 0.3 (0.6-1.8).

Control (n=17) GAP-43 mean ± SEM IENF/mm was 0.6 ± 0.3 (0.0-5.3), excluding 10 zero values control (n=7) GAP-43 mean ± SEM IENF/mm was 1.5 ± 0.7 (0.3-5.3).
**Figure 4.9** DPN group: Intra-epidermal nerve fibres per mm length of tissue section (30µm thickness) vs. control. PGP 9.5 (***p <0.0001) and GAP-43 (**p=0.0032) fibres are significantly reduced.

A summary of the diabetic first visit intra-epidermal nerve fibre counts is shown in Table 4.2.

<table>
<thead>
<tr>
<th>DPN group (Section thickness µm))</th>
<th>Patients (n)</th>
<th>Mean ± SEM (range) IENF/mm including zero values</th>
<th>(n) zero values excluded</th>
<th>Mean ± SEM (range) IENF/mm excluding zero values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5 (15)</td>
<td>40</td>
<td>1.7 ±0.4 (0.0-9.5)</td>
<td>15</td>
<td>4.6 ± 0.6 (1.7-9.5)</td>
</tr>
<tr>
<td>TRPV1 (15)</td>
<td>40</td>
<td>1.2 ± 0.3 (0.0-9.1)</td>
<td>15</td>
<td>3.1 ± 0.6 (0.3-9.1)</td>
</tr>
<tr>
<td>PGP 9.5 (30)</td>
<td>39</td>
<td>2.4 ± 0.5 (0.0-10.5)</td>
<td>18</td>
<td>5.2 ± 0.7 (0.5-10.5)</td>
</tr>
<tr>
<td>GAP-43 (30)</td>
<td>40</td>
<td>0.1 ± 0.1 (0.0 – 1.8)</td>
<td>3</td>
<td>1.2 ± 0.3 (0.6-1.8)</td>
</tr>
</tbody>
</table>

**Table 4.2** DPN group: A summary of intra-epidermal nerve fibre counts from the first visit.
DPN group: Sub-epidermal nerve fibre (SENF) counts

PGP 9.5 (15μm section thickness, Figure 4.10)

Sub-epidermal nerve fibres for PGP 9.5 were significantly decreased in the DPN group in comparison with controls at 15μm section thickness (p = 0.0012).

Patient (n=40) PGP 9.5 mean ± SEM SENF % area (range) was 0.46 ± 0.05 (0.01-1.11). Out of 40, 5 patients had undetectable PGP 9.5 and were given a nominal value of 0.010 % area, which have been included in the mean, excluding the zero values PGP 9.5 mean ± SEM SENF % area (range) was 0.52 ± 0.05 (0.12-1.11). Control (n=12) PGP 9.5 mean ± SEM SENF % area was 1.00 ± 0.18 (0.35-2.64).

TRPV1 (15μm section thickness, Figure 4.10)

Sub-epidermal nerve fibres for TRPV1 were significantly decreased in the DPN group in comparison with controls (p<0.0001).

Patient (n=40) TRPV1 mean ± SEM SENF % area (range) was 0.11 ± 0.02 (0.01-0.53). Out of 40, 10 patients had undetectable TRPV1 and were given a nominal value of 0.010 % area, which have been included in the mean, excluding the zero values TRPV1 mean ± SEM SENF % area (range) was 0.15 ± 0.03 (0.02-0.53). Control (n=12) TRPV1 mean ± SEM SENF % area was 0.52 ± 0.11 (0.18-1.38).
**Figure 4.10** DPN group: Sub-epidermal nerve fibres per mm length of tissue section (15\(\mu\)m thickness) vs. control. PGP 9.5 (**p= 0.0012) and TRPV1 (***p < 0.0001) fibres are significantly reduced.

PGP 9.5 (30\(\mu\)m section thickness, Figure 4.11)

Sub-epidermal nerve fibres for PGP 9.5 were significantly decreased in the DPN group in comparison with controls at 30\(\mu\)m section thickness (p=0.0051).

Patient (n=40) PGP 9.5 mean ± SEM SENF %area (range) was 1.02 ± 0.11 (0.01-3.42). Out of 40, 2 patients had undetectable PGP 9.5 and were given a nominal value of 0.010 % area, which have been included in the mean, excluding the zero values PGP 9.5 mean ± SEM SENF % area (range) was 1.10 ± 0.11 (0.08-3.42). Control (n=16) PGP 9.5 mean ± SEM SENF % area was 1.47 ± 0.13 (0.13-2.33).
GAP-43 (30μm section thickness Figure 4.11)

Sub-epidermal nerve fibres for GAP-43 were not significantly decreased in the DPN group in comparison with controls (p=0.0551).

Patient (n=40) GAP-43 mean ± SEM %area (range) was 0.17 ± 0.04 (0.01-0.93). Out of 40, 12 patients had undetectable GAP-43 and were given a nominal value of 0.010 % area, which have been included in the mean, excluding the zero values GAP-43 mean SEM % area (range) was 0.23 ± 0.05 (0.01-0.93). Control (n=18) GAP-43 mean ± SEM % area was 0.17 ± 0.12 (0.01-0.37), excluding one zero value GAP-43 mean ± SEM % area was 0.18± 0.02 (0.01-0.93).

Further analysis of the sub-epidermal GAP (% area) in the DPN group reveals a range of values (0.0%-1.34%). The group with higher than average GAP-43 (n=7, those with values greater than 2SD above the control mean) were looked at as a group and compared to those diabetic patients who had a low sub-epidermal GAP-43 (n=12, those with values less than 2SD below the control mean). Interesting results were found in that the high GAP-43 group had a smaller duration of symptoms (2.2 yrs vs. 4.7 yrs), and had less severe neuropathy, for example McGill pain score was less (12.8 vs. 19.2), Neuropathy Impairment Score was less (8.2 vs. 16.0) and Sural Nerve amplitude was higher in this group (5.4mV vs. 2.0mV), however no differences were found to be significant using a two-tailed Mann-Whitney test, and this is likely to be because of the small numbers in each group. Age, duration of diabetes and HbA1c level did not show an obvious difference.
**Figure 4.11** DPN group: Sub-epidermal nerve fibres per mm length of tissue section (30µm thickness) vs. control. PGP 9.5 (**p=0.0051**) and GAP-43 (p=0.0551) fibres are significantly reduced.

A summary of the sub-epidermal nerve fibre counts from the first visit is shown in Table 4.3.

<table>
<thead>
<tr>
<th>DPN group (Section thickness µm)</th>
<th>Patients (n)</th>
<th>Mean ± SEM (range) SENF % area</th>
<th>(n) zero values excluded</th>
<th>Mean ± SEM (range) SENF % area excluding zero values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5 (15)</td>
<td>40</td>
<td>0.46 ± 0.05 (0.01-1.11)</td>
<td>35</td>
<td>0.52 ± 0.05 (0.12-1.11)</td>
</tr>
<tr>
<td>TRPV1 (15)</td>
<td>40</td>
<td>0.11 ± 0.02 (0.01-0.53)</td>
<td>30</td>
<td>0.15 ± 0.03 (0.02-0.53)</td>
</tr>
<tr>
<td>PGP 9.5 (30)</td>
<td>40</td>
<td>1.02 ± 0.11 (0.01-3.42)</td>
<td>38</td>
<td>1.10 ± 0.11 (0.08-3.42)</td>
</tr>
<tr>
<td>GAP-43 (30)</td>
<td>40</td>
<td>0.17 ± 0.04 (0.01-0.93)</td>
<td>28</td>
<td>0.23 ± 0.05 (0.01-0.93)</td>
</tr>
</tbody>
</table>

**Table 4.3** DPN group: A summary of sub-epidermal nerve fibre counts from the first visit.
**DPN group: Correlations**

A number of significant correlations were found between the diabetic patient test results at the baseline visit. Histamine invoked flare area (Figure 4.12) correlated with PGP9.5 IENF ($r=0.3711$, $p=0.0282$).

![Graph showing correlation between PGP 9.5 IENF and flare area](image)

**Figure 4.12** DPN group: Histamine flare area was significantly correlated with intra-epidermal PGP 9.5 ($r=0.3711$, $p=0.0282$).

Contact Heat Evoked Potential (Aδ) N2-P2 amplitude from the face correlated with PGP 9.5 ($r=0.466$, $p=0.0332$) and TRPV1 ($r=0.451$, $p=0.0401$) IENF; there was no significant correlation seen with amplitudes from the leg or arm perhaps because of the number of patients who had un-recordable amplitudes from those regions (Figure 13).
Figure 4.13 DPN group: Contact Heat Evoked Potential (Aδ) N2-P2 amplitudes from the face significantly correlated with counts of intra-epidermal a) PGP 9.5 (r=0.466, p=0.0332) and b) TRPV1 nerve fibres (r=0.451, p=0.0401). Data includes zero value IENF/mm counts and nominal values of 0.1μV given to patients who had un-recordable amplitudes from the face.
4.1.2 DPN group: Follow up visits

DPN group follow up: Quantitative Sensory Testing

Touch impairment did not significantly change over the year (Figure 4.14). Vibration threshold mean did not significantly change, although there was a trend to increase over the year (Figure 4.14).

![Figure 4.14](image.png)

**Figure 4.14** DPN group: Mean ± SEM at each visit for a) Touch perception (values >No. 3 monofilament (0.0479 g) were considered abnormal b)Vibration threshold (values >12V were considered abnormal). Results shown are of the 22 patients who attended all 4 visits.

Cool perception threshold mean did not significantly change over the year (Figure 4.15).

Warm perception threshold mean did not significantly change over the year (Figure 4.15).

Heat pain threshold mean did not significantly change over the year (Figure 4.15). Cold pain threshold mean did not significantly change over the year (Figure 4.15).
Figure 4.15  DPN group: Mean ± SEM at each visit for Thermal threshold temperature change from baseline (32°C) for a) Cool, b) Warm, c) Heat pain and d) Cold pain. Results shown are of the 22 patients who attended all 4 visits. Values >6.4°C for warm sensation, >2.3°C for cool sensation and >10.4°C for heat pain, were considered abnormal.

DPN group: Histamine Skin Flare

Histamine flare area mean did not significantly change over the year (Figure 4.16).

DPN group: Sweat Rate

Sweat rate mean did not significantly change over the year (Figure 4.16).
Figure 4.16 DPN group: Mean ± SEM at each visit for a) Histamine Flare area, results shown are of the 16 patients who had histamine flare area performed on all 4 visits) b) Sweat rate, results shown are of the 22 patients who attended all 4 visits. Abnormal skin flare was taken to be <14.9 cm$^2$ and abnormal sweat rate values <10 g/m$^2$/h were considered abnormally low and >30 g/m$^2$/h considered abnormally high.

**DPN group follow up: Nerve Conduction Studies**

Nerve conduction studies were performed once only – please see DPN group: First visit section for results.

**DPN group follow up: Contact Heat Evoked Potentials**

Of the 22 patients who attended all 4 visits, for logistical reasons and patient preference only 7 patients had recordings from all 3 sites (leg, arm and face) at each visit.

Contact heat evoked potentials were measured from the leg. Mean leg amplitude did not significantly change, although there was a trend to decrease over the year (Figure 4.17).

Contact heat evoked potentials were measured from the arm. Mean arm amplitude did not significantly change, although there was a trend to decrease over the year (Figure 4.17).
Contact heat evoked potentials were recorded from the face. Mean face amplitude did not significantly change, although there was a trend to decrease over the year (Figure 4.17).

**Figure 4.17** DPN group: Mean ± SEM at each visit for Contact Heat Evoked Potential (Aδ) N2-P2 amplitudes from a) Leg, b) Arm, c) Face. Results shown are of the 7 patients who attended all 4 visits and who had CHEPS in all 3 areas.

DPN group follow up: Intra-epidermal nerve fibre (IENF) counts

Repeat skin punch biopsies were taken after 6 months in 29 patients. Biopsies taken from the same patients at their first (0 months) and third (6 months) visits showed a significant reduction of IENF for both PGP 9.5 and TRPV1 (zero values removed at baseline).
Rate of loss of IENF was calculated to be $0.010 \pm 0.004$ fibres/mm/day or $3.76 \pm 1.46$ mm/yr for PGP 9.5, and $0.009 \pm 0.002$ fibres/mm/day or $3.13 \pm 0.58$ mm/yr for TRPV1.

PGP 9.5 (15μm section thickness, Figure 4.18)

Patient (n=29) PGP 9.5 mean ± SEM IENF/mm (range) at 0 months was $1.5 \pm 0.5$ (0.0-9.5) and at 6 months was $0.9 \pm 0.4$ (0.0-9.7). 20 patients had PGP 9.5 values of 0.0 IENF/mm which have been included in the mean, excluding those with zero values of PGP 9.5 at baseline mean ± SEM IENF/mm (range) at 0 months was $4.7 \pm 0.9$ (1.7-9.5) and at 6 months was $2.8 \pm 1.1$ (0.0-9.7). Analysis of paired repeat samples (n=9) taken at 6 months showed a significantly reduced PGP intra-epidermal nerve fibre count (p=0.0391).

