University of Calgary

PRISM Repository **https://prism.ucalgary.ca**

The Vault Open Theses and Dissertations

2014-07-17

Is Peripheral Neuropathy occurring in association with Idiopathic Parkinson's Disease, an iatrogenic complication?

Podgorny, Peter Jan

Podgorny, P. J. (2014). Is Peripheral Neuropathy occurring in association with Idiopathic Parkinson's Disease, an iatrogenic complication? (Master's thesis, University of Calgary, Calgary, Canada). Retrieved from https://prism.ucalgary.ca. doi:10.11575/PRISM/26681 http://hdl.handle.net/11023/1642

Downloaded from PRISM Repository, University of Calgary

UNIVERSITY OF CALGARY

Is Peripheral Neuropathy occurring in association with Idiopathic Parkinson's Disease, an iatrogenic complication?

by

Peter Jan Podgorny

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN NEUROSCIENCE

CALGARY, ALBERTA

JULY, 2014

© Peter Jan Podgorny 2014

Abstract

Idiopathic Parkinson's disease (PD) is primarily a movement disorder resulting from the degeneration of dopaminergic neurons within the basal ganglia. However, it is not known if changes occur in PD other than traditional motor signs and symptoms, manifesting as non-motor problems such as autonomic or sensory abnormalities. The additional involvement of the peripheral nervous system in PD has only recently gained appreciation, particularly with a higher prevalence of peripheral neuropathy (PN). The concomitant occurrence of PN in PD has prompted speculation about the cause of PN, whether it has been an overlooked phenomenon of PD or a side effect of treatment with levodopa. The current study will investigate the occurrence of PN in newly diagnosed PD patients not yet treated with levodopa. In addition to gold-standard tests for PN, including neurological physical examination and nerve conduction studies, we will study new and sensitive techniques namely corneal confocal microscopy and skin biopsy, to search for subclinical indications of PN occurring in this patient population.

Acknowledgements

I would like to thank Dr. Cory Toth for his ongoing support throughout my studies, since I began working with him as a summer student in the summer of 2011 – Thank you for your mentorship and direction on my career and life path. Thank you to Dr. Tom Feasby, who was willing to substitute as primary supervisor, when times were getting stressful for all us at the lab – thank you for your support and guidance in my preparations for my defence and post-graduate plans. Thank you to the members of the Toth and Zochodne labs, many of which provided me with support and help with my project, as well as the friendship that made every day at the lab enjoyable and the entire graduate experience a good one. Thank you to my supervisory committee, Drs. Sarah Furtado, Ken Romanchuk and Roy Gravel for your help in learning and understanding my project techniques and for your advice and critiques which helped improve the project and my understanding of the concepts involved. Thank you to the Movement Disorders Clinic doctors and staff, who facilitated patient recruitment without which this project could not have taken place – thank you for your time and willingness to help. Thank you to Christine Millar who captured the corneal confocal images for our patients, for her patience with me as I attempted to schedule the patients to be seen by our teams as soon as possible. And finally, thank you to the patients who have all been very kind and cooperative with me, for their time taken to come in for the assessments so that we may advance knowledge about these important clinical problems and help optimize treatment for future patients.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Definition
AAAD	Aromatic amino acid decarboxylase
Ado-Cbl	Adenosyl cobalamin
BG	Basal Ganglia
BM	Basement membrane (epidermal-dermal junction)
CCM	Corneal confocal microscopy
CLS	Calgary Laboratory Services
CMAP	Compound muscle action potential
CNBD	Corneal nerve branching density
CNFD	Corneal nerve fiber density
CNFL	Corneal nerve fiber length
COMT	Cathecol-O-methyl transferase
CTRL	Control subject
DLI	Duodenal levodopa infusion
EFNS	European Federation of Neurological Sciences
ENFD	Epidermal nerve fiber density (also intra-epidermal nerve fiber density)
GGT	Gamma-glutamyl transpeptidase
GPi	Globus pallidus interna
Hcy	Homocysteine
IHC	Immunohistochemistry
IV	Intravenous
LB	Lewy bodies
MAO	Monoamine oxidase
MAT	Methionine adenosyl transferase
Met-Cbl	Methyl cobalamin
MMA	Methylmalonic acid
MS	Methionine synthase
MTHF	Methyl-tetrahydrofolate
MTHFR	Methylene-tetrahydrofolate reductase
NCS	Nerve conduction studies
NMS	Non-motor symptom(s)
OCT	Optimal cutting temperature medium
OMD	O-methyl dopa
PD	Parkinson’s Disease
PFA	Paraformaldehyde
PLP	Paraformaldehyde-lysine-periodate (fixative)
PN	Peripheral Neuropathy
QST	Quantitative sensory testing
REM	Rapid eye movement
RLS	Restless leg syndrome
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SNAP	Sensory nerve action potential

Abbreviation	Definition
SNc	Substantia nigra par compacta
SNr	Substantia nigra pars reticulata
THF	tetrahydrofolate
UENS	Utah Early Neuropathy Scale

Introduction

Parkinson's disease (PD) is a condition classically characterized by slowness of movement (bradykinesia), resting tremor, cogwheel rigidity and postural instability (Calne et al., 1992, Jankovic, 2008). There are several other diseases (eg. progressive supranuclear palsy, multiple systems atrophy) in which patients exhibit some or several of these parkinsonian symptoms, hence a diagnosis of PD often requires exclusion of other alternate disease states (de Lau and Breteler, 2006). The typical signs and symptoms associated with PD have been attributed to the degeneration of dopaminergic cells in the substantia nigra, pars compacta of the basal ganglia (BG). The decreased levels of dopamine resulting from this degeneration lead to reduced function of the direct and indirect pathways in the BG and subsequent decrease in thalamic excitation, thereby suppressing the normal execution of movements (Figure 1). The mechanisms of this degeneration are not understood but several mechanisms have been postulated (Greenamyre and Hastings, 2004).

Correspondingly, the etiology of PD is largely unknown, however a small proportion of PD cases are recognized to have genetic mutations leading to familial or hereditary forms of PD. Otherwise, the majority of PD cases do not have a clearly defined cause and are termed idiopathic PD. The literature reports that although a proportion of idiopathic PD cases may exhibit a genetic cause (and are not truly sporadic) as suggested by increased occurrence among relatives (Warner and Schapira, 1998), the majority seem to result from an interaction of genetic and environmental factors. The evidence for risk factors for PD has largely been inconsistent and only age and gender appear to have a clear association with PD, as they do for many other neurodegenerative diseases (Kieburtz and Wunderle, 2013, Smith and Dahodwala, 2014).

Additional risk factors with less significant evidence for causation include occupational exposures to toxins such as pesticides and herbicides, depression, brain injury and low exercise levels. On the other hand, caffeine consumption and tobacco use paradoxically appear to have a protective function in PD, but no concrete mechanisms have yet been delineated and competing risks may be a factor in this observed phenomenon.

Many PD patients will also experience a number of non-motor symptoms including autonomic dysfunction and cognitive changes which often result in dementia in late stages of the disease. Cognitive and psychiatric disturbances include memory loss, anxiety, depression, and fatigue, and may not be related to dopaminergic dysfunction in PD (Park and Stacy, 2009). Autonomic dysfunction also appears to be prevalent in PD, manifesting mostly in the forms of constipation, orthostatic hypotension, and urogenital dysfunction (Cersosimo and Benarroch, 2012). In addition, PD patients may experience several sensory abnormalities such as hyposmia, visual disturbances, dysphagia and pain, which often precede motor dysfunction (Park and Stacy, 2009, Lin et al., 2013). This is reinforced by the finding of alpha-synuclein pathology, a pathological hallmark of PD, within peripheral sensory and autonomic neurons in PD patients (Mu et al., 2013b, Wang et al., 2013).

Last, but not least, a large proportion of Parkinson's patients can also display features of peripheral neuropathy (PN), a disease characterized predominantly by sensory loss beginning in the distal extremities (namely the legs) that can lead to problems with balance. When PN is compounded with the movement problems intrinsically affecting PD patients, it can lead to more severe loss of mobility and risk of falling. Previously, the occurrence of PN in PD has been

thought to be coincidental, however recently an association has been demonstrated by separate research groups (Toth et al., 2008, Toth et al., 2010, Rajabally and Martey, 2011). Incidentally, the occurrence of PN in PD in these studies appeared to be related to patient exposure to levodopa, the gold-standard treatment for PD (Rajabally and Martey, 2013). These findings are reinforced by studies of duodenal delivery of levodopa (which provides a more stable plasma concentration of levodopa over time) in which patients developed an acute and severe polyneuropathy (Antonini et al., 2007, Manca et al., 2009, Urban et al., 2010, Santos-Garcia et al., 2012). Thus, it has been suggested that PN in PD may be an iatrogenic effect of levodopa treatment, in a vitamin B12 (cobalamin) deficiency-dependent manner, as evidenced by lower levels of cobalamin in treated patients (Ceravolo et al., 2013). Conversely, the finding that high levels of methylmalonic acid (MMA), an important metabolite of the cobalamin pathway and common surrogate marker of cobalamin deficiency, are associated with levodopa-treatment and PN in PD patients has led to the speculation that this molecule may be responsible for inducing PN (Levin et al., 2010, Toth et al., 2010). If these speculations are true, it follows that *de novo* PD patients (newly diagnosed or untreated) should not have any indications of PN. This will be the focus of the current study.

Hypothesis and Aims

Main Hypothesis: Individuals with Parkinson's disease who have not begun treatment with levodopa will have the same frequency of neuropathy as a control cohort of individuals, and serum levels of cobalamin, homocysteine and MMA will be comparable between these cohorts.

Specific Aim 1. To assess the structure and function of peripheral nerves in PD patients prior to levodopa exposure and in control subjects.