TRPV1 (15μm section thickness, Figure 4.18)

Patient (n=28) TRPV1 mean ± SEM IENF/mm (range) at 0 months was $0.9 \pm 0.3$ (0.0-6.4) and at 6 months was $0.5 \pm 0.2$ (0.0-3.7). 19 patients had TRPV1 values of 0.0 IENF/mm which have been included in the mean, excluding those with zero values of TRPV1 at baseline mean ± SEM IENF/mm (range) at 0 months was $2.8 \pm 0.6$ (0.9-6.4) and at 6 months was $1.2 \pm 0.4$ (0.0-3.7). Analysis of paired repeat samples (n=9) taken at 6 months showed a significantly reduced TRPV1 intra-epidermal nerve fibre count (p = 0.0091).

PGP 9.5 (30μm section thickness)

Patient (n=29) PGP 9.5 mean ± SEM IENF/mm (range) at 0 months was $1.8 \pm 0.5$ (0.0-10.5) and at 6 months was $1.4 \pm 0.6$ (0.0-11.2). 18 patients had PGP 9.5 values of 0.0 IENF/mm which have been included in the mean, excluding those with zero values of PGP 9.5 at baseline mean ± SEM IENF/mm (range) at 0 months was $4.7 \pm 0.9$ (0.5-10.5) and at 6 months
was 3.6 ±1.2 (0.0-11.2). Analysis of paired repeat samples (n=11) taken at 6 months did not show a significantly reduced PGP intra-epidermal nerve fibre count (p=0.1971).

GAP-43 (30μm diameter section thickness)

Patient (n=29) GAP-43 mean ± SEM IENF/mm (range) at 0 months was 0.1 ± 0.1 (0.0-1.8). At 6 months no intra-epidermal GAP-43 was detectable thus precluding any comparison of first and second biopsies.

A Summary of diabetic patient intra-epidermal nerve fibre counts at 0 and 6 months is shown in Table 4.4.

<table>
<thead>
<tr>
<th>(Section thickness μm)</th>
<th>Patients (n)</th>
<th>Mean ± SEM (range) IENF/mm including zero values</th>
<th>(n) zero values at baseline excluded</th>
<th>Mean ± SEM (range) IENF/mm excluding zero values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5 0m (15)</td>
<td>29</td>
<td>1.5 ± 0.5 (0.0-9.5)</td>
<td>9</td>
<td>4.7 ± 0.9 (1.7-9.5)</td>
</tr>
<tr>
<td>PGP 9.5 6m (15)</td>
<td>29</td>
<td>0.9 ± 0.4 (0.0-9.7)</td>
<td>9</td>
<td>2.8 ± 1.1 (0.0-9.7)</td>
</tr>
<tr>
<td>Control PGP 9.5 (15)</td>
<td>11</td>
<td>6.1 ± 0.9 (1.6-11.3)</td>
<td>9</td>
<td>1.8 ± 0.8 (0.0-9.7)</td>
</tr>
<tr>
<td>TRPV1 0m (15)</td>
<td>28</td>
<td>0.9 ± 0.3 (0.0-6.4)</td>
<td>9</td>
<td>2.8 ± 0.6 (0.9-6.4)</td>
</tr>
<tr>
<td>TRPV1 6m (15)</td>
<td>28</td>
<td>0.5 ± 0.2 (0.0-3.7)</td>
<td>9</td>
<td>1.2 ± 0.4 (0.0-3.7)</td>
</tr>
<tr>
<td>Control TRPV1 (15)</td>
<td>13</td>
<td>5.9 ± 0.5 (2.0-8.7)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PGP 9.5 0m (30)</td>
<td>29</td>
<td>1.8 ± 0.5 (0.0-10.5)</td>
<td>11</td>
<td>4.7 ± 0.9 (0.5-10.5)</td>
</tr>
<tr>
<td>PGP 9.5 6m (30)</td>
<td>29</td>
<td>1.4 ± 0.6 (0.0-11.2)</td>
<td>11</td>
<td>3.6 ± 1.2 (0.0-11.2)</td>
</tr>
<tr>
<td>Control PGP 9.5 (30)</td>
<td>16</td>
<td>7.6 ± 0.9 (0.0-12.8)</td>
<td>15</td>
<td>8.1 ± 0.8 (2.3-12.8)</td>
</tr>
<tr>
<td>GAP-43 0m (30)</td>
<td>29</td>
<td>0.1 ± 0.1 (0.0-1.8)</td>
<td>1</td>
<td>1.8 ± 0.0 (1.8-1.8)</td>
</tr>
<tr>
<td>GAP-43 6m (30)</td>
<td>29</td>
<td>0.0 ± 0.0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Control GAP-43 (30)</td>
<td>17</td>
<td>0.6 ± 0.3 (0.0-5.3)</td>
<td>7</td>
<td>1.5 ± 0.7 (0.3-5.3)</td>
</tr>
</tbody>
</table>

Table 4.4 DPN group: A summary of intra-epidermal nerve fibre counts at 0 and 6 months.
DPN group follow up: Sub-epidermal nerve fibre (SENF) counts

PGP 9.5 (15μm section thickness, Figure 4.18)

Patient (n=29) PGP 9.5 mean ± SEM SENF % area (range) at 0 months was 0.42 ± 0.06 (0.01-1.10) and at 6 months was 0.29 ± 0.04 (0.01-0.78). 4 patients had PGP 9.5 values of 0.0 % area which have been included in the mean, excluding those with zero values of PGP 9.5 at baseline mean ± SEM SENF % area (range) at 0 months was 0.51 ± 0.06 (0.12-1.11) and at 6 months was 0.31 ±0.04 (0.01-0.78).

Analysis of paired repeat samples (n=25) taken at 6 months showed a significantly reduced PGP sub-epidermal nerve fibre count (p = 0.0043).

TRPV1 (15μm section thickness, Figure 4.18)

Patient (n=28) TRPV1 mean ± SEM SENF % area (range) at 0 months was 0.11 ± 0.03 (0.01-0.53) and at 6 months was 0.03 ± 0.00 (0.01-0.20). 8 patients had TRPV1 values of 0.0 % area, which have been included in the mean, excluding those with zero values of TRPV1 at baseline mean ± SEM IENF/mm (range) at 0 months was 0.14 ± 0.03 (0.02-0.53) and at 6 months was 0.04 ± 0.01 (0.01-0.20).

Analysis of paired repeat samples (n=21) taken at 6 months showed a significantly reduced TRPV1 sub-epidermal nerve fibre count (p =0.0006).

PGP 9.5 (30μm section thickness)

Sub-epidermal fibres were not analysed in repeat sections at this section thickness.
GAP-43 (30μm section thickness)

Patient (n=29) GAP-43 mean ± SEM SENF % area (range) at 0 months was 0.17 ± 0.04 (0.01-0.93) and at 6 months was 0.15 ± 0.02 (0.01-0.36). 8 patients had GAP-43 values of 0.0 % area, which have been included in the mean, excluding those with zero values of GAP-43 at baseline mean ± SEM IENF/mm (range) at 0 months was 0.23 ± 0.06 (0.01-0.93) and at 6 months was 0.13 ± 0.02 (0.01-0.36).

Analysis of paired repeat samples (n=21) taken at 6 months did not show a significantly reduced GAP-43 sub-epidermal nerve fibre count (p = 0.2238).

A Summary of diabetic patient sub-epidermal nerve fibre counts at 0 and 6 months is shown in Table 4.5.

<table>
<thead>
<tr>
<th>(Section thickness [μm]) m = months</th>
<th>(n)</th>
<th>Mean ± SEM (range) SENF % area including zero values</th>
<th>(n) zero values at baseline excluded</th>
<th>Mean ± SEM (range) SENF % area excluding zero values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5 0 m (15)</td>
<td>29</td>
<td>0.42 ± 0.06 (0.01-1.10)</td>
<td>25</td>
<td>0.51 ± 0.06 (0.12-1.11)</td>
</tr>
<tr>
<td>PGP 9.5 6 m (15)</td>
<td>29</td>
<td>0.29 ± 0.04 (0.01-0.78)</td>
<td>25</td>
<td>0.31 ± 0.04 (0.01-0.78)</td>
</tr>
<tr>
<td>Control PGP 9.5 (15)</td>
<td>12</td>
<td>1.00 ± 0.18 (0.35-2.64)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TRPV1 0 m (15)</td>
<td>29</td>
<td>0.11 ± 0.03 (0.01-0.53)</td>
<td>21</td>
<td>0.14 ± 0.03 (0.02-0.53)</td>
</tr>
<tr>
<td>TRPV1 6 m (15)</td>
<td>29</td>
<td>0.03 ± 0.00 (0.01-0.20)</td>
<td>21</td>
<td>0.04 ± 0.01 (0.01-0.20)</td>
</tr>
<tr>
<td>Control TRPV1 (15)</td>
<td>12</td>
<td>0.52 ± 0.11 (0.18-1.38)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GAP-43 0 m (30)</td>
<td>29</td>
<td>0.17 ± 0.04 (0.01-0.93)</td>
<td>21</td>
<td>0.23 ± 0.06 (0.01-0.93)</td>
</tr>
<tr>
<td>GAP-43 6 m (30)</td>
<td>29</td>
<td>0.15 ± 0.02 (0.01-0.36)</td>
<td>21</td>
<td>0.13 ± 0.02 (0.01-0.36)</td>
</tr>
<tr>
<td>Control GAP-43 (30)</td>
<td>18</td>
<td>0.17 ± 0.12 (0.01-0.37)</td>
<td>17</td>
<td>0.18 ± 0.02 (0.01-0.93)</td>
</tr>
</tbody>
</table>

**Table 4.5** DPN group: A summary of sub-epidermal nerve fibre counts at 0 and 6 months.
Figure 4.18 DPN group: Counts of paired intra-epidermal nerve fibres per mm length for PGP 9.5 (a), TRPV1 (b) \( *p = 0.00391, \; **p=0.0091 \), and paired % area for sub-epidermal nerve fibres for PGP 9.5 (c) and TRPV1 (d) \( *p=0.0043, \; **p=0.0006 \). Patients with zero values at baseline removed. Control mean values ± SEM are indicated by dotted rectangle.
4.2 CIPN group

4.2.1 CIPN group: First Visit

CIPN group: Quantitative Sensory Testing

Out of 31, 18 (58%) of patients had an abnormal touch threshold [Monofilament No. >3, 0.0479 g; Semmes-Weinstein hairs No.1 (0.0174 g) to No.20 (263.0 g)], mean ± SEM (range) monofilament number at the first visit was 5.1 ± 0.6 (1-15) (Figure 4.19).

Out of 33, 25 (76%) patients had an abnormal vibration threshold (>12mV), mean ± SEM (range) at the first visit was 25.6 ± 2.6 (8-50) mV (Figure 4.19).

Figure 4.19 CIPN group: Touch perception (values >No. 3 monofilament (0.0479 g) were considered abnormal and Vibration threshold (values >12V were considered abnormal).

The chemotherapy-induced neuropathy group was divided into two groups for analysis of thermal thresholds in order to look for any differences in patients receiving the chemotherapy agent Oxaliplatin (Figure 4.20).
Oxaliplatin group: Thermal thresholds performed on the sole of the right foot showed abnormal cool perception thresholds in 10/15 (67%) patients mean ± SEM (range) at first visit was 4.3 ± 0.7 (1.2-9.7) °C, abnormal warm perception thresholds in 8/15 (53%) patients mean ± SEM (range) at first visit was 7.4 ± 1.0 (1.8-12.3) °C and abnormal heat pain thresholds in 11/15 (73%) patients mean ± SEM (range) at first visit was 13.3 ± 0.9 (5.3-18.0) °C. Cold pain threshold mean ± SEM (range) at first visit was 19.7 ± 2.5 (8.9-32.0) °C.

Non-Oxaliplatin group: Thermal thresholds performed on the sole of the right foot showed abnormal cool perception thresholds in 12/18 (67%) patients mean ± SEM (range) at first visit was 4.5 ± 0.7 (1.0-11.0) °C, abnormal warm perception thresholds in 9/18 (50%) patients mean ± SEM (range) at first visit was 7.1 ± 1.0 (1.7-15.9) °C and abnormal heat pain thresholds in 12/17 (71%) patients mean ± SEM (range) at first visit was 12.4 ± 0.9 (5.0-18.0) °C. Cold pain threshold mean ± SEM (range) at first visit was 21.9 ± 2.3 (7.5-32.0) °C.
Figure 4.20 CIPN group: Thermal thresholds for a) Cool, b) Warm, c) Heat pain and d) Cold pain. O = Oxaliplatin; NO = Non-oxaliplatin. Values >6.4°C for warm sensation, >2.3°C for cool sensation and >10.4°C for heat pain, were considered abnormal.

CIPN group: Histamine Skin Flare

Out of 28, 17 (61%) patients had an abnormal histamine skin flare, mean ± SEM (range) at the first visit was 14.7 ± 1.2 (5.8-29.3) cm² (Figure 4.21).

CIPN group: Sweat Rate
Out of 33, 1 (3%) patient had an abnormally low sweat rate and 2 (6%) patients had an abnormally high sweat rate, mean ± SEM (range) at the first visit was 18.3 ± 1.1 (7.0-32.0) g/m2/h (Figure 4.21).

Figure 4.21 CIPN group: Histamine flare area and sweat rate. Abnormal skin flare was taken to be <14.9 cm² and abnormal sweat rate values <10 g/m²/h were considered abnormally low and >30 g/m²/h considered abnormally high.

CIPN group: Nerve Conduction Studies

Out of 21, 15 (71%) patients had at least one abnormality on nerve conduction study (Figure 4.22).

Out of 21, 3 patients had reduced amplitude recorded of the common peroneal motor action potential and was absent in 2 patients. The mean ± SEM (range) peroneal motor action potential was 4.7 ± 0.6 (0.0-8.2) μV. Of the 19 patients in where an amplitude was recorded, 1 had reduced common peroneal conduction velocity recorded. The mean ± SEM (range) peroneal conduction velocity was 48.7± 1.1 (38.6-59.5) m/s. F-wave latency was absent in 2/21 patients. The mean ± SEM (range) F-wave latency was 47.2 ± 1.2 (38.9-56.8) ms (all patients <185cm²).
Out of 21, 5 patients had reduced amplitude recorded of the sural sensory action potential and was absent in 4 patients. The mean ± SEM (range) sural sensory action potential was 8.7 ± 1.6 (0.0-22.0) μV. Of the 17 patients where an amplitude was recorded, all were in the normal range. The mean ± SEM (range) sural conduction velocity was 52.7 ± 1.6 (46.2-67) m/s.

**Figure 4.22** CIPN group: Peroneal (upper panels) and sural (lower panels) nerve conduction amplitude (left panels) and velocity (right panels). Peroneal motor action potential values < 3 mV amplitude and <40 m/s conduction velocity were considered abnormal and Sural sensory action potential values <5 μV amplitude and <40 m/s conduction velocity were considered abnormal.
A summary of results is shown in Table 4.6.

<table>
<thead>
<tr>
<th>Clinical Test</th>
<th>(n)</th>
<th>Mean ± SEM (range)</th>
<th>% patients in abnormal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Touch Threshold *</td>
<td>31</td>
<td>5.1±0.6 (1-15)</td>
<td>58%</td>
</tr>
<tr>
<td>Vibration threshold (mV)</td>
<td>33</td>
<td>25.6±2.6 (8-50)</td>
<td>76%</td>
</tr>
<tr>
<td>Cool perception threshold (°C)**</td>
<td>33</td>
<td>4.4±0.5 (1.0-11.0)</td>
<td>67%</td>
</tr>
<tr>
<td>Warm perception threshold (°C)**</td>
<td>33</td>
<td>7.2±0.7(1.7-15.9)</td>
<td>52%</td>
</tr>
<tr>
<td>Heat Pain Threshold (°C)**</td>
<td>32</td>
<td>12.8±0.6 (5.0-18.0)</td>
<td>75%</td>
</tr>
<tr>
<td>Cold Pain Threshold (°C)**</td>
<td>32</td>
<td>20.9±1.7 (7.5-32.0)</td>
<td>-</td>
</tr>
<tr>
<td>Histamine skin flare (cm²)</td>
<td>28</td>
<td>14.7 ± 1.2 (5.8-29.3)</td>
<td>64%</td>
</tr>
<tr>
<td>Sweat rate (g/m²/h)</td>
<td>30</td>
<td>18.3±1.1 (7.0-32.0)</td>
<td>3%</td>
</tr>
<tr>
<td>Peroneal action potential (μV)</td>
<td>21</td>
<td>4.7 ± 0.6 (0.0-8.2)</td>
<td>24%</td>
</tr>
<tr>
<td>Peroneal conduction velocity (m/s)</td>
<td>21</td>
<td># 48.7± 1.1 (38.6-59.5)</td>
<td>14%</td>
</tr>
<tr>
<td>F-wave latency (ms)</td>
<td>21</td>
<td>##47.2± 1.2 (38.9-56.8)</td>
<td>10%</td>
</tr>
<tr>
<td>Sural action potential (μV)</td>
<td>21</td>
<td>8.7 ± 1.6 (0.0-22.0)</td>
<td>43%</td>
</tr>
<tr>
<td>Sural conduction velocity (m/s)</td>
<td>21</td>
<td>###52.7 ± 1.6 (46.2-67)</td>
<td>19%</td>
</tr>
</tbody>
</table>

Table 4.6 CIPN group: A summary of findings from clinical tests performed. * Monofilament No.1 (0.0174 g) to No.20 (263.0 g) ** temperature change from baseline (°C). # Mean from 19 patients where a value was recorded (absent in 2 patients). ##Mean from 19 patients where a value was recorded. ###Mean from 17 patients where value was recorded (absent in 4 patients).

**CIPN group: Contact Heat Evoked Potentials**

Contact heat evoked potentials were measured from the leg (Figure 4.23). Out of 15 patients, responses were recorded in 10. No response could be recorded in the remaining 5 patients. These patients were given a nominal value of 0.1 μV for analysis. Mean ± SEM (range) leg amplitude 7.5 ± 0.0(0.1-28.3) μV. Age-matched control data from other studies in the Peripheral Neuropathy Unit (n=6) had a mean ± SEM (range) leg amplitude 14.6 ± 7.8 (0.1-47.2) μV. CHEPS (Aδ) N2-P2 amplitudes were not significantly reduced from the compared with control values (p=0.4051). Leg amplitude in the DPN group was significantly lower (p=0.0006) compared with the CIPN group.
Contact heat evoked potentials were measured from the arm (Figure 4.23). Out of 15 patients, responses were recorded in 13. No response could be recorded in the remaining 2 patients. These patients were given a nominal value of 0.1 μV for analysis. Mean ± SEM (range) arm amplitude 10.1 ± 2.1 (0.1-28.8) μV. Age-matched control data from other studies in the Unit (n=8) had a mean ± SEM (range) arm amplitude 18.3 ± 2.7 (4.5-27.0) μV. CHEPS (Aδ) N2-P2 amplitudes were significantly reduced from the arm (p= 0.0130) compared with control values. Arm amplitude in the DPN group was significantly lower (p=0.0040) compared with the CIPN group.

Contact heat evoked potentials were measured from the face (Figure 4.23). Out of 10 patients, responses were recorded in 8. No response could be recorded in the remaining 2 patients. These patients were given a nominal value of 0.1 μV for analysis. Mean ± SEM (range) leg amplitude 11.4 ± 2.7 (0.1-32.1) μV. Age-matched control data from other studies in the Unit (n=6) had a mean ± SEM (range) face amplitude 18.0 ± 3.6 (9.5-32.0) μV. CHEPS (Aδ) N2-P2 amplitudes were not significantly reduced from the face (p=0.0736) compared with control values. A significant difference was not found when comparing DPN and CIPN Face Amplitude.
Figure 4.23 CIPN group: Contact Heat Evoked Potential (A6) N2-P2 Amplitude was significantly reduced as follows; Leg Amplitude: DPN vs. control (p=0.0260), DPN vs. CIPN (p=0.0006); Arm amplitude: CIPN vs. control (p=0.0130), DPN vs. control (p<0.0001), DPN vs. CIPN (p=0.0040); Face Amplitude: DPN vs. control (p=0.0353). An example of leg, arm and face recordings from a control subject (d) and patient with chemotherapy-induced neuropathy (e)
Comparison of sensory tests between the DPN and CIPN groups

Quantitative Sensory Testing, Histamine Skin Flare and Sweat Rate measurement

A symmetrical distribution of signs was found in all patients from both groups. A summary of findings is shown in Figure 4.24. The majority of patients showed abnormalities of sensory nerve function.

Figure 4.24 Incidence of (a) abnormal sensory tests and (b) abnormal thermal thresholds in the CIPN and DPN groups.
**CIPN group: Immunohistology**

Immunostaining in the chemotherapy-induced neuropathy group showed marked changes when compared with control and diabetic sections.

Immunostaining for PGP 9.5 showed a preserved number of intra-epidermal nerve fibres (Figure 4.25), however thick fascicles were seen in the sub-epidermis adjacent to and coursing along the basal epidermis. These nerve fibres were better visualised at thicker 30μm sections.

Immunostaining for TRPV1 showed a preserved number of intra-epidermal nerve fibres (Figure 4.26), however there was a trend to a decreased number of fibres which reached significance in the Oxaliplatin group (p=0.0476). Slightly thicker sub-epidermal fibres were seen near the basal epidermis, but the thick, branching fibres seen with immunostaining for PGP 9.5 was not seen.

Immunostaining for GAP-43 showed an increased number of intra-epidermal nerve fibres, although this did not reach significance. Again thicker sub-epidermal fibres were seen adjacent to and coursing along the basal epidermis (Figure 4.28). GAP-43 was also seen in nerves supplying the sweat glands, blood vessels and within deep nerve fascicles in the deeper dermis as in the DPN group.
**CIPN group: Intra-epithelial nerve fibre (IENF) counts**

Results are shown for the total chemotherapy group, with sub-divisions also analysed in those patients who received Oxaliplatin chemotherapy and those patients who received other chemotherapy to look for any differences.

PGP 9.5 (15μm section thickness, Figure 4.25)

Patient (n=33) PGP 9.5 mean ± SEM IENF/mm (range) was 6.6 ± 0.7 (0.0-13.8). Out of 33, 2 patients had PGP 9.5 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values PGP 9.5 mean ± SEM IENF/mm (range) was 7.0 ± 0.7 (0.9-13.8).

Oxaliplatin patient group (n=15) PGP 9.5 mean ± SEM IENF/mm (range) was 6.7 ± 1.0 (0.9-13.8). Non-Oxaliplatin patient group (n=18) PGP 9.5 mean ± SEM IENF/mm (range) was 6.5 ± 1.0 (1.0-13.7). Out of 18, 2 patients in this group had PGP 9.5 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values PGP 9.5 mean ± SEM IENF/mm (range) was 7.3 ± 0.9 (2.8-13.7).

Control (n=11) PGP 9.5 mean ± SEM IENF/mm (range) was 6.1 ± 0.9 (1.6-11.3).

TRPV1(15μm section thickness, Figure 4.26)

Patient (n=33) TRPV1 mean ± SEM IENF/mm (range) was 4.6 ± 0.7 (0.0-17.5). Out of 33, 3 patients had TRPV1 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values TRPV1 mean ± SEM IENF/mm (range) was 5.0 ± 0.7 (0.5-17.5).

Oxaliplatin patient group (n=15) TRPV1 mean ± SEM IENF/mm (range) was 3.9 ± 0.8 (0.0–9.9). Out of 15, 1 patient in this group had a TRPV1 value of 0.0 IENF/mm which has been
included in the mean, excluding the zero value: TRPV1 mean ± SEM IENF/mm (range) was 4.2 ± 0.8 (0.5-9.9). Non-Oxaliplatin patient group (n=18) TRPV1 mean ± SEM IENF/mm (range) was 5.1 ± 1.0 (0.0-17.5). Out of 18, 2 patients in this group had TRPV1 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values TRPV1 mean ± SEM IENF/mm (range) was 5.8 ± 1.0 (1.3-17.5).

Control (n=13) TRPV1 mean ± SEM IENF/mm (range) was 5.9 ± 0.5 (2.0-8.7).

PGP 9.5 (30μm section thickness, Figure 4.27)
Patient (n=33) PGP 9.5 mean ± SEM IENF/mm (range) was 8.7 ± 0.9 (0.0-22.2). Out of 33, 1 patient had a PGP 9.5 value of 0.0 IENF/mm which has been included in the mean, excluding the zero value PGP 9.5 mean ± SEM IENF/mm (range) was 9.0 ± 0.9 (0.3-22.2). Control (n=16) PGP 9.5 mean ± SEM IENF/mm was 7.6 ± 0.9 (0.0-12.8), excluding one zero value control (n=15) PGP 9.5 mean ± SEM IENF/mm was 8.1 ± 0.8 (2.3-12.8).

Oxaliplatin patient group (n=15) PGP 9.5 mean ± SEM IENF/mm (range) was 8.5±1.1 (2.1-14.3). Non-Oxaliplatin patient group (n=18) PGP 9.5 mean ± SEM IENF/mm (range) was 8.9 ±1.5 (0.0-22.2). Out of 18, 1 patient in this group had PGP 9.5 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values PGP 9.5 mean ± SEM IENF/mm (range) was 9.5 ±1.5 (0.3-22.2).

Control (n=16) PGP 9.5 mean ± SEM IENF/mm was 7.6 ± 0.9 (0.0-12.8), excluding one zero value control (n=15) PGP 9.5 mean ± SEM IENF/mm was 8.1 ± 0.8 (2.3-12.8).

GAP-43 (30μm section thickness, Figure 4.28)
Patient (n=33) GAP-43 mean ± SEM IENF/mm (range) was 0.4 ±0.2 (0.0-4.1). Out of 33, 24 patients had GAP-43 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values GAP-43 mean SEM IENF/mm (range) was 1.5 ± 0.4 (0.3-4.1).