Specific Aim 2. To determine cobalamin, homocysteine and MMA serum levels in patients with PD prior to levodopa exposure.

Rationale

Levodopa is an effective treatment for PD, however its use requires continual adjustment as the disease progresses such as to delay significant hyperkinetic motor side-effects that can occur after 5-10 years of use (Lewis et al., 2003). Additionally, non-motor symptoms which do not respond to treatment with levodopa or dopamine agonists, must be managed in other ways. It is also important to recognize that the symptoms associated with PD may have unique etiologies that may need to be managed independently.

It will be important to perform diagnostic tests that will identify those patients with asymptomatic or subclinical neuropathy. These patients may have a peripheral neuropathy related to the overall neurodegenerative condition, and as such are of pivotal importance to this study.

Background

1 Parkinson's Disease

1.1 History and epidemiology

PD was first formally described in 1817 in a landmark paper by James Parkinson, where he fittingly described the disease as a "shaking palsy" after observations of patients with involuntary tremulous motion. French neurologist Jean-Martin Charcot also played a pivotal role in further characterizing the disease, highlighting a central feature of slowness of movement, and differentiating the appearance of the tremor from other neurological disorders (Goetz, 2011). PD appears to be an age-related disease as occurs less commonly before the age of 50 years. Its prevalence is such that it is the second most common neurodegenerative disorder with a prevalence of 1% in people over 60 years of age (de Lau and Breteler, 2006), at least in the Caucasian population. Patients with PD are reported to have between 2-5 times higher mortality than age-matched controls. Quality of life is markedly impaired and symptoms can be particularly disabling, which draws much attention and research to improve care and more effective treatment options.

1.2 Clinical Presentation

To this day, PD remains a clinical diagnosis for which the cardinal symptoms are resting tremor, bradykinesia, cogwheel rigidity and postural instability. PD tremor typically occurs in the distal limbs (usually the arms) and may also involve areas of the face but not the head and neck, as can occur with essential tremor. The name *resting* tremor itself is important as it implies an essential feature of PD tremor – it is prominent during relaxation of the limb, and diminishes upon

movement of the limb. Additionally, PD tremor can be often unilateral, occurring at a frequency of 4-6 Hz in a supinating-pronating fashion that is often labelled as *pill-rolling* tremor (Jankovic, 2008). Bradykinesia in PD manifests as an overall slowness of activity (including pre-motor planning processes) and specifically a shuffling walk, decreased gesturing, reduced facial expression (hypomimia), smaller writing (micrographia), and increased reaction times; however, and interestingly, PD patients may exhibit close to normal movement and reaction times when experiencing heightened emotional states or when triggered by a startling external cue. Rigidity is often manifested as the *cogwheel* phenomenon, such that the increased resistance to passive limb movement is of a jerking nature. Rigidity in PD can occur throughout the body across different joints. Similarly, PD patients exhibit postural abnormalities, most commonly flexion across a particular joint, and instability in maintaining an upright posture which can lead to an increased risk of injuries from falling. Problems with posture typically manifest later in the disease, and may lead to difficulties with ambulation and requirement of walking aids or a wheelchair.

Although, the aforementioned cardinal features are of significant importance to the diagnosis of PD, it is important to acknowledge that a proportion of patients will not exhibit all of them, especially at disease onset. It has become widely accepted that a range of non-motor symptoms (NMS) may precede the development of the cardinal motor features of PD; often termed "prodromal features" they constitute a "premotor phase" which is most evident in the decade before PD diagnosis but likely begins much sooner (Tolosa and Pont-Sunyer, 2011). These symptoms include constipation, REM sleep behavioral disorder, hyposmia and depression; these occur with various degrees of severity and in differing combinations in PD patients (NMS are

discussed in section 1.3). However, these early mostly non-motor symptoms are non-specific and common in a variety of individuals and thus must be carefully considered in making a PD diagnosis, especially at early stages of the disease (Marti and Tolosa, 2013). The diagnosis of PD is therefore often made as a diagnosis of exclusion, by ruling out other conditions that cause tremor as well as both atypical and secondary forms of Parkinsonism. Standardized criteria, and in particular the UK Parkinson's Disease Society Brain Bank's clinical criteria, appear to have provide a good and accurate guide for the diagnosis of PD (Tolosa and Pont-Sunyer, 2011). The additional use of disability ratings scales in PD is valuable for tracking disease progression and response to treatment, which may be particularly important in research studies. Likely the most reliable and extensively validated scale is the Unified Parkinson's Disease Rating Scale, which has recently been modified to incorporate additional criteria (Goetz et al., 2008). Besides the post-mortem pathological confirmation of PD, success with sustained levodopa therapy appears to be the most reliable confirmation of a clinical suspicion of PD, even more so upon the additional exclusion of atypical signs and symptoms (Merello et al., 2002).

1.3 Peripheral nervous system abnormalities of PD

PD patients may exhibit an array of features, which do not appear to be directly associated with the motor complications of PD, and can present at various stages of the disease (see Table 1). These non-motor symptoms (NMS) associated with PD appear to be more prevalent in the late-onset form of the disease (Spica et al., 2013). Perhaps the most commonly found NMS in PD is autonomic dysfunction, which may affect over 70% of patients and presents most commonly as constipation (or general sphincter dysfunction), orthostatic hypotension and increased sweating, (Martignoni et al., 1995, Sung and Nicholas, 2013). Orthostatic hypotension may be of particular

concern as it is related to cardiovascular dysfunction and can be debilitating; interestingly, several studies have suggested abnormal autonomic innervation in PD, in addition to central degeneration, as the root cause of this problem (Biaggioni, 2007). Sensory abnormalities also occur in PD and include pain, paresthesia and hyposmia (Jankovic, 2008). Some studies have reported pain to be present in close to 80% of PD patients; several pain types were noted, but of particular interest is neuropathic-type pain which occurred in 17% of all PD patients (Beiske et al., 2009). Although there are reports of sensory abnormalities in PD dating back almost 30 years (Schneider et al., 1987), it appears that these have largely been overlooked and only recently have been acknowledged as important and disabling features of PD (Maetzler, 2014). Of note, convincing evidence for an association between PD and a nerve disease, termed peripheral neuropathy, has surfaced in recent years as possibly related to the current mainstay treatment, Levodopa (Toth et al., 2008). PD patients may also develop ocular abnormalities, namely decreased tear film production and increased corneal sensitivity, although it has not been confirmed if this is related to corneal innervation (Reddy et al., 2013). It is unclear to what extent these additional symptoms are inherent features of PD itself, as they do not occur consistently in all patients. Some NMS are most likely true PD effects, rather than comorbidities secondary to PD treatment, as they occur in a high proportion of patients before initiation of therapies (Kim et al., 2009, Mollenhauer et al., 2013). If indeed these symptoms are a result of pathological changes associated with PD, they may suggest the occurrence of peripheral changes in these patients. In fact, there has been renewed interest in examining other causes for the compendium of symptoms associated with PD (Willis et al., 2012).

1.4 Other non-motor symptoms of PD

Sleep disorders occur in the context of PD, the most common being REM sleep behavioral disorder as it may predict the development of PD, as described earlier. PD patients also report excessive daytime sleepiness, which may be a result of increased sleep fragmentation as well as a factor of the normal aging process (Stocchi et al., 1998). Restless leg syndrome (RLS) is often diagnosed in patients with PD, and appears to be a unrelated to peripheral neuropathy in PD (Rajabally and Martey, 2013); importantly, RLS can also result in sleep disturbances. PD patients also experience cognitive disturbances, including that of dementia. There is much discussion of the association between PD and dementia, to the extent that some groups have suggested that the current definitions and criteria for PD merit redefinition (Berg et al., 2014).

Executive dysfunction, even in the absence of dementia, is also commonly reported in PD, even in early stages of the disease, such that patients experience difficulties with control of attention, planning and decision making (Dirnberger and Jahanshahi, 2013). Psychiatric symptoms have also been described, ranging from depression and apathy, to psychosis and hallucinations.

A number of NMS associated with PD are also known to occur secondary to treatment. With respect to the above described NMS, orthostatic hypotension and psychosis are often reported to worsen with PD treatment, especially with the use of dopamine agonists and may also occur with other medications, as levodopa (Sung and Nicholas, 2013). Impulse control disorders may also develop, such that family members of previously normal PD patients make note of compulsive shopping, gambling and eating behaviors, following the start of treatment.

1.5 Parkinson`s disease pathways and neuroanatomy

Parkinson`s disease is fundamentally an extrapyramidal movement disorder, which results from neuropathological changes in the basal ganglia (BG), a cluster of four nuclei within the forebrain and extending into the rostral midbrain. They are composed of the caudate nucleus and putamen (collectively the striatum they are grouped this way due to overlapping function), globus pallidus (externa, GPe; and interna, GPi), subthalamic nucleus (STN) and the substantia nigra (pars compacta, SNc; and pars reticulata, SNr). The amygdala is sometimes considered to be a part of the BG, however as it plays mostly a limbic system role, it is not classically included when discussing the BG's role in movement.

Many cortical areas responsible for the production of movement project to the striatum; the projections eventually return to the motor cortex via the thalamus (no direct descending pathways to spinal cord; see Figure 1). Glutamate and GABA are the primary excitatory and inhibitory neurotransmitters, respectively, used by this pathway, although the only excitatory structure utilizing glutamate in the BG is the STN. Additionally, dopamine acts as the major transmitter in the nigrostriatal pathway, modulating striatal output, and is critical in PD. The striatum projects to the output nuclei of the BG (SNr and GPi) via two pathways, the indirect pathway which stimulates movement, and the direct pathways which inhibits movement. Thus, the signals received at the striatum are subsequently modified by dopaminergic secretions from the SNc to regulate movement. The effect of dopamine upon striatal cells occurs either via D1 receptors to stimulate the direct pathway, or via D2 receptors to inhibit the indirect pathway and ultimately facilitate motor activity. It follows, that decreased dopamine secretion in PD would result in a dampening of these effects, leading to increased inhibition of thalamocortical inputs

and the result being hypokinesia. It is important to acknowledge that, in addition to these pathways, the BG play roles in several other functions including involuntary motor functions, as well as cognitive and behavioral functions and through other neurotransmitters such as noradrenaline, adrenaline and acetylcholine. Many PD patients display disturbances in these multiple clinical spheres.

1.6 Neuropathology of PD

A defining feature of PD is the degeneration and loss of dopaminergic neurons of the substantia nigra pars compacta (SNc); the reason for this selective loss of neurons is not known. Modern studies have shown that symptoms do not typically present until degeneration of close to 50-60% of nigrostriatal neurons occurs, or 85-90% of dopamine is depleted from the brain (Wirdefeldt et al., 2011). This is of critical importance, as it suggests there are significant subclinical neurodegenerative changes that occur early and prior to clinical diagnosis of PD.

The majority of nigrostriatal neurons project to the putamen; hence, it is no surprise that the greatest drop in dopamine levels occurs within this structure (Dauer and Przedborski, 2003). Associated with the degeneration of the nigrostriatal neurons is the presence of α-synuclein deposition, which upon aggregation forms Lewy bodies (LB); these inclusions are considered a pathological hallmark of PD (Wakabayashi et al., 2007). The pathogenesis of LB in PD has long been under debate, as it appears that the presence of LB may not be the direct cause of cell death, and some have suggested they may even serve a cytoprotective function (Olanow et al., 2004). In addition, there is no clear-cut Lewy pathology that defines PD, as different genetic cases of PD can show quite varied Lewy pathology (Zimprich et al., 2004). Interestingly, synuclein

deposition is by all means not restricted to the SN and has been found in other CNS and PNS structures, as the olfactory nuclei, vagal nuclei, other brainstem nuclei, and in enteric and cardiovascular autonomic nervous system tissue (Jellinger, 2011). It is perhaps not a coincidence that synuclein deposits in these areas and that their dysfunction would be expected to contribute to at least some of the NMS occurring in PD.

1.7 Etiology of PD

The exact cause of nigrostriatal degeneration in PD is unknown, but may relate to several different pathogenic mechanisms, including genetic and environmental associations. Sex hormonal function (Miller and Cronin-Golomb, 2010), toxin exposure (Wirdefeldt et al., 2011)), and the interaction of environmental stimuli with susceptibility genes (Jenner and Olanow, 2006) may each have important contributions. Genetic mutations in alpha-synuclein, leucine-rich-repeat kinase, parkin, PINK1 and DJ1 (Hattori et al., 2000, Houlden and Singleton, 2012, Singleton et al., 2013) have all been identified as risk factors for clinical development of PD. Nevertheless, 90% of PD cases occur sporadically, and are thereafter idiopathic (without known cause) (de Lau and Breteler, 2006).

1.8 Treatment of motor deficits in PD

There are a number of available treatments for PD and thus they are most often compared on the basis of relative “ON time” (or “on state”; when medication appears to be working, patient is having good response to levodopa, and symptoms are controlled) and “OFF time” (or “off state”; when medication is not working, and parkinsonian symptoms re-emerge), in addition to the nature and number of side effects; ON/OFF periods are particularly disabling as they are

unpredictable, and concerning to patients and caregivers. Since the identification of dopamine loss as the cause of motor dysfunction in PD, dopamine replacement has been the mainstay of PD therapy. The most well-known and current mainstay treatment is the drug Levodopa (also known as L-DOPA), a precursor molecule to dopamine that can traverse the blood brain barrier to act at the dopaminergic centres of the brain. The development and use of Levodopa as treatment in PD is often cited as one of the greatest success stories of modern neuroscience (Hornykiewicz, 2010). In humans and other animals, Levodopa is otherwise a natural intermediate product in the biosynthetic pathway of dopamine from the amino acid L-tyrosine (Fahn, 2008). Levodopa is then metabolized by either of two enzymes, aromatic L-amino acid decarboxylase (AAAD) to make dopamine, or catechol-O-methyl transferase (COMT). Levodopa is most commonly administered orally, in pill form, and patients are commonly required to take up to 3 or more tablets per day in order to achieve and maintain an effective dose. It is important for patients to be regular and consistent about taking oral levodopa, especially in later stages of the disease when the emergence of "end-of-dose" wearing-off phenomena and motor complications are particularly sensitive to alterations in plasma levodopa levels (Pahwa and Lyons, 2009). For this reason, efforts have been made to develop "controlled-release" levodopa formulations with a longer half-life, however these have not been shown to be significantly more effective (Wright and Waters, 2013). Levodopa is classically administered in conjunction with Carbidopa, a peripheral AAAD inhibitor which itself cannot cross the blood-brain barrier, thus increasing the bioavailability of Levodopa to the brain; this treatment has been trademarked under the name Sinemet® or Prolopa ®.

Other routes of administration of levodopa have been considered, each with notable considerations. Intravenous (IV) Levodopa, for one, may not be administered for a prolonged period of time as the acidic solution appears to irritate the blood vessels (Wright and Waters, 2013). More importantly, IV administration is not practical and does not offer significantly superior benefit to oral treatment to justify its use. Intranasal and transdermal levodopa are more recently considered routes that allow circumvention first-pass metabolism and provide constant plasma levels, and are being evaluated for safety and efficacy (Ngwuluka et al., 2010). Perhaps the most widely considered alternate to oral Levodopa has been duodenal delivery (duodenal Levodopa infusion, DLI; Levodopa/Carbidopa Intestinal Gel, LCIG; duodenal delivery of levodopa, DUODOPA). The main evidence supporting this method has been arisen from clinical trials showing that DIL allows for a continual supply of Levodopa which appears to be critical in reducing "OFF time" (Devos, 2009, Antonini et al., 2013). Due to the involvement of surgery to insert the pump, DIL is often a last line of treatment (and may even be considered secondary to deep brain stimulation) and has been studied mostly in patients with advanced PD. DIL appears to be an effective treatment, the only major complication being related to enteric tube malfunction, and then potential development of psychiatric side-effects and a sub-acute polyneuropathy.

Likely the second most effective single drug treatment for PD are dopamine agonists (ergot and non-ergot), the major advantage being that they do not produce the well-known disabling levodopa-associated dyskinesias. Also used are COMT and MAO inhibitors, which aim to reduce the catabolism of levodopa and dopamine, respectively; a well-known MAO inhibitor is selegiline (also known as Deprenyl), which may additionally exhibit neuroprotective effects that

merited a randomized double blind placebo controlled trial for the drug (DATATOP study), which was inconclusive. Rasagiline (or Azilect®) is another MAO inhibitor more commonly used in recent years, that appears to be well tolerated by PD patients and can be used as monotherapy or adjunctive therapy (Hoy and Keating, 2012). Similarly, the COMT inhibitor Entacapone (trademark name, Comtan ®), may be as effective for PD as dopamine agonists, with much fewer side effects. Nowadays, these drug treatments are rarely used on their own due to incomplete efficacy, and instead are used in conjunction with Levodopa to better control "OFF-times", especially late in the disease of interest, European neurologists use agonist monotherapy (Gazewood et al., 2013). Of note, surgical therapy may also be available for PD patients, most importantly deep brain stimulation, which involves implanting electrodes into the brain to stimulate specific areas of the basal ganglia as the STN or GPi.

2 Peripheral Neuropathy

2.1 Characteristics of peripheral neuropathy

Peripheral neuropathy (PN) is a common neurologic disorder encompassing any form of damage or abnormal function in peripheral nerve fibers. The prevalence of PN in the general population is estimated between 2% and 8%, with higher incidence in the elderly (Martyn and Hughes, 1997). There are hundreds of different causes of PN, many of which are known. These range from hereditary disorders to traumatic afflictions, chronic disease, metabolic disorders, immune disorders, nutritional deficiencies and environmental toxins. Among these, diabetes is known as a major cause of PN, and has a unique spot on the list of PN taxonomy as diabetic peripheral neuropathy (DPN). Other etiologies include vitamin deficiencies, endocrinological or hormonal abnormalities, autoimmune diseases, toxins, and hereditary causes (England and Asbury, 2004,

Lozeron et al., 2013, Saporta and Shy, 2013). However, despite recognition of many individual causes of PN, 20-25% of PN cases (and upwards of 50% of small fiber PN cases) do not have an identifiable cause and are termed as idiopathic PN (PN) (Brannagan, 2012).

Symptoms of PN usually begin at the location where the longest nerve fibers of the body innervate the skin and muscle targets, therefore manifesting at the distal extremities such as the feet and later in the lower legs and hands. Manifestations present as numbness, parasthesias (tingling, pins and needles), allodynia or pain, weakness, incoordination, or any combination of these symptoms. This leads to the typical stocking-glove pattern (Figure 2) that is observed in idiopathic PN.

PN can affect any of sensory, autonomic or motor fibers, thus explaining the wide variety of features that can present with PN. Weakness and fasciculations are common signs of a PN affecting the motor nerves, which rarely occurs without concurrent sensory involvement, manifesting as paresthesias or pain. PN affects nerves fibers of all size and can affect the axon and/or myelin component of a nerve fiber. The largest fibers, which are always myelinated, convey motor and sensory signals, such as proprioception, vibration and light touch, whereas smaller thinly myelinated fibers convey light touch, pain, temperature and pre-ganglionic autonomic signals. The smallest, unmyelinated fibers are those which convey temperature, pain and post-ganglionic autonomic signals. Thus, abnormalities in any or all of these sensory modalities are clinical features of specific types of PN (Alport and Sander, 2012).