Oxaliplatin patient group (n=15) GAP-43 mean ± SEM IENF/mm (range) was 0.4 ± 0.3 (0.0-4.1). Out of 15, 12 patients in this group had GAP-43 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values GAP-43 mean ± SEM IENF/mm (range) was 2.0 ± 1.1 (0.8-4.1). Non-Oxaliplatin patient group (n=18) GAP-43 mean ± SEM IENF/mm (range) was 0.4 ± 0.2 (0.0-2.4). Out of 18, 12 patients in this group had GAP-43 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values: GAP-43 mean ± SEM IENF/mm (range) was 1.3± 0.3 (0.3-2.4). Control (n=17) GAP-43 mean ± SEM IENF/mm was 0.6 ± 0.3 (0.0-5.3), excluding 10 zero values control (n=7) GAP-43 mean ± SEM IENF/mm was 1.5 ± 0.7 (0.3-5.3).

A summary of the Oxaliplatin and Non-Oxaliplatin group first visit intra-epidermal nerve fibre counts is shown in Tables 2 and 3 respectively. A summary of total counts is shown in Table 4.7.
<table>
<thead>
<tr>
<th><strong>Oxaliplatin group (Section thickness µm)</strong></th>
<th><strong>Patients (n)</strong></th>
<th><strong>Mean ± SEM (range) IENF/mm including zero values</strong></th>
<th><strong>Patients (n) zero values excluded</strong></th>
<th><strong>Mean ± SEM (range) IENF/mm excluding zero values</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5 (15)</td>
<td>15</td>
<td>6.7 ± 1.0 (0.9-13.8)</td>
<td>15</td>
<td>6.7 ± 1.0 (0.9-13.8)</td>
</tr>
<tr>
<td>TRPV1 (15)</td>
<td>15</td>
<td>3.9 ± 0.8 (0.0-9.9)</td>
<td>14</td>
<td>4.2 ± 0.8 (0.5-9.9)</td>
</tr>
<tr>
<td>PGP 9.5 (30)</td>
<td>15</td>
<td>8.5 ± 1.1 (2.1-14.3)</td>
<td>15</td>
<td>8.5 ± 1.1 (2.1-14.3)</td>
</tr>
<tr>
<td>GAP-43 (30)</td>
<td>15</td>
<td>0.4 ± 0.3 (0.0-4.1)</td>
<td>3</td>
<td>2.0 ± 1.1 (0.8-4.1)</td>
</tr>
</tbody>
</table>

Table 4.7 CIPN oxaliplatin group: Summary of intra-epidermal nerve fibre counts from the first visit.

<table>
<thead>
<tr>
<th><strong>Non-oxaliplatin group (Section thickness µm)</strong></th>
<th><strong>Patients (n)</strong></th>
<th><strong>Mean ± SEM (range) IENF/mm including zero values</strong></th>
<th><strong>Patients (n) zero values excluded</strong></th>
<th><strong>Mean ± SEM (range) IENF/mm excluding zero values</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5 (15)</td>
<td>18</td>
<td>6.5 ± 1.0 (0.0-13.7)</td>
<td>13</td>
<td>7.3 ± 0.9 (2.8-13.7)</td>
</tr>
<tr>
<td>TRPV1 (15)</td>
<td>18</td>
<td>5.1 ± 0.7 (0.0-17.5)</td>
<td>16</td>
<td>5.8 ± 1.0 (1.3-17.5)</td>
</tr>
<tr>
<td>PGP 9.5 (30)</td>
<td>18</td>
<td>8.9 ± 1.5 (0.0-22.2)</td>
<td>17</td>
<td>9.5 ± 1.5 (0.3-22.2)</td>
</tr>
<tr>
<td>GAP-43 (30)</td>
<td>18</td>
<td>0.4 ± 0.2 (0.0-2.4)</td>
<td>6</td>
<td>1.3 ± 0.3 (0.3-2.4)</td>
</tr>
</tbody>
</table>

Table 4.8 CIPN non-oxaliplatin group: Summary of intra-epidermal nerve fibre counts from the first visit.

<table>
<thead>
<tr>
<th><strong>CIPN group (Section thickness µm)</strong></th>
<th><strong>Patients (n)</strong></th>
<th><strong>Mean ± SEM (range) IENF % area</strong></th>
<th><strong>Patients (n) zero values excluded</strong></th>
<th><strong>Mean ± SEM (range) IENF % area excluding zero values</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5 (15)</td>
<td>33</td>
<td>6.6 ± 0.7 (0.0-13.8)</td>
<td>31</td>
<td>7.0 ± 0.7 (0.9-13.8)</td>
</tr>
<tr>
<td>TRPV1 (15)</td>
<td>33</td>
<td>4.6 ± 0.7 (0.0-17.5)</td>
<td>30</td>
<td>5.0 ± 0.7 (0.5-17.5)</td>
</tr>
<tr>
<td>PGP 9.5 (30)</td>
<td>33</td>
<td>8.7 ± 0.9 (0.0-22.2)</td>
<td>32</td>
<td>9.0 ± 0.9 (0.3-22.2)</td>
</tr>
<tr>
<td>GAP-43 (30)</td>
<td>33</td>
<td>0.4 ± 0.2 (0.0-4.1)</td>
<td>9</td>
<td>1.5 ± 0.4 (0.3-4.1)</td>
</tr>
</tbody>
</table>

Table 4.9 CIPN group: Summary of intra-epidermal nerve fibre counts from the first visit.
**Figure 4.25**  
I Comparison of PGP 9.5 (15µm section thickness) in control, CIPN and DPN (vs control p=0.0003) groups  
II Photomicrographs at 15µm section thickness showing PGP 9.5 immunoreactive intra-epidermal nerve fibre staining (control, a patient who had received Oxaliplatin, a patient who had received Cisplatin, 2 patients who had received Carbo Taxol and a diabetic patient) for comparison, magnification x 40.
Figure 4.26  I Comparison of TRPV1 (15µm section thickness) in control, CIPN and DPN (vs control p<0.0001) groups. II Photomicrographs 15µm section thickness showing TRPV1 immunoreactive intra-epidermal nerve fibre staining (control, 2 patients who had received Oxaliplatin, 2 patients who had received Carbo Taxol and a diabetic patient) for comparison, magnification x 40.
Figure 4.27 I Comparison of PGP 9.5 (30µm section thickness) in control, CIPN and DPN (vs control p<0.0001) groups II Photomicrographs at 30µm section thickness showing PGP 9.5 immunoreactive intra-epidermal nerve fibre staining (control, 2 patients who had received Oxaliplatin, 2 patients who had received CarboTaxol and a diabetic patient) for comparison, magnification x 40.
Figure 4.28  I Comparison of GAP-43 (30µm section thickness) in control, CIPN and DPN groups. II Photomicrographs at 30µm section thickness showing GAP-43 immunoreactive intra-epidermal nerve fibre staining (control and patient who had received CarboTaxol) for comparison, magnification x 40.
CIPN group: *Sub-epidermal nerve fibre (SENF) counts*

PGP 9.5 (15μm section thickness, Figure 4.29)

Sub-epidermal nerve fibres for PGP 9.5 were not significantly different in the CIPN group in comparison with controls at 15μm section thickness. DPN SENF were significantly reduced when compared to the CIPN group (p<0.0001).

Patient (n=32) PGP 9.5 mean ± SEM SENF % area (range) was 1.09 ± 0.09 (0.34-2.60).
Control (n=12) PGP 9.5 mean ± SEM SENF % area was 1.00 ± 0.18 (0.35-2.64).

TRPV1 (15μm section thickness, Figure 4.30)

Sub-epidermal nerve fibres for TRPV1 were not significantly different in the CIPN group in comparison with controls at 15μm section thickness. DPN SENF were significantly reduced when compared to the CIPN group (p<0.0001).

Patient (n=33) TRPV1 mean ± SEM SENF % area (range) was 0.39 ± 0.06 (0.01-1.41). Out of 33, 1 patient had undetectable TRPV1 and was given a nominal value of 0.010 % area, which has been included in the mean, excluding the zero value TRPV1 mean ± SEM SENF % area (range) was 0.42 ± 0.06 (0.03 -1.41). Control (n=12) TRPV1 mean ± SEM SENF % area was 0.52 ± 0.11 (0.18-1.38).

PGP 9.5 (30μm section thickness, Figure 4.31)

Sub-epidermal nerve fibres for PGP 9.5 were significantly increased in the CIPN group in comparison with controls at 30μm section thickness (p=0.0138). DPN SENF were significantly reduced when compared to the CIPN group (p<0.0001).
Patient (n=33) PGP 9.5 mean ± SEM SENF % area (range) was 1.97 ± 0.13 (0.12-3.40).

Control (n=16) PGP 9.5 mean ± SEM SENF % area was 1.47 ± 0.13 (0.13-2.33).

GAP-43 (30μm section thickness Figure 4.32)
Sub-epidermal nerve fibres for GAP-43 were significantly increased in the CIPN group in comparison with controls at 30μm section thickness (p=0.0033). DPN SENF were significantly reduced when compared to the CIPN group (p<0.0001).

Patient (n=33) GAP-43 mean ± SEM % area (range) was 0.39 ± 0.05 (0.01-0.99). Out of 33, 1 patient had undetectable GAP-43 and was given a nominal value of 0.010 % area, which has been included in the mean, excluding the zero value GAP-43 mean SEM % area (range) was 0.40 ± 0.05 (0.02-0.99). Control (n=18) GAP-43 mean ± SEM % area was 0.17 ± 0.12 (0.01-0.37), excluding one zero value GAP-43 mean ± SEM % area was 0.18± 0.02 (0.01-0.93).

A summary of the diabetic first visit sub-epidermal nerve fibre counts is shown in Table 4.10.

<table>
<thead>
<tr>
<th>CIPN patient group (Section thickness µm)</th>
<th>Patients (n)</th>
<th>Mean ± SEM (range) SENF % area</th>
<th>Patients (n) zero values excluded</th>
<th>Mean ± SEM (range) SENF % area excluding zero values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5 (15)</td>
<td>32</td>
<td>1.09 ± 0.09 (0.34-2.60)</td>
<td>32</td>
<td>1.09 ± 0.09 (0.34-2.60)</td>
</tr>
<tr>
<td>TRPV1 (15)</td>
<td>33</td>
<td>0.39 ± 0.06 (0.01-1.41)</td>
<td>31</td>
<td>0.42 ± 0.06 (0.03-1.41)</td>
</tr>
<tr>
<td>PGP 9.5 (30)</td>
<td>33</td>
<td>1.97 ± 0.13 (0.12-3.40)</td>
<td>33</td>
<td>1.97 ± 0.13 (0.12-3.40)</td>
</tr>
<tr>
<td>GAP-43 (30)</td>
<td>33</td>
<td>0.39 ± 0.05 (0.01-0.99)</td>
<td>32</td>
<td>0.40 ± 0.05 (0.02-0.99)</td>
</tr>
</tbody>
</table>

Table 4.10 CIPN group: Summary of sub-epidermal nerve fibre counts.
Figure 4.29 CIPN group: Sub-epidermal nerve fibres per mm length of tissue section PGP 9.5 (15μm thickness), DPN vs. control (p=0.0012).

Figure 4.30 CIPN group: Sub-epidermal nerve fibres per mm length of tissue section TRPV1, DPN vs. control (p<0.0001).
Figure 4.31 CIPN group: Sub-epidermal nerve fibres per mm length of tissue section PGP 9.5 (30μm thickness), CIPN vs. control (p=0.0138), DPN vs. control (p=0.0051).

Figure 4.32 CIPN group: Sub-epidermal nerve fibres per mm length of tissue section GAP-43, CIPN vs. control (p=0.0033), DPN vs. control (p<0.0001).
CIPN group: Correlations

Histamine Flare area correlated significantly with PGP 9.5 intra-epidermal nerve fibre counts (Figure 4.33).

![Graph showing correlation between Flare Area (cm²) and PGP 9.5 IENF/mm](image)

**Figure 4.33** CIPN group: Chemotherapy-induced neuropathy group: Histamine flare area was significantly correlated with PGP 9.5 IENF/mm counts ($r=0.326$, $p=0.00455$).

TRPV1 intra-epidermal nerve fibre counts did not correlate significantly with histamine flare area. Contact Heat Evoked Potentials ($Aδ$) N2-P2 from Leg, Arm and Face did not correlate significantly with PGP 9.5 or TRPV1 intra-epidermal nerve fibre counts.