2.2 Diagnosis and testing for peripheral neuropathy

There are a number of clinical tests to aid in the diagnosis of suspected PN. The workup for identification of a cause for PN can be extensive, and requires a holistic approach with several blood tests required for diagnosis. The gold standard test for detection of PN is a physical examination which includes an assessment of motor function through strength testing and examination for muscle atrophy, sensory function though vibration threshold, joint position, touch and pinprick testing, light touch assessment, and assessment of reflexes (Alport and Sander, 2012). The physical exam has the additional benefit of being able to provide an indication as to whether a PN is affecting small or large fibers.

Clinical scales for defining the severity of a neuropathy

A number of different scales have been developed to help detect and characterize neuropathy; each scale may emphasize particular aspects of the disorder such as symptoms, neurologic deficits, findings on clinical examination and presence of pain, or may utilize any combination of these (Herrmann, 2008). Such scales are commonly used as endpoint measures in clinical trials, as they often take into account motor and sensory abnormalities and do not require specialized equipment to perform, as with electrophysiological assessments. The most commonly used scales are the Michigan Diabetes Neuropathy Scale (MDNS) and the Neuropathy Impairment Score as it concerns the lower limbs (NIS-LL). However, these scales are motor-dominant scales, lacking sensitivity to detect early presence of small fiber neuropathy and also failing to take into account the anatomical spread of symptoms. Thus, the Utah Early Neuropathy Scale (UENS) was developed and validated for determining the presence and severity of small fiber PN (Singleton et al., 2008). First designed to assess for early diabetic neuropathy, this protocol is

applicable to other forms of PN also, and provides a sensorimotor assessment including evaluation of both small and large nerve fibers.

Nerve conduction study

Nerve conduction study (NCS) is a non-invasive and widely used electrophysiological test for the diagnosis and characterization of PN (Herrmann, 2008). NCS are a reliable measure of large fiber PN and are sometimes used as a primary measure in therapeutic trials. However, NCS fail to detect small fiber PN whereas clinical examination can detect such features easily. To perform NCS, a stimulating electrode is placed on the surface of the skin overlying a nerve of interest and a recording electrode is placed over either the nerve at a distal or proximal site, or over the muscle distally relative to the site of stimulation. Depending on the placement of electrodes and the nerve being studied one can record either the sensory nerve action potential (SNAP) or compound muscle action potential (CMAP), which represent the sum of the individual sensory or motor unit activation, respectively (Bromberg, 2013). Abnormalities in SNAP or CMAP amplitude reflect axonal loss or degeneration, whereas timing and therefore conduction velocity abnormalities reflect changes in myelin. The SNAP and CMAP waveforms provide additional insight into the nature of the abnormalities in a PN case. As alluded to earlier, a major limitation of NCS is that they allow only for the examination of large fiber function, as the contribution of small fibers to the measured potentials is largely restricted to clinical evaluation or different techniques.

Techniques which allow for testing of small fiber damage are the analysis of epidermal innervation from skin biopsies and corneal innervation with corneal confocal microscopy.

However, depending on the clinical location, these may not be routinely used in the clinical evaluation as they require a far greater investment of time, expertise and resources. An advantage to these techniques, however, is that they may be far less subjective and more specific than NCS or quantitative sensory testing (QST).

Skin biopsy

In the past decade, the analysis of skin biopsies has become prevalent in studies of small fiber neuropathy, to the extent that there are now guidelines and normative ranges for the determination of an epidermal nerve fiber density (ENFD; summarized by a European Federation of Neurological Societies Task Force (Lauria et al., 2005, Lauria et al., 2010a, Lauria et al., 2010b). The majority of nerve fibers crossing into the epidermis and therefore contributing to the ENFD are free nerve endings composed of small unmyelinated fibers, termed C fibers, and some small thinly myelinated fibers, termed Aδ, which are largely responsible for temperature and pain sensation (Kennedy and Wendelschafer-Crabb, 1993). As would be expected, the ENFD differs depending on the location from which the biopsy is taken, the distal leg being the most common site in most studies. Additionally, ENFD loss has been observed in animal models of PN and DPN, rendering it a good measure of small fiber damage in animal research models as well (Johnson et al., 2008, Dacci et al., 2010). ENFD appears to be a specific marker for small fiber PN, although it lacks in sensitivity and is unable to detect those cases of PN which may only affect large fibers (Herrmann, 2008). Besides ENFD, there are other features of PN that can be examined in skin biopsies including morphological abnormalities such as axonal swellings or branching patterns, although these measures have received significantly less attention (Lauria et al., 2003).

Corneal Confocal Microscopy

Corneal confocal microscopy (CCM) is an emerging technique which allows the visualization of the main nerve plexus supplying the corneal epithelium, situated within Bowman's membrane (Figure 3); this is the sensitive portion of the eye and not the portion of the eye where visual information is processed. The cornea is the most densely innervated surface tissue in the body, rendering it an interesting subject for the study of PN. As in the innervation of the epidermis, corneal innervation is supplied by Aδ and C fibers, but may exhibit a more equal distribution of these fibers than the skin which is innervated primarily by unmyelinated C fibers (Tavakoli et al., 2012). CCM has most extensively been studied in the setting of DPN, where corneal innervation has been shown to have a positive correlation with ENFD, cold and hot temperature thresholds (as determined by QST) and a negative correlation with the severity of neuropathy (Malik et al., 2003, Quattrini et al., 2007, Tavakoli and Malik, 2011). The outcome measures most commonly used for assessment of neuropathy in DPN are corneal nerve fiber density (CNFD), branching density (CNBD) and fiber length (CNFL). Nerve fiber tortuosity has also been assessed, but has been determined to be less robust and thus, this is not as commonly used as the former measures. In one study, based on Receiver Operating Characteristic (ROC) curve analysis CNFL was the best measure to diagnose DPN, with the highest specificity, but was not as sensitive as CNBD and CNFD measures (Tavakoli et al., 2010a). CNFL also appears to be a more reliable and consistent biomarker in the diagnosis of diabetic sensory polyneuropathy (Hertz et al., 2011). Nevertheless, there has been inconsistency across CCM studies in the determination of these measures; the use of automated software to make these measurements in the future may partially resolve this problem, although the software is still dependent on subjective user input (Dabbah et al., 2011). In addition, the presence of CCM abnormalities in diabetic patients without

neuropathy suggests this technique may also be able to detect early, preclinical neurodegenerative changes (Tavakoli et al., 2010b, Ziegler et al., 2014).

2.3 Peripheral Neuropathy in Idiopathic Parkinson's disease

In 1992 Taly & Muthane set-out to explore the occurrence of dysautonomia in a cohort of juvenile PD patients, an early-onset form of PD (Taly and Muthane, 1992). In the absence of clinically present neuropathy, they performed nerve conduction studies and described both motor and sensory nerve abnormalities in 13.8% and 31% of patients, respectively. This was likely the first tangible evidence of neuropathy-like features existing in PD patients. Merely two years later, Tranchant et al. reported cases of a dopa-sensitive parkinsonian syndrome developing in association with a neuropathy (which he described was more alike to Charcot-Marie-Tooth type 2 disease, a primary axonal neuropathy of genetic origin), highlighting the unlikely chance that this was a coincidence (Tranchant et al., 1994). Nevertheless, it has only been in recent years that an increased prevalence of PN has been explicitly shown to exist in PD patients as compared to individuals without PD (Toth et al., 2008, Toth et al., 2010, Rajabally and Martey, 2011); this has been supported by several case reports with similar findings that have surfaced in the same period of time (Manca et al., 2009, Gondim Fde et al., 2010, Urban et al., 2010).

The Rajabally and Toth groups reported a prevalence of PN between 38% and 58% in their PD patients, as compared to rates of 8-9% in controls. Other emergent studies report a less dramatic but nevertheless higher prevalence of PN in PD as compared to controls, particularly in those with long term exposure to treatment (19% versus 9% in controls, (Ceravolo et al., 2013)). The neuropathy noted was mostly of sub-acute onset with progressive development of numbness and

tingling; it also appeared to be of a sensory axonal-predominant type, although the motor and myelinated nerves were affected in some patients as well. Until recently, the occurrence of PN may have been overshadowed by the motor symptoms occurring in PD. Identifying PN as concomitant entity in PD, necessitating additional attention, allows us to acknowledge the unique contribution it makes to a PD patient's weakness and risk of falling, and additional set of positive symptoms.

The authors of the associative studies, cited above, have additionally noted that the occurrence and severity of PN appeared to correlate positively with exposure to the aforementioned PD treatment Levodopa. However, disease duration and patient age are confounding factors for the development of PN, and must be considered when interpreting the results of these studies; most importantly, PD patients with longer-term levodopa exposure have longer duration of PD and thus PN could simply be manifesting at a later stage of the disease. Interestingly, these studies have also reported alterations in the pathways of cobalamin metabolism, namely abnormalities in levels of serum cobalamin, homocysteine and/or MMA. These findings are supported by smaller case studies of PN developing in high-dose chronic levodopa-treated PD patients (Kimber et al., 2013).

The importance of these findings has also been heightened by reports of PN developing in patients receiving duodenal delivery of levodopa (Urban et al., 2010, Santos-Garcia et al., 2012, Muller et al., 2013). Patients on DLI in these studies developed a sensory predominant sub-acute PN within periods of weeks to months after beginning such therapy. PD patients appeared to be

at greater risk of developing PN when treated with DLI versus oral levodopa, and when treated with levodopa (any form) versus other dopaminergic therapy (Mancini et al., 2014).

Neuropathy has also been reported in a limited number of cases of levodopa-treated early-onset familial PD with *parkin* mutations (Abbruzzese et al., 2004). Interestingly, in these patients the expression of the *parkin* gene was not limited to the CNS, but was also localized to the peripheral nerve.

Furthermore, there is evidence of PN developing in similar neurodegenerative disorders to PD, as in amyotrophic lateral sclerosis (Weis et al., 2011), progressive supranuclear palsy (Levin et al., 2010), or multiple system atrophy (Rossi et al., 1986). Thus, peripheral nerves may be subject to neurodegeneration in a number of conditions that were previously believed to be localized forms of pathological disease.

2.4 Other evidence for peripheral nervous system involvement in PD

The classic CNS pathology associated with PD is only evident upon post-mortem examination, rendering it an ineffective early marker of disease and spurring new interest in identifying biomarkers to accompany the clinical diagnosis of PD. Although some CNS imaging techniques such as Positron Emission tomography appear to show dopaminergic degeneration as it correlates with PD severity (Hsiao et al., 2014), some research groups have turned to the PNS in attempt to identify biomarkers, greatly widening our understanding of and appreciation for the peripheral manifestations of the disease. For one, it has been shown that the synuclein deposition that occurs in PD is in fact not restricted to the striatum but also localizes to peripheral nerve

fibers (Cersosimo and Benarroch, 2012). Synuclein deposits have been found throughout the autonomic systemic, ranging from cardiac sympathetic fibers and the enteric nervous system to cutaneous autonomic nerve fibers (Wang et al., 2013). A recent study has unequivocally demonstrated that α-synuclein is present in cutaneous nerve fibers of PD patients with great specificity, such that it may even be a helpful biomarker to differentiate between PD and other forms of parkinsonism (Donadio et al., 2014). Synuclein is also present in peripheral motor fibers of PD patients, specifically the nerves innervating the pharyngeal muscles where it may be pathogenic (Mu et al., 2013a). However, any implication that synuclein is pathogenic has not been confirmed

Additionally, some PD patients exhibit frank degeneration of peripheral nerve fibers, specifically cardiac sympathetic deafferentation (Goldstein et al., 2000) and cutaneous denervation which may account for the sensory disturbances that occur with PD (Nolano et al., 2008). Although inherently a central nervous system tract (and not a peripheral nervous system structure as other cranial nerves), the optic nerve also exhibits damage in PD as evidenced by increased retinal thinning (Inzelberg et al., 2004), which appears to also correlate with functional disability in PD (Satue et al., 2014).

3 Etiology of Peripheral Neuropathy in PD

3.1 Proposed Etiology of PN in PD

The etiology of PN in PD is still not clear; however, a hypothesis has been proposed by Toth and colleagues (Toth et al., 2010) implicating the metabolism of levodopa. The idea that Levodopa

may somehow contribute to PN has been further supported in the latest study, showing an increased prevalence of PN in Levodopa-treated patients as compared to patients treated with dopamine agonists (Mancini et al., 2014). To examine the potential pathogenesis of Levodopa in PN, we will consider its metabolism in humans: as previously mentioned, upon uptake of Levodopa into systemic circulation, it is metabolized into dopamine via AAAD, or alternatively, degraded by COMT into 3-O-methyldopa (3-OMD), after which it can no longer exert its intended beneficial effects in PD. The degradation of Levodopa via COMT, requires methylation by S-adenosyl methionine (SAM) which is converted to S-adenosyl homocysteine in the process, and eventually homocysteine (Figure 4). Homocysteine is then recycled to methionine, the precursor to SAM, via a cobalamin (Vitamin B12) dependent enzyme methionine synthase, which transfers a methyl group from a pool of methyl-tetrahydrofolate (MTHF) molecules. This pool is replenished by the enzyme methylene-tetrahydrofolate reductase (MTHFR). Alternatively, if any of the required co-factors or enzymes (ie. cobalamin, MTHFR) are lacking or dysfunctional, an accumulation of homocysteine could result. Physiologically, homocysteine may also be shuttled down a second pathway where it is converted to cystathionine via the vitamin B6 dependent enzyme cystathionine β-synthase (transulfuration pathway); thus deficiency of vitamin B6, and associated accumulation of homocysteine, may be responsible for some cases of PN in PD (Urban et al., 2010, Klostermann et al., 2012).

Whether elevated homocysteine itself could directly contribute to development of PN is unknown, although homocysteine does not appear to be toxic to neuronal cells (unpublished results, Dr. C. Toth Lab). Several studies have noted elevated homocysteine levels in PD patients ((Blandini et al., 2001), reviewed in (Postuma and Lang, 2004)). Hyperhomocystenuria is also

known to occur in patients with MTHFR polymorphisms (Cortese and Motti, 2001), which coincidentally may be common in PD (Vallelunga et al., 2013).

Additionally, elevated MMA levels were noted by Toth et al. (2010) in PD patients taking levodopa. It is unlikely that the aforementioned MTHFR polymorphisms are responsible for these altered levels of MMA (Fredriksen et al., 2007, Barbosa et al., 2008). Clinically, elevated MMA serves as a surrogate marker of cobalamin deficiency, and may be more accurate than the serum cobalamin level itself (Weir and Scott, 1999). The buildup of MMA is most important when considering that at high levels it may be a neurotoxin, and thus capable of producing a PN *in vitro* (unpublished results, Dr. C. Toth Lab). If PN is occurring in PD as a result of levodopa treatment and is related to altered MMA levels, it may be possible to treat this problem through supplementation with cobalamin, altering functional levels of cobalamin and thus normalizing levels of MMA (Nakamura et al., 2002).

3.2 Other considerations for implicating levodopa in the development of PN

There are other possible mechanisms which could be postulated regarding how levodopa may act to induce PN in PD patients. As Levodopa is generally administered in conjunction with carbidopa, the AAAD inhibitor, the blockage of this pathway forces the metabolism of Levodopa onto COMT. Presumably, the high rates of methylation that occur in order to convert levodopa to 3-OMD by way of COMT, draw on the pool of the methyl donor SAM and in turn the folate pool required to produce SAM, which exists primarily in the form of 5-methyltetrahydrofolate (5-MTHF). In this form, folate is fated to the pathway of homocysteine methylation as the enzyme methionine synthase (MS), which converts homocysteine to methionine, is the only enzyme in

humans that can utilize 5-MTHF (Blom and Smulders, 2011). Thus, any blocks to the activity of this enzyme result in what is known as the "methyl-trap" hypothesis, which predicts that folate is trapped in a 5-MTHF pool. This trap would then lead to a reduction of availability of other forms of folate, namely tetrahydrofolate (THF) and all of its intermediate methylated forms which are important particularly for purine and pyrimidine synthesis. It is possible that levodopa may act indirectly to reduce availability of important factors in the folate-methionine pathway that could lead to abnormalities in peripheral nervous tissue.

Studies have demonstrated that levodopa is able to directly reduce the SAM/SAH ratio, an indicator of methylation potential within a cell (Liu et al., 2000, Obeid et al., 2009), which in turn decreases SAM-induced inhibition from MTHFR. It is possible then, that alteration of the methylation potential in peripheral nerve contributes to the development of peripheral neuropathy in PD patients. One study addressed this possibility by examination of the methylation status in myelin basic protein, however no association with the development of neuropathy was noted (Deacon et al., 1986). Methylation is also important for the clearance and/or alteration of activity of endogenous and exogenous compounds that could be toxic at high concentrations. It follows that dysregulation of methylation pathways, as with chronic methylation of levodopa by COMT, could lead to weakening of detoxification mechanisms and the resulting buildup of an unrecognized peripheral neurotoxin (Muller, 2010).

Remarkably, PD patients appear to exhibit an inherent increased rate of methylation of certain exogenous compounds (ie. nicotinamide) (Williams et al., 1993). Whether this increase is pathological and whether the acute effects of levodopa to decrease SAM are therapeutic is not

clear. However, Liu et al. (2000) caution that the chronic effects of levodopa to induce increased expression of the enzymes methionine adenosyl transferase and COMT, may lead to aggressive transmethylation reactions responsible for producing unwanted side-effects in levodopa treated PD patients. Also, nicotine appears to affect levodopa pharmacokinetics, suggesting that similar catabolic pathways are being used by both compounds (Kyaw et al., 2013).

Upon entry into the body, levodopa is metabolized into various products raising the possibility that one of these metabolites may exhibit peripheral neurotoxicity. In particular, Lee and colleagues have shown that 3-OMD, the product of levodopa breakdown by COMT, may be neurotoxic (Lee et al., 2008). Others have suggested that the products of L-DOPA and dopamine breakdown, which may otherwise be sequestered by neuromelanin as a protective mechanism, are toxic at high concentrations (Sulzer et al., 2000).

With regards to cobalamin and its involvement in the levodopa and methionine pathway, an important distinction must be made. The MS enzyme, which undertakes the methylation of homocysteine to methionine utilizes the methylated form of cobalamin (Met-Cbl), whereas the other important enzyme which requires cobalamin as co-factor, methylmalonyl-CoA mutase utilizes an adenosylated form (Ado-Cbl) (Fenton et al., 2001). One may hypothesize that an imbalance between the different forms of cobalamin and/or a deficiency in the availability of either to their respective coenzyme, may contribute to the development of abnormalities as in the case of a peripheral neuropathy.

Another consideration is the molecular structure of levodopa. Interestingly, levodopa is a close structural analog of the amino acid L-tyrosine. It appears that at pharmacological levels, it may even be incorporated into normal protein, which may have detrimental effects (Rodgers et al., 2006, Chan et al., 2012). It is possible, that these effects could contribute to peripheral neurotoxicity and result in neuropathy.

An overall view

The occurrence of PN in PD is a new finding, for which the cause is unclear and may have significant implications for the treatment of PD. The purpose of this research will be to determine if peripheral neuropathy (PN) is present in patients with newly diagnosed Idiopathic Parkinson's disease (PD) to a greater extent than in the general population, and if PN in PD may be an iatrogenic complication of levodopa use.