4.2.2 CIPN group: Follow up visits

Figures shown below are for general information. Given the small number (n=10) of patients who attended all three visits, results were not analysed.
CIPN group follow up: Quantitative Sensory Testing

**Figure 4.34** CIPN group follow up (n=10): Touch perception (values >No. 3 monofilament (0.0479 g) were considered abnormal and Vibration threshold (values >12V were considered abnormal).
Figure 4.35 CIPN group follow up (n=10): Thermal thresholds for a) Cool, b) Warm, c) Heat pain and d) Cold pain. Values >6.4°C for warm sensation, >2.3°C for cool sensation and >10.4 °C for heat pain, were considered abnormal.
CIPN group follow up: Histamine Flare area and Sweat rate

![Histogram of Flare area and Sweat rate](image)

**Figure 4.36** CIPN group follow up: Histamine flare area (n=8) and sweat rate (n=6). Abnormal skin flare was taken to be <14.9 cm² and abnormal sweat rate values <10 g/m²/h were considered abnormally low and >30 g/m²/h considered abnormally high.

CIPN group follow up: Contact Heat Evoked Potentials

Out of 15, 6 patients had repeat contact heat evoked potential measurement, making comparison of repeat testing difficult. Many patients declined to have contact heat evoked potentials recorded because they felt it was too uncomfortable. Others did not like the discomfort on the scalp following hair loss with chemotherapy and some who were wearing wigs did not wish to remove them. Others felt the length of time taken to perform CHEPS was too great given the amount of time already spent in hospital having chemotherapy, follow up and the other tests.

CIPN group follow up: Intra-epithelial nerve fibre (IENF) counts

Repeat skin punch biopsy was performed in 13 patients; taken after 6 months in 5 patients who had been treated with chemotherapy previously and in 8 patients after 3 months, at the
end of chemotherapy treatment. Results are presented together for general information, there were no discernable trends found during analysis of either group separately

PGP 9.5 (15μm section thickness, Figure 4.37)
Analysis of paired repeat samples (n=13) did not show a significant difference in the mean intra-epidermal nerve fibre count.

Patient (n=13) PGP 9.5 Mean ± SEM (range) IENF/mm at the 1st visit 6.9 ± 1.2 (0.0-13.8). 1 patient had a PGP 9.5 value of 0.0 IENF/mm which has been included in the mean, excluding the zero value PGP 9.5 Mean ± SEM (range) IENF/mm at the 1st visit 7.5 ± 1.1 (0.9-13.8).
Patient (n=13) PGP 9.5 Mean ± SEM (range) IENF/mm at the 3rd visit 5.7 ± 0.9 (0.4-11.6).

TRPV1 (15μm section thickness, Figure 4.37)
Analysis of paired repeat samples (n=13) did not show a significant difference in the mean intra-epidermal nerve fibre count.

Patient (n=13) TRPV1 Mean ± SEM (range) IENF/mm at 3.9 ± 0.9 (0.0-9.9)
2 patients had TRPV1 values of 0.0 IENF/mm which have been included in the mean, excluding the zero values TRPV1 Mean ± SEM (range) IENF/mm at the 1st visit 4.6 ± 0.9 (1.1-9.9). Patient (n=13) TRPV1 Mean ± SEM (range) IENF/mm at the 3rd visit 4.8 ± 1.1 (0.1-13.5)

GAP-43 (30μm diameter section thickness)
Patient (n=13) GAP-43 Mean ± SEM (range) IENF/mm at the 1st visit 0.2 ± 0.1 (0.0-1.7). 11 patients had GAP-43 value of 0.0 IENF/mm which have been included in the mean,
excluding the zero values GAP-43 Mean ± SEM (range) IENF/mm at the 1st visit 1.3 ± 0.5 (0.8-1.7). There were no intra-epidermal nerve fibres present in this patient group (n=13) at the 3rd visit.

A Summary of chemotherapy-induced neuropathy patient intra-epidermal nerve fibre counts is shown in Table 4.1.

<table>
<thead>
<tr>
<th>CIPN patient group (Section thickness µm)</th>
<th>Patients (n)</th>
<th>Mean ± SEM (range) IENF/mm including zero values</th>
<th>Patients (n) zero values excluded</th>
<th>Mean ± SEM (range) IENF/mm excluding zero values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5 (15) 1st visit</td>
<td>13</td>
<td>6.9 ± 1.2 (0.0-13.8)</td>
<td>12</td>
<td>7.5 ± 1.1 (0.9-13.8)</td>
</tr>
<tr>
<td>PGP 9.5 (15) 3rd visit</td>
<td>13</td>
<td>5.7 ± 0.9 (0.4-11.6)</td>
<td>13</td>
<td>5.7 ± 0.9 (0.4-11.6)</td>
</tr>
<tr>
<td>Control PGP 9.5 (15)</td>
<td>11</td>
<td>6.1 ± 0.9 (1.6-11.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRPV1 (15) 1st visit</td>
<td>13</td>
<td>3.9 ± 0.9 (0.0-9.9)</td>
<td>11</td>
<td>4.6 ± 0.9 (1.1-9.9)</td>
</tr>
<tr>
<td>TRPV1 (15) 3rd visit</td>
<td>13</td>
<td>4.8 ± 1.1 (0.1-13.5)</td>
<td>3</td>
<td>4.8 ± 1.1 (0.1-13.5)</td>
</tr>
<tr>
<td>Control TRPV1 (15)</td>
<td>13</td>
<td>5.9 ± 0.5 (2.0-8.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAP-43 (30) 1st visit</td>
<td>13</td>
<td>0.2 ± 0.1 (0.0-1.7)</td>
<td>2</td>
<td>1.3 ± 0.5 (0.8-1.7)</td>
</tr>
<tr>
<td>GAP-43 (30) 3rd visit</td>
<td>13</td>
<td>0.0 ± 0.0</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Control GAP-43 (30)</td>
<td>17</td>
<td>0.6 ± 0.3 (0.0-5.3)</td>
<td>7</td>
<td>1.5 ± 0.7 (0.3-5.3)</td>
</tr>
</tbody>
</table>

Table 4.1 CIPN group follow up: Summary of intra-epidermal nerve fibre counts.
Figure 4.37 CIPN group follow up (n=13): Intra-epiderma nerve fibres per mm length of tissue section. Control mean values ± SEM are indicated by dotted rectangle.
5.0 Discussion

5.1 DPN group

This study has shown a lack of significant progression in clinical symptoms, Neuropathy Impairment score (Lower Limbs) and sensory tests over one year in a cohort of patients with diabetic polyneuropathy (DPN), confirming previous findings in placebo arms of clinical trials (Dyck et al. 2007; Tesfaye et al. 2007; Ziegler et al. 2011). Baseline sensory tests were in keeping with expected values and significant reductions in all markers (PGP 9.5, TRPV1 and GAP-43) were seen compared with healthy controls at baseline, again as expected (Kennedy et al. 1996; Coppini et al. 2001; Face et al. 2007).

A significant decrease in intra- and sub-epidermal nerve fibres was found in patients with diabetic sensory neuropathy after 6 months for PGP 9.5 and TRPV1 immunoreactive fibres. This decrease has been quantified in this study and the rate of loss of IENF calculated to be 0.010 ± 0.004 fibres/mm/day or 3.76 ± 1.46 mm/year for PGP 9.5, and 0.009 ± 0.002 fibres/mm/day or 3.13 ± 0.58 mm/year for TRPV1. Repeat skin biopsy in a small select group of patients with painful diabetic neuropathy, has been studied once previously where there was no intervening intervention. The study was performed in 15 patients with persistent pain in their feet, and included patients with several different aetiologies of neuropathy, six of whom had diabetes. Skin biopsy was repeated after a mean of 19.2 months (range of 12-28 months). A decrease in intra-epidermal nerve fibre counts was detected in all patients. A crude extrapolation of their published data would give a loss of intra-epidermal fibres of 2.14 mm/year, which is of the same order as what this study has shown.
Interestingly, the rate of IENF regeneration following topical capsaicin application in (non-neuropathic) diabetic skin has been reported as 0.074 fibres/mm/day, and 0.04 fibres/mm/day in diabetic neuropathy (Polydefkis et al. 2004). The rate of spontaneous IENF degeneration in patients with diabetic neuropathy thus appears slower, but of similar magnitude to IENF regeneration after capsaicin application.

CHEPS amplitudes were reduced as expected from previous studies (Atherton et al. 2007; Chao et al. 2010). CHEPS face stimulation amplitudes correlated significantly with PGP 9.5 and TRPV1 IENF. As lumbosacral and cervical spondylosis with associated radiculopathy are common and may reduce limb stimulation-evoked potentials, face stimulation may provide an objective, sensitive and non-invasive marker in future trials. Heat evoked cerebral potentials have also been shown to track GAP-43 SENF following topical capsaicin in human volunteers (Rage et al. 2010). Histamine skin flare area was reduced in this group and correlated with PGP 9.5 IENF, confirming that it is another useful objective non-invasive measure of small sensory fibre dysfunction. In this population of patients with mostly painful neuropathy it is interesting to note that sudomotor function was intact.

The diabetic cohort studied were volunteers from a general hospital, diabetic outpatient clinic and as such included those with both type 1 and type 2 diabetes, early and more advanced DPN. This should be borne in mind when interpreting the findings. It is interesting to note that almost twice as many men volunteered for the study as compared to women which may in part be related to findings that diabetic neuropathy is more common in males (Adler et al. 1997).
The majority of patients (85%) in this study complained of pain, which is higher than other reports; for example, Daousi et al. report 16-26% prevalence of chronic neuropathic pain in diabetic patients (Daousi et al. 2004). The presence of pain could have been a motivational factor in taking part in the study and may explain the high prevalence in this group. Further studies in those patients without painful diabetic neuropathy and those with diabetes, but without neuropathy deserve study. Reassuringly glycaemic control as measured by HbA1c did not vary significantly over the year. This is important given the pathogenic implications of glycaemia and the fact that change in HbA1c was thought to contribute to change in outcome during a trial previously (Ziegler et al. 2011).

This study shows that measuring sub-epidermal nerve fibres is practical and useful in patients with diabetic neuropathy. Out of 29 patients where sub-epidermal fibres were stained with PGP 9.5, only 4 had zero values, whereas with intra-epidermal nerve fibres, the standard method, 20 patients had zero values. Measuring sub-epidermal fibres will allow more patients to be included in future trials. In addition, this method allows the quantification of GAP-43 fibres which were only present in one out of 29 patients in the epidermal layer. Out of 29, 21 patients had detectable GAP-43 in the sub-epidermal layer. This finding will be very useful in future trials of regenerative agents. GAP-43 fibres did not significantly change over 6 months and this may reflect ongoing degeneration/regeneration.

Previously it has been shown that a decrease in TRPV1 immunoreactivity, which is regulated by nerve growth factor (NGF), preceded a decrease in PGP 9.5 innervation, and that NGF is itself decreased in diabetic neuropathy (Anand et al. 1996). TRPV1 IENF may thus be a particularly useful marker in future clinical trials with rhNGF.
There are many new agents being developed which may prevent degeneration or encourage regeneration of nerve fibres in patients with neuropathy such as NGF agonists (Colangelo et al. 2008), agents that enhance endogenous NGF production such as Neotrofin (Calcutt et al. 2006), and trials of other neurotrophic factors such as Enovin (Masure et al. 1999). Given the lack of marked success of trials of neurotrophic agents so far, any putative intervention should be given the best possible chance of showing efficacy in human clinical trials by optimizing study design. A number of studies have so far been performed that show little or no change in neuropathy. The information gleaned from these studies (Dyck et al. 1997; Apfel et al. 2001; Dyck et al. 2007; Tesfaye et al. 2007), along with observations from this study should be collated to provide the best possible chance of a positive outcome being achieved.

Demographical and clinical information is important to collect given that age, sex, height, blood pressure, smoking, type of diabetes, presence of retinopathy, and presence of microalbuminuria have an impact (Tesfaye et al. 1996). A clear definition of neuropathy must be used for inclusion, such as in this study (Dyck 2003) and a measure of severity such as that proposed by Dyck (Dyck 2003) would be useful. Duration of neuropathy (presence of symptoms) and duration of diabetes, although not correlated here, do reflect severity of neuropathy and must be recorded (Shun et al. 2004). Glycaemic control and lipid profile are also important in pathogenesis and must be recorded, with glycaemia monitored during the trial (Tesfaye et al. 2010). In order to prevent variation in HbA1c, patients with well-controlled, stable diabetes, for example those with insignificant variation in HbA1c in the last 6-12 months could be considered.
In terms of study design, a dropout rate of approximately 25% should be considered as experienced in this study, when performing power calculations to estimate numbers of participants. A placebo arm is vital to assess the natural history of the process being modified and will add to the data gathered in this study. Study visits as set out in this study were feasible and practical. The assessments detailed here tested a wide range of sensory nerve fibres and should be included. Contact heat evoked potential measurement was time-consuming, but measurement at the face could be included as this yielded the most information.

The most obvious measures of the effect of an intervention would be seen in patients with early diabetic neuropathy as quite simply they have more nerve fibres present to show a change (Shun et al. 2004). Those with a higher number of detectable intra and/or sub-epidermal fibres were also those who tended to have a shorter duration of diabetes and also positive symptoms, although not statistically significant with the numbers in this study. Demographical, clinical information and sensory tests provide an indication of which patients are likely to have early neuropathy, but from this study it would appear that it is not until results of the skin biopsies are seen that the degree of nerve damage is clear. Patients could be stratified into different groups on the basis of their baseline skin biopsy. Few patients with diabetic neuropathy have detectable intra-epidermal nerve fibres, but we have still shown a meaningful change over 6 months, which is a practical time period for an intervention trial.