Specific Aim 1. To assess the structure and function of peripheral nerves in PD patients prior to levodopa exposure and in control subjects. We will be examining a cohort of PD patients as well as an age-matched cohort of control subjects without PD. We will use a standard clinical exam to detect PN, using the Utah Early Neuropathy Scale (UENS) as a guide, accompanied by nerve conduction studies to examine the lower extremity nerves within subjects. We will also use new and sensitive techniques to identify the extent of PN including skin biopsies to determine intra-epidermal nerve-fiber density (ENFD) and corneal confocal microscopy (CCM) to determine corneal nerve fiber length, branching and density. Both techniques have shown to be sensitive and able to detect PN at early stages (Lauria et al., 2009).

We will compare the results of these tests between the PD cohort of patients and control subjects in order to determine if the occurrences of features of PN differ between these individuals.

Specific Aim 2. Determine cobalamin, homcysteine and MMA serum levels in patients with PD prior to levodopa exposure. Blood will be collected via routine phlebotomy by Calgary Laboratory Services, and analyzed for cobalamin, homocysteine and MMA via standardized protocols. The purpose of these blood tests is to determine if indeed increased levels of MMA are correlated with the development of neuropathy in PD patients and if it is related to a deficiency in cobalamin. If this is the case, we suggest that PN is an iatrogenic complication in PD, and may be easily prophylaxed through the simultaneous administration of cobalamin with Levodopa.

Methods

4 Patient enrollment and eligibility

Patients were recruited prospectively, as they were being seen at regular clinic appointments at the University of Calgary Movement Disorders Clinic. To be eligible, patients must have had ‘de novo’ idiopathic Parkinson’s, meaning they were either newly diagnosed with PD or had PD but were not yet treated with levodopa. For practical purposes, patients were permitted to enroll in the study if they had begun levodopa treatment less than one week before being seen for the study. It was reasoned that at this point levodopa levels would still be low, as patients typically begin with a low dose that is progressively increased, and thus unlikely to exert any possible toxic effects. Additionally, the occurrence of PN in oral levodopa-treated PD patients does not appear to be an acute event, except for the reported case where duodenal delivery of levodopa

occurred; as such, clinically relevant effects are unlikely to occur in this window of time (Mancini et al., 2014). In all cases, the diagnosis of PD in these patients must have been confirmed by a specialized Movement Disorders Neurologist to be further eligible for the study. Due to the multiple existing hypotheses regarding the etiology of PD, it is unclear whether PD is a single disorder with a common etiology or a group of similar diseases with different etiologies (Jenner and Olanow, 2006). For this reason, our study considered only idiopathic PD, without known genetic cause. Additional eligibility criteria included patient consent to participate. Exclusion criteria included previous treatment with levodopa, early onset PD, diabetes or other risk factors for PN, and PN of a known cause. The presence of pre-existing idiopathic PN did not exclude participation in the study. Control subjects were recruited by word of mouth and PD participants were asked to recommend a family member, spouse or friend that was willing to participate in the study; this facilitated recruitment of individuals of a similar demographic to our PD cohort. The exclusion criteria used for the PD cohort was also applied to control participants.

5 Testing

5.1 Physical exam and history

An initial screening was performed over the phone to assure patient eligibility, as per the criteria above. In addition, a patient history was collected including patient demographics, past medical history, current medications and supplements (namely vitamin B supplementation), family medical history (specifically presence of PD and PN) and social history. A standard neurological evaluation for PN in the lower limbs was performed using the Utah Early Neuropathy Scale (UENS); this was performed by a specialized Neuromuscular Neurologist (Dr. C. Toth). The UENS is composed of 5 sections which characterize detect various characteristics of large fiber

neuropathy (Figure 5). This includes motor examination for toe weakness, testing for pin sensation, signs of hyperesthesia and allodynia, great toe joint position and vibration detection, and tendon reflexes at the ankle. A score is given for each of these tests, for each leg; a score above zero is indicative of abnormalities, and in most cases a higher score is given in the case of complete absence of a response versus a decreased response as compared to normal. Patients with a total score of ≥4 out of 42 were considered to have a large fiber neuropathy and additional blood tests were ordered to exclude potential known causes of neuropathy (see below, section 5.5). This cut-off was selected based on empirical reasoning that in the mildest case of PN, a patient would experience at least decreased pinprick sensation in the feet and up to the level of the ankle, resulting in a score of 4 on the UENS. Additionally, this score is also very near the cut-off of 5 selected in another study of PN in PD (Rajabally and Martey, 2011).

5.2 Nerve conduction

Nerve conduction studies (NCS) were performed for each patient by a specialized neurologist with neurophysiological training and certification (Dr. C. Toth). The XLTEK NeuroMax 1002 EMG System was used to perform NCS (Natus Medical Inc., San Carlos, CA). Orthodromic compound muscle action potentials (CMAPs) were recorded from the common peroneal and tibial nerves. For the common peroneal nerve, signal was recorded from the extensor digitorum brevis muscle (dorsal aspect of foot) with stimulation of the nerve proximally at the ankle, fibular head and popliteal fossa. For the tibial nerve, signal was recorded from the abductor hallucis muscle (medial aspect of foot) upon stimulation of the nerve proximally at the ankle and at the popliteal fossa. Antidromic sensory nerve action potentials (SNAPs) were recorded from the superficial peroneal and sural nerves. For the superficial peroneal nerve, signal was recorded

from the dorsum of the foot at the level of the malleoli with proximal stimulation of the nerve 14 mm proximal at the lateral leg. For the sural nerve, signal was recorded from a location dorsal to the lateral melleoulus with proximal stimulation of the nerve 14 mm proximal at the posterior lower leg. NCS were performed on the right leg unless patient preference dictated assessment of the left leg or if there were asymmetrical or abnormal findings on the physical exam, then both legs were tested. SNAPs and CMAPs were later analyzed for conduction velocity, latency and amplitude. The temperature of each patient's leg was verified prior to NCS with an infrared thermometer; in the case where the limb temperature was below 30ºC, blankets were used to warm the legs.

5.3 Skin biopsy and ENFD determination

For each patient, a 3mm punch skin biopsy was collected from the region approximately 15cm above the lateral malleolus, on the right leg unless patient preference dictated assessment of the left leg. This was performed under antiseptic conditions with local anesthetic provided (1% lidocaine) Additionally, if skin abnormalities were present or abnormal findings on the physical exam occurred, the skin biopsy had to be taken from the left leg instead of the right. Two biopsies were taken, with the extra biopsy to be used for any unforeseen testing. Upon collection, samples were immediately submerged in 2% paraformaldehyde-lysine-periodate (PLP) solution for 12-24 hrs at 4ºC. Following this, samples were washed 2x with 0.1M Sorrenson's phosphate buffer for 5 minutes and cryoprotected by immersion in 20% glycerol/0.1M Sorrenson's phosphate buffer overnight at 4ºC. Finally, the samples were embedded in optimal cutting temperature (OCT) medium (VWR cat#95057-838) and frozen at -30ºC for up to one year, or until processed further. Embedded tissues were then sectioned at 50µm using a sliding

microtome with freezing unit (MICROM HM 430 w/ MICROM Gmbh KS-34-S, Thermo Scientific) and immediately placed in a phosphate-buffered antifreeze solution (30% glycerol/30% ethylene glycol/sodium phosphate buffer) following a floating-sections method, as executed previously elsewhere (McCarthy et al., 1995). The free-floating sections in antifreeze solution were further stored in microcentrifuge tubes at -30ºC until immunohistochemistry (IHC) could be performed batch wise, as follows: samples were brought to room temperature and washed 2x with PBS for 5 minutes. The tissue sections were then immersed in 1% TritonX100/PBS for 6 minutes and then rinsed again, 3x with PBS for 5 minutes. Sections were then placed in blocking solution for 1 hour at room temperature, and then immersed in a 1:1000 dilution of rabbit anti-UCHL1 primary antibody (EnCor Biotechnology Inc. cat#RPCA-UCHL1) overnight at 4 ºC. Tissue sections were then washed 3x with PBS for 5 minutes and immersed in 1:100 dilution of Cy3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch cat#111-165-003) to visualize the nerve fibers. Tissue sections were then transferred onto glass slides and excess solution was removed, allowing the sections to lie flat. Coverslips were then mounted onto the slides using a Mowiol®4-88 mounting solution (Sigma-Aldrich cat#81381; according to an NIH biological imaging protocol).

Tissue sections were then imaged at 60x magnification using a confocal microscope (Olympus Fluoview BX50); the entire thickness of tissue sections was imaged at 1µm intervals, creating a *z-stack*, which was saved as a single flattened 8-bit *.tif* image (512x512 pixels). Five images were taken from each of five tissue sections per patient, constituting at least 1220µm of epidermis per section, and ENFD was determined as per the guidelines and recommendations set-out by the European Federation of Neurological Sciences (Lauria et al., 2005). Briefly, epidermal nerve

fibers were counted if they crossed the basement membrane (BM), the junction between epidermis and dermis, as delineated by a sharp change in background staining (Figure 6). If branching of a fiber occurred deep to the epidermis, branches were counted as separate fibers; if branching occurred within the BM or superficial to it, this was counted as a single nerve fiber crossing. Number of fibers were summated across a total of 5 images per tissue section and divided by the total length of epidermis, measured along its superficial aspect using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014). Of note, imaging of the outer edges of epidermis was avoided as this area is most susceptible to damage artifact upon handling of tissues during collection and processing.

5.4 Corneal confocal microscopy (CCM)

CCM was performed by an orthoptist (C. Millar, OC(C)), experienced and trained in operating this unique microscope (Heidelberg Retina Tomograph Rostock Corneal Module, Heidelberg Engineering). During the imaging process, the centre of the cornea was targeted, all the way through the thickness of the sub-basal nerve plexus in Bowman's layer. Many images were taken of each eye, at the discretion of the orthoptist, such as to provide a pool of images of good clarity and minimal movement artifact. Eight representative images of the best quality were selected for each eye under the guidance of an ophthalmologist experienced with CCM and blinded to the disease profile of the patient (Dr. K. Romanchuk, Ophthalmologist). The average CNFL, CNFD and CNBD were determined for each patient, using a new prototype automated analysis program which recently became available (Tavakoli et al., 2010a). In addition to calculating the statistics of interest, the program outputs the processed images overlayed with the nerve fiber traces and

branching points which were used to make the calculations. Unpublished data from separate CCM research groups in Calgary and the UK suggest strongly that the results from automated analysis is correlated strongly with numbers from manual analysis of CCM images, and thus are a valid replacement for the latter.

5.5 Blood collection and analysis

Patients were asked to have their blood collected at any Calgary Laboratory Services (CLS) clinic. Blood was then be analyzed by CLS for 12 hour fasting levels of methylmalonic acid and homocysteine (determined by high performance liquid chromatography and chemiluminescence, respectively), as well as non-fasting cobalamin and folate (determined by immunoassay). Patients with findings suggestive of neuropathy on the physical exam and NCS had additional tests ordered to identify potential causes of PN. These analyses include complete blood count with differential, levels of alkaline phosphatase, alanine aminotransferase, calcium, creatine kinase, fasting-glucose, gamma-glutamyl transpeptidase, hemoglobin A1C, serum protein electrophoresis, anti-nuclear antibodies, thyroid stimulating hormone, and screen for anti-nuclear antibodies and extractable nuclear antibodies. If a clear cause for PN was identified, these patients were excluded from the study.

6 Sample size

We aimed to recruit a total of 60 participants (30 in each of the PD and control cohorts), a number derived from power calculations based on previously reported prevalence of PN in PD and control populations. As described previously, the studies of Rajabally et al. (2011) and Toth

et al. (2010) report a prevalence of PN between 38% and 58% in PD patients, and 8% to 9% in matched controls. Assuming a conservative prevalence of PN in our PD and control cohorts of 30% and 10%, respectively, we performed calculations based on the recommendations for sample size estimation in clinical research studies set out in a recent publication (Suresh and Chandrashekara, 2012). Here the authors suggest the below formula to estimate sample size based on expected proportions of the outcome of interest, PN.

$$N = \frac{\left(Z_{\alpha}\sqrt{2p(1-p)} + Z_{1-\beta}\sqrt{p_1(1-p_1)p_2(1-p_2)}\right)^2}{(p_1 - p_2)^2}$$

***N** = total sample size estimation (assuming equal size cohorts)*
p_1 *= expected proportion of PN in CTRL*
p_2 *= expected proportion of PN in PD*
p *= difference in proportions*
Z_{α} *= normal deviate for a one-tailed, α=0.01 level of significance (2.33)*
Z_{1-B} *= normal deviate for 90% power (1.28)*

Based on this formula, enrollment of a total of 30 participants in each cohort would be more than enough to give us 90% power to detect a difference of 20% in the prevalence of PN between cohorts, with a level of significance of α=0.01. Important considerations in utilizing the aforementioned estimates are the associated age and duration of disease of the participants, which may be considerably shorter in our cohort of 'de novo' PD patients.

7 Statistics

We performed the standard student's t-test to compare corneal confocal measures, ENFD and NCS measures between control and PD cohorts; we used a one-tailed p-value as we expected that PD patients would have decreased measures. We performed Fisher's exact test to compare patient characteristics and UENS scores between cohorts. We performed the Mann-Whitney test to compare patient age between cohorts. Finally, we performed Pearson's correlation to determine intra-observer reliability for ENFD and additional linear regression for comparison of

patient's age with blood levels of metabolites and vitamins. For all statistics, we assumed a p value <0.05 to indicate a significant comparison.

Results

8 Patient population

We studied a total of 49 patients of which 27 where 'de novo' PD patients and 22 where control subjects without PD. A total of 8 patients could not be enrolled in the study, due to presence of risk factors predisposing to PN (diabetes, n=3), unwillingness lack of willingness to undergo study procedure(s) (n=2), or difficulty with travel from a rural location for the study visit (n=3). Patient mean age and age distributions were similar between cohorts (Table 2). In the control cohort, there was a predominance of female participants (64%) as compared to the PD cohort (41%), which was not significant (Table 2).

No one in our study had a significantly restricted diet (ie. vegetarian or vegan); one control subject had a diet consisting entirely of organic food and one PD patient was celiac. Many patients had mild-to-moderate drinking habits, but we did not find any of the patients to be at risk of chronic alcohol intoxication (self-reported). 7 (26%) PD patients and 5 (23%) control subjects were on either oral cobalamin or B-complex supplementation (see Table 2). Patients from both cohorts were also on other common medications including but not limited to statins, blood thinners, antihypertensives and sleeping pills. None of our patients were contact lens users. Four PD patients had begun low-dose levodopa shortly (≤1 week) prior to our assessment (3, 5, 6, and 7 days before) due to either delays in assessment or misunderstanding of instructions provided. One PD patient was on dopamine agonist medication for PD (Pramipexole).

9 Clinical and Laboratory Findings

9.1 Clinical examination

Using the UENS with a cutoff score set at ≥4 out of 42, we identified a neuropathy in 5 control subjects and in 10 PD patients (23% and 37%, respectively; p=NS see Table 2). We did not identify a cause for PN in any of these patients, based on the patient history and our panel of blood tests. The majority of abnormalities manifested as decreased pinprick sensation (31% of all patients) and/or decreased vibration threshold detection (41% of all patients; see Figure 7 for proportions per cohort). There was a significant difference between cohorts for presence of vibration sensation abnormalities (Fisher's p=0.008). The remaining sections of the UENS did not reveal any significant abnormalities with only one patient showing diminished joint position sensation and one patient with mildly diminished reflexes. All findings were symmetrical, with the exception of two patients (4%) who had mildly increased severity of abnormalities in one limb and two patients who had mild findings in only one limb.

9.2 Nerve conduction results

Nerve conduction studies were performed on the right leg, except in four patients who asked that we perform all studies on the left leg. One patient did not have NCS performed, due to technical difficulties at the time of the visit. We were not able to obtain peroneal nerve potentials in two patients (1 CTRL and 1 PD) due to marked edema in the legs. One PD patient had absent peroneal sensory nerve potentials. We compared conduction velocities, base-to-peak amplitudes and distal latencies in each of the sensory nerves, and velocities and amplitudes in the motor nerves examined (described in methods). We did not find any significant difference between PD patients and control subjects in any of the NCS parameters examined (see Figure 8). PD patients

had near-significantly lower sural sensory velocities and amplitudes than control subjects (p=0.056 and p=0.061, respectively; Figure 8). Three patients (2 control and 1 PD) had a decreased peroneal motor amplitude (>20%) across the knee, suggesting a potential focal lesion at this site (Marciniak, 2013).

9.3 Epidermal nerve fiber density

The mean ENFD was higher in control subjects (4.86 ±0.48 mm^{-1}) than in PD subjects (3.99 ±0.38 mm^{-1}), but this difference was not significant (p=0.079; Figure 9). Interestingly, the ENFD among control subjects appeared to have a bimodal distribution, perhaps suggesting a subpopulation of controls with a PN. Thus, we divided our control cohort into patients scoring ≥4 on the UENS (presumably having PN) and those scoring <4, but found that there was no significant difference in ENFD between these groups (p=0.123). As the determination of ENFD is subject to observer error, we also determined intra-observer reliability by comparing the ENFD determined at two separate occasions for 39 samples; the Pearson's correlation coefficient was 0.93 (95%CI 0.88-0.97 and R^2 0.87).

9.4 Corneal nerve fiber analysis

CCM was performed on all but two participants (1 CTRL, 1PD). The mean CNFL was significantly higher in control subjects (16.75 ±0.70 mm) than in PD patients (14.14 ±0.78 mm; p=0.010, Figure 10). CNFD was also higher in control subjects than in PD patients (25.56 ±1.33 mm^{-1} versus 22.16 ±1.41 mm^{-1}; p=0.046). Finally, CNBD was also compared between cohorts and was found to be significantly higher in control subjects (43.63 ±4.54 mm^{-1}) than in PD patients (31.39 ±2.70 $mm^{-1;}$ p=0.010). Automated analysis of CCM images had been previously

compared to manual analysis and was found to be highly correlated (Petropoulos et al., 2014); in some cases, automated analysis yielded slightly lower numbers yet in a consistent fashion such that group differences remained true (Dr. K. Romanchuk, personal communications).

We performed additional analysis in which we compared the parameters obtained from CCM analysis and other measures of PN severity, the ENFD and the UENS. We did not find any significant correlations.

9.5 Blood tests

Blood tests were obtained for most patients (n=18 (82%) for control cohort and n=23 (85%) for PD cohort); some patients had incomplete blood tests due to a lab mistake or simply not presenting to the lab. We did not find any significant differences between PD and control cohorts for blood levels of folate, cobalamin, Hcy or MMA. Generally, we did not find any abnormalities in blood levels of folate. However, the following abnormalities were identified in our patients (see also cutoffs Figure 11): six patients (2 CTRL and 4 PD) had very mildly increased levels of Hcy (≥13 μmol/L); two patients had abnormally low levels of cobalamin (1 CTRL and 1PD; <200 ng/L) and several patients had low-normal cobalamin levels (200-300 ng/L). Additionally, three patients had mildly high levels of cobalamin (CTRLs; one of these was on oral B-complex supplementation and two were on multivitamins). Finally, one patient (PD) had high levels of MMA (this patient had begun levodopa prior to assessment). Patients in both cohorts had abnormalities in other blood parameters, most of which were near the limits of normal and included abnormalities in lipid profiles, mildly elevated HgbA1C and abnormalities in blood cell

quantities. Two patients (1PD and 1 CTRL) had elevated serum creatinine levels. There were also sporadic individual cases of elevated gamma-glutamyl transpeptidase (GGT).