Repeat skin punch biopsy is not ideal, given the invasiveness of the test, but is warranted, at least initially, to show efficacy. Repeat skin biopsy in the lower lateral calf was very well tolerated in this study with no adverse events. The fact that changes in IENF and SENF were present without a change in QST may explain why trials so far have seemed to be ineffective.
As skin biopsy was not performed, initial changes may not have been detected, and the trial drug abandoned rather than developed. Non-invasive tests did not correlate longitudinally in this study. Corneal confocal microscopy has been shown to be a non-invasive measure of intra-epidermal nerve fibres (Quattrini et al. 2007). It is not clear whether it would correlate with staining for nerve fibres other than PGP 9.5 or sub-epidermal fibres. Also, intervention may change the morphology of nerve fibres which would only be seen when assessed directly (Lauria et al. 2003; Gibbons et al. 2006), so this technique is not likely to be useful in the initial investigation of new treatments.

There are some limitations to a descriptive longitudinal study, such as this. Results need to be interpreted in terms of the characteristics of cohort studied, as already highlighted. Patients were taking analgesia and new treatments or optimisation of dose were undertaken during the study as part of standard care. This needs to borne in mind when interpreting the findings, particularly of results of pain scores and symptom assessment; however there was no significant difference in pain score overall, despite therapy.

In summary, this study suggests PGP 9.5 and TRPV1 IENF and SENF in skin biopsies are useful markers of progression in DPN, while GAP-43 SENF could potentially detect nerve fibre regeneration in future trials of disease-modifying agents.
5.2 CIPN group

This study has shown the presence of a length-dependent, symmetrical, predominantly sensory neuropathy in patients who had prior treatment with chemotherapy, confirming findings in other studies (Grunberg et al. 1989; Lipton et al. 1989; Rowinsky et al. 1993; Hilkens et al. 1997; Quasthoff et al. 2002). Despite treatment with different chemotherapeutic agents, and testing carried out at various time points post-chemotherapy in this cross-sectional study, the clinical symptoms were similar across the group indicating that taxol and platinum based-chemotherapeutic agents seem to produce a similar sensory polyneuropathy.

The DPN and CIPN groups were similar in terms of age, however it is worth noting that the majority of chemotherapy patients were female (82%), compared to the diabetic group in which the majority were male (65%). The female preponderance in the CIPN group relates to the fact that patients were recruited mainly from clinics treating ovarian cancer. The two groups also differ in terms of duration of symptoms. The DPN group had a significantly longer duration of symptoms (4.0 years) compared with the CIPN group (9.3 months), although the latter is a measure of time since last chemotherapy treatment. Despite these differences, both groups had a similar range of symptoms and a significant proportion of both groups reported neuropathic pain, although the Likert and McGill pain scores were significantly higher in the DPN group. Interestingly, the Histamine flare area results showed that a greater proportion of the CIPN group (64%) compared with the DPN group (37%) had abnormal values. This may indicate greater involvement of a specific subset of C-fibres in the CIPN group. Nerve conduction studies showed more frequent abnormalities of sural sensory amplitude reduction than peroneal motor amplitude reduction. This indicates a
mainly sensory axonal polyneuropathy, and is in keeping with previous studies (Hilkens et al. 1997).

Contact heat evoked potentials showed a reduction from each site, leg, arm and face; however, the reduction reached significance only from the arm. A lack of significance from the leg is surprising given the length-dependent nature of the neuropathy, but may relate to the large standard error measurement in the leg control group, which in turn may be the result of lumbar spondylosis.

Intra- and sub-epidermal nerve fibres have been quantified in patients with CIPN for the first time. A reduction of skin innervation was expected given that the symptoms, signs and sensory tests in patients with CIPN were similar to patients with DPN, although those with DPN were presumed to have more advanced neuropathy. Two studies that had investigated PGP 9.5 IENF in rats have also shown a decrease (Siau et al. 2006; Liu et al. 2010). This study has demonstrated relative preservation of intra-epidermal nerve fibres and an increase in sub-epidermal nerve fibres in patients with chemotherapy-induced neuropathy. Usually abnormal sensory tests are associated with nerve fibre loss. Here, fibres were detected and even increased in the sub-epidermal layer. These nerve fibres must, therefore, be dysfunctional in some way.

One of the striking findings in this study relates to the morphological changes seen in the sub-epidermal layer. These thick fibres could indicate regenerating fibres as they showed immunostaining for GAP-43, a marker of nerve regeneration. This may be an explanation for the phenomenon known as ‘coasting’, in which neuropathy can develop or worsen after cessation of the chemotherapeutic agent. The mechanism for this has not previously been
understood, but was thought potentially to relate to accumulation of the chemotherapeutic agent in the DRG (Grunberg et al. 1989; Quasthoff et al. 2002; Windebank et al. 2008). It could be postulated that ongoing changes in the micro-environment of the DRG, or epigenetic changes, may result in the persistent abnormal regeneration. Presumably after the cessation of chemotherapy, once the DRG has recovered, normal regeneration occurs and the neuropathy therefore resolves. It would be interesting in a future study to look longitudinally at changes in intra- and sub-epidermal innervation in patients receiving chemotherapy, and to correlate symptoms and sensory tests with changes in intra- and sub-epidermal fibres.

Given the unique neurotoxic action of oxaliplatin (Binder et al. 2007) it was felt that it would be worth looking at this group of patients separately. There was no difference observed in this subset of patients in the clinical tests, although these tests were not carried out during the acute phase of symptoms after oxaliplatin infusion. A difference was found however when looking at intra-epidermal nerve fibres stained for the heat and capsaicin receptor TRPV1, which showed a significant decrease in number compared with controls (p=0.0476). This subset of patients is the only one to have shown a decrease in intra-epidermal nerve fibre count in the chemotherapy group. This is interesting because oxaliplatin has marked acute effects causing heat and cold hyperalgesia in patients, and in an animal models oxaliplatin treatment has been shown to cause TRP sensitization (Anand et al. 2010). This needs to be studied further.

Histamine flare area was found to be a sensitive biomarker of intra-epidermal nerve function as it correlated with PGP 9.5 immunostaining in both groups.
A longitudinal study was not successfully carried out due to lack of patients able and willing to participate. Clinical studies are particularly difficult in this group of patients when participation is not directly related to treatment for cancer. Patients are already spending extended periods of time at hospital for treatment, investigation and follow up, plus numerous visits for blood tests and radiological investigation. Skin biopsy was well tolerated in this group, despite use of chemotherapeutic agents however, the majority of patients were recruited once the course of chemotherapy was finished. Ten patients attended all three visits and were followed up over 6 months. There was no significant change over this time in this small number. Histamine flare area seemed to increase by the second visit, although this was not a significant increase. Repeat skin biopsies were difficult to interpret given the cross-sectional nature of patients included and in future studies patients at a similar chronological stage post-chemotherapy treatment should be included for follow up.

A number of agents have been shown to alleviate acute symptoms or improve some electrophysiological measures of neuropathy (Penz et al. 2001; Bianchi et al. 2005; Flatters et al. 2006; Gibson et al. 2006; Lu et al. 2008), mostly in animal models; however, further studies are needed to investigate their effect on epidermal innervation, now that these significant abnormalities have been found, and to elucidate their long term effects.

In summary, despite abnormal sensory function in both the DPN and CIPN groups, we see a relatively preserved number of intra-epidermal nerve fibres and even an increase in sub-epidermal nerve fibres in the CIPN group. It can therefore be inferred that the intra- and sub-epidermal nerve fibres must be dysfunctional in some way. Rather than aiming for treatment with a regenerative neurotrophic agent, research should be directed towards prevention of
degeneration initially and preservation of function, though the latter may be more difficult to achieve.

For future studies, important lessons can be learned. The least number of tests possible should be arranged, as length of study appointment was a major factor in declining participation to the study. Contact heat evoked potential measurement may not be feasible given time pressures, and constraints such as the wearing of wigs post-chemotherapy.

A prospective study in chemotherapy-naive patients would be the ultimate aim, and very useful in determining when the changes found in this study start to occur. Numbers of patients recruited could be improved by involving patients from several centres, but testing performed at one site.
6.0 Conclusions

This study has indicated that immunostaining of nerve markers in skin biopsies over a six month period in patients with DPN is sufficient to show changes in PGP 9.5 and TRPV1 intra- and sub-epidermal nerve fibres. These are useful markers of progression in DPN, whilst preserved GAP-43 sub-epidermal nerve fibres could potentially detect nerve fibre regeneration.

Despite abnormal sensory function in both the DPN and CIPN groups, there are a relatively preserved number of fibres with abnormal morphology in the CIPN group, which has been shown for the first time. It can therefore be inferred from the sensory test findings that the intra- and sub-epidermal nerve fibres in these patients must be dysfunctional. This study indicates that the treatment strategy should be directed towards restoration of function in patients with chemotherapy-induced neuropathy, rather than enhancing regeneration of nerve fibres as in patients with DPN.
References


Appendices

Appendix A

A copy of the short-form McGill pain questionnaire (SF-MPQ) \cite{Melzack87}.

Appendix IV (i)

SHORT FORM McGill Pain QUESTIONNAIRE and PAIN DIAGRAM

(Reproduced with permission of author © Dr. Ron Melzack, for publication and distribution)

Date: ____________________________

Name: ___________________________

Check the column to indicate the level of your pain for each word, or leave blank if it does not apply to you._

<table>
<thead>
<tr>
<th></th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Throbbing</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>2</td>
<td>Shooting</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>3</td>
<td>Stabbing</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>4</td>
<td>Sharp</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>5</td>
<td>Cramping</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>6</td>
<td>Gnawing</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>7</td>
<td>Hot-burning</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>8</td>
<td>Aching</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>9</td>
<td>Heavy</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>10</td>
<td>Tender</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>11</td>
<td>Splitting</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>12</td>
<td>Tiring, Exhausting</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>13</td>
<td>Sickening</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>14</td>
<td>Fearful</td>
<td>———</td>
<td>———</td>
</tr>
<tr>
<td>15</td>
<td>Cruel, Punishing</td>
<td>———</td>
<td>———</td>
</tr>
</tbody>
</table>

Mark or comment on the sparse figure where you have your pain or problems.

Indicate on this line how bad your pain is—at the left end of line means no pain at all, at right end means worst pain possible.

<table>
<thead>
<tr>
<th>No Pain</th>
<th>Worst Possible Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 /33</td>
<td>A /12 VAS5 /10</td>
</tr>
</tbody>
</table>
Appendix B

The Neuropathy Impairment Score Lower Limbs (NIS-LL) is shown here (Dyck et al. 1997; Bril 1999).

**NEUROPATHY IMPAIRMENT SCORE (NIS)**

**OBJECTIVE:** To provide a single score of neuropathic deficits and subset scores: cranial nerve, muscle weakness, reflexes and sensation. Abnormalities are abstracted from a neurologic examination in which all of the assessments are made.

**SCORING:** The examiner scores deficits by what he (she) considers to be normal considering test, anatomical site, age, gender, height, weight, and physical fitness.

**SCORING, MUSCLE WEAKNESS**

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NORMAL</td>
</tr>
<tr>
<td>1</td>
<td>25% WEAK</td>
</tr>
<tr>
<td>2</td>
<td>50% WEAK</td>
</tr>
<tr>
<td>3</td>
<td>75% WEAK</td>
</tr>
<tr>
<td>3.25</td>
<td>MOVE AGAINST GRAVITY</td>
</tr>
<tr>
<td>3.5</td>
<td>MOVEMENT, GRAVITY ELIMINATED</td>
</tr>
<tr>
<td>3.75</td>
<td>MUSCLE FLICKER, NO MOVEMENT</td>
</tr>
<tr>
<td>4</td>
<td>PARALYSED</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RIGHT</th>
<th>LEFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>0</td>
</tr>
</tbody>
</table>

**Muscle Weakness**

17. Hip flexion
18. Hip extension
19. Knee flexion
20. Knee extension
21. Ankle dorsiflexors
22. Ankle plantar flexors
23. Toe extensors
24. Toe flexors

Subset Score: ________
NEUROPATHY IMPAIRMENT SCORE (NIS)

For patients 50-69 years old, ankle reflexes which are decreased are graded 0 and when absent are graded 1. For patients ≥70 years, absent ankle reflexes are graded 0.

### SCORING, REFLEXES

<table>
<thead>
<tr>
<th>Reflexes</th>
<th>RIGHT</th>
<th>LEFT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>28. Quadriceps femoris</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>29. Triceps surae</td>
<td>○</td>
<td>○</td>
</tr>
</tbody>
</table>

Subset Score: __________

Touch-pressure, pin-prick and vibration sensation are tested on the dorsal surface, at the base of the nail, of the terminal phalanx of the index finger and great toe. Touch-pressure is assessed with a long fiber cotton wool. Pin-prick is assessed with straight pins. Vibration sensation is tested with a 165Hz tuning fork (V. Mueller, Chicago, length 25 cm, made from 1/2" x 1 1/4" stock; 165Hz with counterweights). Joint motion is tested by moving the terminal phalanx of the index finger and great toe.

### SCORING, SENSATION

<table>
<thead>
<tr>
<th>Sensation - G. Toe</th>
<th>RIGHT</th>
<th>LEFT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>34. Touch pressure</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>35. Pin-prick</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>36. Vibration</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>37. Joint position</td>
<td>○</td>
<td>○</td>
</tr>
</tbody>
</table>

Subset Score: __________

Total Score: __________
Appendix C

Assessment of Neuropathy

1. Semmes-Weinstein Monofilaments. 2. Biosthesiometer used to measure vibration sense.

3. Equipment for measuring thermal thresholds. Paddle that is placed under the sole of the foot is on the top left of picture.

4. Evaporimeter used for sweat assessment.

5. Images produced by Laser Doppler Imaging; control on left and patient with DPN on right.
6. Equipment needed to record contact heat evoked potentials. CHEPS machine is on left, paddle placed on skin is shown on top right. This produces a sensation as if being touched by a ‘hot dinner plate’ in those with normal sensation. Electrodes are placed on the scalp in order to detect the evoked potentials.

7. An example of an evoked potential recorded after stimulation of the Face of a person without neuropathy. The amplitude is a measure of the transmitted signal from skin to the brain.
8. A picture showing the lateral calf of the right leg and area where two 3mm skin punch biopsies were taken (marked by crosses). The punch biopsies allow a core of skin a few millimetres deep to be taken. The biopsy encompasses the epidermis, dermis and a little subcutaneous tissue.

9. A cross-section through a skin biopsy section at x40 magnification. Intra-and Sub-epidermal nerve fibres immunoreactive for PGP 9.5, a pan-neuronal marker are stained black.
Appendix D

Manuscript accepted in December 2011 for publication in the Journal of Clinical Neuroscience.

Sensory biomarkers of progression in diabetic peripheral neuropathy: a longitudinal clinical study using skin biopsies

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Sources of support in the form of grants:

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Abstract
Aims: Identification of biomarkers in skin punch biopsies which could be used to monitor progression of diabetic peripheral neuropathy (DPN), and to assess efficacy of agents that may reduce progression in future studies. Methods: Patients with DPN were studied with clinical assessments, skin biopsies, quantitative sensory testing (QST), histamine-induced skin flare, nerve conduction studies and contact heat evoked potentials (CHEPS). Skin biopsies were performed on 2 visits with a 6 month interval (n=29 patients) to quantify intra- (IENF) and sub-epidermal (SENF) nerve fibres immunoreactive for PGP9.5 (pan-neuronal marker), TRPV1 (heat and capsaicin receptor) and GAP-43 (marker of regenerating fibres). IENF were counted along the length of four non-consecutive sections, and results expressed as fibres/mm length of section. SENF were measured by image analysis, and the area of highlighted immunoreactivity obtained as a percentage (% area) of the field scanned. QST, skin flare and CHEPS were also performed at the 2 visits. Results: There was a statistically significant reduction in IENF and SENF for both PGP9.5 and TRPV1 between the first and second skin biopsy over 6 months. The rate of IENF loss was 3.76±1.46 fibres/mm/yr for PGP9.5, and 3.13±0.58 fibres/mm/yr for TRPV1. The other tests did not show significant changes. Strongly positive GAP-43 nerve fibres were found in deep dermis in the diabetic patients, even in those with absence of IENF. Conclusions: PGP9.5 and TRPV1 IENF and SENF in skin biopsies are useful markers of progression in DPN, while GAP-43 SENF could potentially help detect nerve regeneration in severe neuropathy.

Keywords: diabetes, neuropathy, skin biopsy, IENF, biomarker
Introduction

Diabetic peripheral neuropathy (DPN) is a common complication of Diabetes mellitus, particularly distal symmetric polyneuropathy. The prevalence of DPN may range between 30% and 54%\(^1,2\). The clinical spectrum can vary from asymptomatic or mild in the majority to a severe, painful and disabling condition. Treatments are available to alleviate pain in DPN, but these do not impact on progression of the polyneuropathy.

A number of studies have investigated the risk factors associated with DPN, and the underlying pathological changes. There are, however, no interventions currently available to reverse or halt the development of the condition, apart from improving glycaemic control, which is effective to only a small degree\(^3-5\). Clinical trials with neuroprotective agents including neurotrophic factors have failed to show meaningful efficacy, perhaps because more sensitive and robust objective markers are needed to detect changes produced by the relatively short duration of clinical trials. Recombinant human nerve growth factor trials showed an improvement in global symptom assessment, but failed to show a significant change in neuropathy impairment score or quantitative sensory testing\(^6,7\). Recombinant human brain-derived neurotrophic factor was trialled in our Unit\(^8\); a sub-set of patients showed an improvement of cool detection threshold. Thus, despite the known biological roles of neurotrophic factors and their efficacy in animal models\(^9-11\), these trials have not shown a significant clinical benefit in the treatment of DPN. A number of factors may explain this, including lack of suitable biomarkers, and have recently been discussed elsewhere\(^12,13\).

Small unmyelinated nerve fibres in the skin can show the earliest signs of damage and regeneration in patients with diabetes and impaired glucose tolerance\(^14-17\). IENF are therefore
potentially good biomarker candidates, particularly as their regulatory neurotrophic growth factors are available for clinical trials. Endogenous neurotrophic factors have been shown in animal models and humans to be important for the survival, maintenance and phenotypic expression of small sensory nerve fibres in skin \textsuperscript{9-11, 18}.

In this longitudinal study, we have examined skin biopsy markers at first visit and six months later with tests of sensory nerve function in patients with DPN.

**Patients and Methods**

Patients

Patients (n = 29) with either type 1 or type 2 diabetes mellitus and a history of symptomatic distal symmetric sensory polyneuropathy (affecting lower limbs) for at least 6 months, confirmed on clinical examination and/or sensory testing, were studied. Written informed consent was obtained from all patients in the study, which had approval from the Charing Cross Research Ethics Committee, London. Patient demographics and characteristics are shown in Table 1. All patients fulfilled criteria for neuropathy outlined by Dyck \textsuperscript{19}.

Clinical symptoms and pain scores

Patients described a number of symptoms in their lower limbs, most commonly numbness, pins and needles, tingling and burning pain. Of the 29 patients, 25 reported pain in their feet and 12 were taking treatment for neuropathic pain at the start of the study (Gabapentin, Pregabalin, Amitriptyline, Tramadol, Oxycontin or a combination of these). If required, patients were commenced on analgesia during the study as part of their care (n = 5) and others had optimization of analgesia. There were no overall differences in test results in the group taking analgesia treatment. The mean ± SEM (range) Likert pain score at the first visit was 5.0 ± 0.6 (0-10) (Figure 1). The mean ± SEM (range) McGill pain questionnaire score at
the first visit was 16.8 ± 2.4 (0.0-47). There was no significant change in pain scores in those tested over 6 months.

Clinical Examination

Clinical examination and tests confirmed that patients had a predominantly sensory, length-dependent neuropathy. Distal weakness (lower limbs) was present in 12/29 patients at MRC grade 4/5 or less. Neurological deficits were recorded using the Neuropathy Impairment Score Lower Limbs \(^{20}\) which is a summed score of muscle power, reflex loss and sensation loss (maximum score 88, indicating severe neuropathy). The mean ± SEM NIS-LL (range) at the first visit was 13.6 ± 1.9 (2.0-48.0) with no significant change in score after 6 months (Figure 1). All patients had palpable peripheral pulses at the first visit.

Assessment of neuropathy

Two study visits were performed at first visit (0 months) and 6 months in 29 patients. Clinical examination and tests were performed in both lower limbs, and the right lower limb values were used for analyses. All tests were carried out in the same clinic room by the same assessor in a standardized manner as described below. Nerve conduction studies were performed in the standard manner by a senior consultant neurophysiologist, with controlled room and limb temperature. The following assessments and tests were carried out at each study visit unless otherwise stated:
Symptoms were recorded using the short-form McGill Pain Questionnaire\textsuperscript{21} (maximum score 55, indicating severe symptoms) and by recording a numerical pain score (Likert pain score 0 = no pain and 10 = maximum pain).

Neurological deficits were recorded using the Neuropathy Impairment Score Lower Limbs\textsuperscript{20}.

Quantitative Sensory Testing (QST)

Thresholds for light touch were measured using Semmes-Weinstein hairs (made by A. Ainsworth, University College London, UK) No.1 (0.0174 g) to No.20 (263.0 g). The number of the hair with the lowest force reliably detected by the patient on the dorsum of the toe was recorded. Values >No. 3 monofilament (0.0479 g) were considered abnormal\textsuperscript{22}.

Vibration perception thresholds were measured using a biothesiometer (Biomedical Instrument Co., Newbury, OH, USA) placed on the metatarsophalangeal joint of the big toe. Three ascending and three descending trials were carried out, and the mean value obtained. Values >12 V were considered abnormal\textsuperscript{23}.

Thermal perception thresholds were performed as previously described\textsuperscript{8,18} using the TSA II – NeuroSensory Analyzer (Medoc Ltd., Israel). A 30 × 30 mm thermode was used and thermal thresholds determined in the soles of the feet (under the instep) for warm perception, cool perception, heat pain and cold pain from a baseline temperature of 32°C, with a change in temperature of 1°C/sec. The mean of three consecutive tests for each modality was recorded. Values >6.4°C for warm sensation, >2.3°C for cool sensation and >10.4 °C for heat pain, were considered abnormal\textsuperscript{8,18,22}. 
Histamine Skin Flare

Intra-dermal injection of Histamine (0.03 ml of 1 mg/ml. Martindale Pharmaceuticals, Romford, UK) was given into the lateral calf of patients as described previously, and the area of flare measured after 10 minutes by scanning laser Doppler imaging (Moor Instruments Ltd, Axminster UK). Abnormal skin flare was taken to be <14.9 cm².

Nerve Conduction Studies

Nerve conduction studies of the common peroneal (including F-wave studies) and sural nerves in the right leg were performed in a standardized manner by the same examiner on a Medtronic Keypoint EMG machine. Nerve conduction studies were performed once at the start of the study. Sural antidromic sensory action potential values of less than 5 μV amplitude and 40 m/s conduction velocity were considered abnormal, and common peroneal nerve (compound muscle action potential from extensor digitorum brevis) values less than 3 mV amplitude and 40 m/s conduction velocity were considered abnormal. F-wave latency greater than 60ms were considered abnormal.

Contact Heat Evoked Potentials (CHEPS)

A Contact Heat Evoked Potential Stimulator (Medoc Ltd, Ramat Yishai, Israel) was used as described previously. The baseline temperature was 32°C, destination temperature 51°C, and stimulus interval 7 seconds. Responses from 10 stimuli were recorded from each patient with the thermode placed over 3 sites (lateral aspect of calf, volar forearm and cheek). An electroencephalograph (EEG) was acquired from eight electrodes (FCz, Cz, C3, C4, Pz, P3, P4,
infra-orbital EOG, reference electrode Fz, ground TP9), with impedance below 5 kΩ. A low- and high cut-off filter (with frequencies of 0.531 Hz and 70 Hz respectively) was applied and the EEG recorded and digitized at a sampling rate of 500 Hz. All EEG data are reported from Cz. The recorded EEG data were analysed using Vision Analyser © Version 1.05.001 (BrainProducts, Munich, Germany) as reported previously. Evoked potential (EP) latency was measured from the first definitive negative peak (N2), and the amplitude measured peak to peak (N2 to P2). N2 to P2 amplitude was considered abnormal if <14.7 µV at the leg, <18.1 µV at the arm and <23.4 µV at the face. When responses could not be recorded, patients were given a nominal value of 0.1µV for statistical analysis.

Calf skin biopsy and immunohistology

Two 3 mm diameter skin punch biopsies were collected on each occasion under local anaesthesia from the lower lateral calf from the 40 diabetic patients, and of these 29 had a repeat biopsy at an interval of 6 months. To avoid any proximo- distal gradient effect, second biopsies were taken as near as possible to the first biopsy site. Control calf skin biopsies (n = 11, for PGP9.5, n = 17 for GAP-43, n = 13, for TRPV1; controls mean age 43 years) were obtained from healthy volunteers or patients undergoing elective sural nerve harvesting for upper limb nerve grafts and were studied with informed consent and Ethics approval. The different numbers of control biopsies reflect the availability of appropriately processed tissue for each marker. The immunohistological methods and antibodies used here have been reported by us previously. Briefly, one of the two skin biopsies was snap frozen and stored at -70°C for TRPV1, whilst the other biopsy was fixed before snap freezing. Routine, 15 µm thickness frozen sections (30µm thickness for GAP-43 to increase the sensitivity of this marker in the epidermis) were collected and unfixed sections post-fixed for 30 minutes.
Sections were incubated overnight with primary antibodies to the structural nerve marker, protein gene product 9.5 (PGP9.5, 1:40,000, Ultraclone Ltd, Isle of Wight, UK), the heat and capsaicin receptor, transient receptor potential cation channel vanilloid 1 (TRPV1, 1:8,000; GlaxoSmithKline, Stevenage, Herts., UK) or the marker of regenerating nerve, growth associated protein (GAP-43, 1:80,000, Sigma-Aldrich Co., Dorset, UK) which were detected using avidin-biotin peroxidase (ABC - Vector Laboratories, Peterborough, UK) methods giving black, positive immunostaining. Tissue sections were counterstained for nuclei in 0.1% w/v aqueous neutral red. Omission of primary antibodies and sequential dilution of antibodies gave appropriate results for specificity.

IENF were counted along the length of four non-consecutive sections. The length of epithelium in each counted section was measured using computerised microscopy software (Olympus ANALYSIS 5.0 Soft, Olympus UK Ltd., Southend, Essex, UK) and results expressed as fibres/mm length of section. SENF were measured by image analysis where digital photomicrographs were captured via video link to an Olympus BX50 microscope. The grey-shade detection threshold was set at a constant level to allow detection of positive immunostaining and the area of highlighted immunoreactivity obtained as a percentage (% area) of the field scanned. Images were captured (x40 objective magnification) along the entire length and the mean values used for statistical analysis. Quantification was performed by two independent blinded observers and there was no significant difference between observers.

Statistical analysis

Data was analysed using GraphPad Prism version 5.0 for Windows (GraphPad Prism Software, San Diego, California, USA). The statistical tests used were: Mann Whitney U
test, Wilcoxon matched pairs test and Spearman’s correlation coefficients. Results of tests for the two visits were analysed using the Wilcoxon matched pair test. Patients’ values were compared with control values using the Mann-Whitney test. Correlation between biopsy results with clinical tests was performed using the Spearman test. For all statistical tests p values <0.05 were considered significant.

Results

All patients showed abnormalities of quantitative sensory tests (data are presented for n=29 patients who completed all tests on the 2 visits). Normal values for QST, nerve conduction studies and CHEPS have been presented in our previous publications\(^8,^{18,22,23}\), and are in accord with reports from other laboratories. There was no significant change (p>0.05) over the 6 month interval in any of these measurements (mean ± SEM (range) values at the first visit are shown): touch threshold monofilament No. 9 ± 1 (3-20), vibration threshold: 35.8 ± 2.6 (10-50) V, cool perception threshold: 14.0 ± 1.6(2.2-32.0) °C, warm perception threshold: 13.0 ± 0.7 (4.2-18.0) °C (Figure 1), heat pain threshold: 17.0±0.4 (10.4-18.0) °C (Figure 1), cold pain threshold: 28.2 ± 1.3 (7.1-32.0) °C, histamine skin flare: 17.3 ±1.3 (6.1-34.6) cm\(^2\).

Nerve Conduction Studies

93% of patients had at least one abnormality on nerve conduction study at first visit. F-wave latency (ms) was absent in 10 and prolonged in 4. The mean ± SEM (range) Peroneal motor action potential was: 3.2 ± 0.7 (0.0 - 14.0) μV, peroneal conduction velocity: 41.3 ±1.3 (31.3 – 54.3) m/s, and was absent in 5 patients. Sural sensory action potential was: 3.1 ± 0.8 (0.0 - 12.0) μV, sural nerve velocity: 42.8 ± 1.6 (31.2 – 52.2) m/s, and was absent in 13 patients.
CHEPS

CHEPS (Aδ) N2-P2 amplitudes were significantly reduced from the leg, arm and face (Mann Whitney U test leg; p<0.0001, arm p<0.0001, face p<0.0001), at first visit, mean ± SEM (range) Amplitude: Leg 2.2 ± 0.9 (0.1-15.3) μV, Arm 5.5 ± 1.0(0.1-15.5) μV, Face 9.3 ± 1.8 (0.1-20.7) μV. Of the 29 patients who attended for both visits, for logistical reasons 9 patients had recordings from leg, arm and face at both visits, and amplitudes of these patients showed a trend to reduced face stimulation amplitude.

Immunohistology

Intra- and sub-epidermal nerve fibres for PGP9.5 and TRPV1 were markedly decreased in the DPN group in comparison with controls (Figure 2a - d). GAP-43 IENF were of very fine calibre in control skin (Figure 2e), but absent or reduced in DPN (Figure 2f). GAP-43 SENF were often close to the dermo-epidermal junction (Figure 2e, f), and were relatively preserved in deep dermis in diabetic skin (see below). In DPN, IENF were significantly reduced for PGP9.5 (Fig. 2 g; p = 0.0003), TRPV1 (Fig 2 g; p < 0.0001), and GAP-43 (Fig. 2 g; p=0.002). Our control values for PGP9.5 IENF at 15 and 30μm compared favourably with those of other authors using similar section thickness in calf skin biopsies 29.

Repeat skin punch biopsy was performed in 29 patients at the 6 month visit. Biopsies taken from the same patients at their first (0 months) and second (6 months) visits showed a significant reduction of IENF and SENF for both PGP9.5 and TRPV1 (Figure 3). Counts and image analysis (% area) of paired, repeat biopsies showed a significant reduction of PGP9.5 IENF (n = 9; omitting zero values at baseline) and SENF (n = 25; omitting zero values, Wilcoxon matched pairs test; IENF p = 0.0391; SENF p = 0.0043; Figures 3, 4). Similarly,
Repeat biopsies showed a significant reduction of TRPV1 IENF (n = 9; omitting zero values at baseline) and SENF (n = 21; omitting zero values at baseline, Wilcoxon matched pairs test; IENF p = 0.0091; SENF p = 0.0006; Figures 3, 4). Only one diabetic patient biopsy showed GAP-43 IENF on the first visit thus precluding any comparison of first and second biopsies. However, image analysis (% area) paired, repeat biopsies for GAP-43 SENF (n = 21; omitting zero values at baseline) showed no statistically significant difference (Figure 4 c).

Strongly positive GAP-43-immunoreactive nerve fibres were found in deep dermal nerve fascicles in the diabetic patients, even in those with marked loss or absence of IENF (Figure 4d).

Rate of loss of IENF was calculated to be 0.010 ± 0.004 fibres/mm/day or 3.76 ± 1.46 fibres/mm/yr for PGP9.5, and 0.009 ± 0.002 fibres/mm/day or 3.13 ± 0.58 fibres/mm/yr for TRPV1, in these patients with established DPN.

Correlations

A number of significant positive correlations were found between the diabetic patient test results at the baseline visit. Histamine invoked flare area correlated with PGP9.5 IENF (Spearman correlation coefficients; r = 0.3711, p = 0.0282). Contact Heat Evoked Potential (Aδ) N2-P2 amplitude from the face correlated with PGP9.5 (Spearman correlation coefficients; r = 0.466, p = 0.0332) and TRPV1 (Spearman correlation coefficients; r = 0.451, p = 0.0401) IENF; there was no significant correlation seen with amplitudes from the leg or arm perhaps because of the number of patients who had un-recordable amplitudes from those regions. These correlations were not seen at the repeat visit, perhaps because of the smaller number of patients, and trend to lower values. There were no correlations between the IENF / SENF and age.
**Discussion**

A lack of significant progression in clinical symptoms, Neuropathy Impairment Score Lower Limbs and a range of sensory tests has been observed in this study, in accord with previous reports\textsuperscript{13,23}. However, we have shown here a significant decrease in intra- and sub-epidermal nerve fibres in patients with diabetic sensory neuropathy over 6 month duration for PGP9.5 and TRPV1. While decreased PGP9.5 and TRPV1 nerve fibres have been reported previously in skin biopsies from patients with diabetic neuropathy by others and ourselves\textsuperscript{23,30,31}, we have now quantified the significant decrease over a 6 month period - the rate of loss of IENF in these patients with established peripheral neuropathy was calculated to be $0.010 \pm 0.004$ fibres/mm/day or $3.76 \pm 1.46$ fibres/mm/year for PGP9.5, and $0.009 \pm 0.002$ fibres/mm/day or $3.13 \pm 0.58$ fibres/mm/year for TRPV1. Interestingly, the rate of IENF regeneration following topical capsaicin application in (non-neuropathic) diabetic skin has been reported as $0.074$ fibres/mm/day, and $0.04$ fibres/mm/day in diabetic neuropathy\textsuperscript{32}. The rate of spontaneous IENF degeneration in patients with diabetic neuropathy thus appears slower, but of similar magnitude to IENF regeneration after capsaicin application. As the majority of the patients had painful diabetic polyneuropathy, further studies in diabetic neuropathy patients without pain, or those with diabetes without neuropathy, deserve study.

The decreases observed in sub-epidermal PGP9.5 and TRPV1 fibres could also be useful in future clinical trials with neuroprotective agents, given the proportion of diabetic neuropathy patients with complete loss of IENF at diagnosis. Patients with detectable IENF tended to
have a shorter duration of diabetes and fewer symptoms, as has been reported previously \textsuperscript{33}. It would therefore be useful to analyse SENF, and thereby include patients who have a longer duration of diabetes and relatively severe neuropathy. Remarkably, sub-epidermal GAP-43 fibres were preserved particularly around blood vessels and sweat glands in deep dermis even in diabetic biopsies with marked loss of PGP9.5 and TRPV1 IENF and SENF, and loss of GAP-43 IENF, without significant change over 6 months; GAP-43 SENF may serve as a potential marker of nerve fibre regeneration. We have previously shown that a decrease in TRPV1 immunoreactivity, which is regulated by nerve growth factor (NGF), preceded a decrease in PGP9.5 innervation \textsuperscript{23}, and that NGF is itself decreased in diabetic neuropathy \textsuperscript{18}; TRPV1 IENF may thus be particularly useful in future clinical trials with rhNGF or its mimics.

CHEPS amplitudes were reduced at baseline in this study, as in our previous study of small fibre neuropathies \textsuperscript{22}. CHEPS face stimulation amplitudes correlated significantly with PGP9.5 and TRPV1 IENF. As lumbosacral and cervical spondylosis with associated radiculopathy are common and may reduce limb stimulation-evoked potentials, face stimulation may provide an objective, sensitive and non-invasive marker in future trials. Heat evoked cerebral potentials have also been shown to track GAP-43 SENF following topical capsaicin in human volunteers \textsuperscript{34}. Histamine skin flare area was reduced in this group and correlated with PGP9.5 IENF, confirming that it is another useful objective non-invasive measure of small sensory fibre dysfunction.

In summary, our findings suggest PGP9.5 and TRPV1 IENF and SENF in skin biopsies are useful markers of progression in DPN, while GAP-43 SENF could potentially detect nerve fibre regeneration.
Tables

**Table 1** Summary of the diabetic patient demographics.

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<tbody>
<tr>
<td>Patients total</td>
<td>n = 29</td>
</tr>
<tr>
<td>Gender</td>
<td>19 male</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>n = 10</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>n = 19</td>
</tr>
<tr>
<td>Mean age yr (range)</td>
<td>57 (36 – 76)</td>
</tr>
<tr>
<td>Mean duration of diabetes yr (range)</td>
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<tr>
<td>Mean duration of symptoms yr (range)</td>
<td>4.4 (0.5-15.0)</td>
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<tr>
<td>HbA1c %/mmol/mol (at first visit)</td>
<td>7.8 (5.5-11.0)/62 (37-97)</td>
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<tr>
<td>HbA1c %/mmol/mol (at patient’s final visit)</td>
<td>8.1 (5.9-12.7)/65 (41-115)</td>
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**Figures**

**Figure 1** Pain scores and selected tests at each visit: a) Likert Pain Score (maximum score 10), b) Neuropathy Impairment Score (LL, maximum score 88), thermal threshold temperature change from baseline (32°C) for c) Warm and d) Heat pain. Control values are indicated by horizontal dotted lines.
Figure 2  Intra- and sub-epidermal nerve fibres immunoreactive for PGP9.5 (a, b), TRPV1 (c, d) and GAP-43 (arrows; e, f) in control subjects (left panels) and diabetic patients (right panels). Magnification x40, a – f. Intra-epidermal nerve fibres per mm length of tissue section (g) vs. control: PGP9.5 ***p = 0.0003; TRPV1 ***p < 0.0001; GAP-43 **p = 0.002.
Figure 3 Counts of paired intra-epidermal nerve fibres per mm length for PGP9.5 (a), TRPV1 (b) $p = 0.00391$, **$p = 0.0091$, and paired % area for sub-epidermal nerve fibres for PGP9.5 (c) and TRPV1 (d) $p = 0.0043$, **$p=0.0006$; Wilcoxon matched pairs test. Control mean values are indicated by horizontal dotted lines.
Figure 4 Image analysis (% area) of sub-epidermal nerve fibres for PGP9.5 (a), TRPV1 (b) and GAP-43 (c) in controls and paired diabetic patients and deep dermal GAP-43-immunoreactive nerve fascicle in diabetic patient (d). *p = 0.0043, ***p = 0.0006.