Discussion

Here we studied a cohort of de novo *levodopa-naïve* PD patients, to eliminate levodopa treatment as a confounding factor and determine if PN is an inherently concomitant entity in PD. In addition to using gold standard tests for PN – the clinical examination and nerve conduction studies – we utilized novel and sensitive tests – CCM and skin biopsy – to detect small fiber changes in these patients. Our results indicate that PD patients may exhibit early peripheral nerve changes, independent of treatment; we identified subtle abnormalities in the neurological exam as well as in the small corneal fibers of PD patients using CCM. However, these abnormalities did not appear to affect all peripheral nerve fibers to the same extent, as we failed to identify any difference in NCS and ENFD between cohorts. Also, and interestingly, we did not find any difference in blood MMA or cobalamin levels between control and PD subjects, suggesting that the mild presence of PN early in PD may either have a different pathogenesis than we hypothesized, or may be an early feature of PD that is aggravated by a buildup of these molecules in later stages of the disease. One could speculate that a buildup of synuclein in these fibers, as shown in cutaneous neurons (Wang et al., 2013), may lead to dysfunction and ultimately neuropathy in some PD patients .

Our patient populations were similar in terms of age, with a non-significant predominance of males in the PD cohort as compared with the control cohort. The impact of this on the interpretation of our data is uncertain, as it is unclear if the prevalence of idiopathic PN differs by

gender. In studies of all PNs, including those of known cause, one study did not find any differences in PN prevalence between sexes (Mold et al., 2004) and another larger community-based study in Egypt found higher prevalence in females (Kandil et al., 2012). However, these studies relied on subjective measures and individual reporting of PN, whereas objective measures would be favored. Our patient cohorts were also free of risk-factors predisposing to PN in order to minimize confounding variables in our analysis.

Upon clinical examination and based on the UENS scores, the proportion of patients with neuropathy was higher in our PD cohort as compared to control subjects, however this was not significant. In all cases, the PN which was identified was an asymptomatic neuropathy such that patients did not report any sensory or motor complaints prior to examination. It is known that PN can develop slowly, especially the idiopathic form of the disease and thus symptoms may not be evident at first presentation (Sachedina and Toth, 2013). Additionally, in many cases PN develops from a subclinical and asymptomatic state; this has been described for many forms of PN, especially in diabetic peripheral neuropathy (Davies et al., 2006).

Our patient sample consisted mostly of elderly individuals which would on the outset be expected to have a higher prevalence of PN as compared to a younger cohort. In any case, we identified PN in an unexpectedly large proportion of control subjects (23%) as compared to previously described estimates (8%) in population-based studies (Martyn and Hughes, 1997, Hoffman et al., 2013). The PN in our control patients could not be explained through other causes identified via results of blood testing, strongly suggesting true idiopathic PN. Rajabally and Martey (2011), who also utilized the UENS in assessing their control cohort of individual,

also report a much lower proportion of PN; this is likely due to selection of a more conservative UENS cutoff for PN than in our study. One research group which investigated sensory neuropathy in healthy elderly patients reported a similar prevalence of PN to our study, however the causes for PN were self-reported by patients and presumably not rigorously investigated though bloodwork or confirmed by a specialist (Mold et al., 2004).

Based on our selected cutoff of ≥4 on the UENS to identify patients with PN, the proportion of PN in our PD cohort was similar to the control cohort. However, most other studies have reported lower rates of PN in control subjects as described above; thus we cannot rule out the possibility that we may in fact be seeing a higher prevalence of PN in our PD patients. Over one third of our PD patients had some neuropathic abnormalities, which is higher than reported in one other study of untreated PD patients (Ceravolo et al., 2013). This could be due to different criteria used by this group, involving the presence of a sign or symptom of neuropathy in order to be classified as PN, whereas our patients were mostly asymptomatic with no clinical signs.

On closer examination, the UENS scores were not equivalent between cohorts in each of the UENS sections. Specifically, a greater proportion of PD individuals had an abnormal vibration sensation threshold, as compared to control subjects (Figure 7), suggesting a large fiber problem. However, no abnormalities were found on other tests of large fiber function, namely joint position sensation. Collectively this may indicate a more specific abnormality at this early stage of the disease such as in the deep epidermal mechanoreceptors, specifically pacinian corpuscles which are responsible for transducing vibration sensation, as tested clinically. This is consistent with a previous study in Italian PD patients that showed denervation of Meissener's corpuscles,

another type of mechanoreceptor in the skin (Nolano et al., 2008). Otherwise, abnormal vibration perception may also be indicative of a central nervous system problem, specifically within the dorsal columns of the spinal cord where vibratory afferent neurons carry signal to the medulla (Gilman, 2002). We do not anticipate this to be a central issue as the dorsal columns are shared for other sensory pathway such as that for proprioception, and an abnormality here would perhaps be expected to affect the other pathways as well, which was not seen in our patients.

In the remaining sections of the UENS, we found that pin sensation was also decreased in both cohorts, but not more significantly in either cohort; as this test is indicative of small fiber damage, it suggests that 'de novo' PD patients may not be exhibiting any functional small fiber abnormalities than control subjects. Notably, only two patients (1 CTRL and 1 PD) had decreased pin sensation all the way to the knee level, evidence of the slow progression and mild presence of abnormalities in our cohort. Ankle reflexes were not significantly affected in either PD or control patients which is not surprising, as reflex abnormalities are least likely to be present in PN, at least at the early stages (Singleton et al., 2008). Finally, motor abnormalities indicative of PN were not evident upon exam in any of our patients, indicating an absence of any more aggressive and global neurodegenerative processes in these patients and suggesting a more sensory-dominant process is occurring.

We did not find a difference in ENFD between control subjects and de novo PD patients, possibly suggesting that epidermal nerve fiber density is not affected early in PD. To our knowledge, this is the first study examining ENFD in untreated PD patients. ENFD has previously been determined and validated for use in detection of small fiber sensory neuropathy,

and tested in this limited setting (Lauria et al., 2005). Our lack of any findings in skin biopsy, however, does not completely rule out occurrence of a neuropathic process that may be occurring in these patients. Neurodegeneration in PN can proceed in different manners, such that motor and/or autonomic fibers can be predominantly affected versus sensory fibers (the opposite is also true) thus sensory free-endings in the skin may not be extensively affected. There are other processes that may be occurring within epidermal fibers which may predispose to developing PN later in PD; one of these may be the accumulation of synuclein, which has been recently described (Donadio et al., 2014). Additionally, our results of ENFD determination may be subject to a type II error, such that we were not able to identify a difference that may in fact exist in the population. Although we were not able to identify a difference in ENFD between cohorts, these findings do appear to reflect the lack of difference in pin sensation abnormalities between cohorts on the UENS, as would be expected since the free nerve endings in the skin are mostly involved in the response to this clinical test.

Our mean ENFD in control patients was lower than the range reported in other studies (summarized in (Lauria et al., 2010b)). There may be several reasons for this, the first being the high higher than anticipated prevalence of PN in our control cohort, as described earlier, which we thought could be responsible for depressing the group mean. To investigate this, we compared the ENFD between control patients that scored <4 on the UENS (thus presumably not having a PN) and those patients who scored ≥4 and found that the means were still similar. Additionally, prior to the EFNS guidelines when most studies determining normative ranges were performed, it appears there was no common standard in processing and preparing tissue sections which inevitably introduced variation into the calculated ENFDs. First, there appears to

be discrepancy as to which preservative to use such as to maximally conserve the morphology of epidermal sections; some studies have utilized Zamboni's and others have utilized PFA. Time spent in the cryoprotective stage can also be modified; varying the amount of time the sample has to be fully penetrated by the preservative may yield different results. Additionally, the use of different markers as growth associate protein 43 may be used to identify additional populations of regenerating axons and thus yield different ENFDs (Rage et al., 2010).

One may also consider the limitations of ENFD determination in the skin biopsy. Based on our supplementary analysis correlating ENFD determined at two separate occasions by the same observer, we found a strong intra-observer correlation, suggesting this is a good method for epidermal nerve fiber quantification if counting is consistent (ie. same observer). However, a recent report tested reliability of ENFD determination in different observers and showed that, particularly in the setting of epidermal sections with a poorly defined basement membrane (BM), ENFD may not be as reliable (Wopking et al., 2009). This may be the case with thicker tissue sections in which the BM may no longer be a linear structure; our chosen thickness of 50μm was such that it allowed visualization of entire nerve endings, yet in some cases the BM was not entirely clear. In future studies, the use of additional markers for BM as type IV collagen may be considered to demarcate this border with greater clarity. Another limitation of ENFD determination from a skin biopsy is that in some cases, the area of skin may have experienced denervation not visible upon to the naked eye, as may be the case with chronic compression from various types of clothing (ie. high socks), blunt force trauma, constant scratching or irritation of the skin by toxic fluids and sun burn. We took precautions to avoid such instances as possible, avoiding areas affect by rash or abnormal skin conditions although such instances were rare.

As we presumed that any PN developing in our patients, if any, would be mostly affecting small fibers early in the disease, we did not expect to identify abnormalities in nerve conduction. Consistent with these expectations, our results from NCS did not reveal any significant differences between cohorts. These results suggest that any neurodegenerative occurring in PD patients are not affecting large nerve fibers at onset. We did observe a nearly significant decrease in sural sensory velocity and distal latency in PD patients which may be indicative of early changes occurring here. To our knowledge only one other recent multicentre study by the Ceravolo research group performed similar electrophysiological investigations in untreated PD patients; here they described decreased SNAP amplitudes and CMAP velocities in PD patients, with SNAP abnormalities also exhibited at the sural nerve (Ceravolo et al., 2013). Of note, the Italian research group performed limited NCS in accordance with some evidence-based recommendations to identify distal symmetric polyneuropathy (England et al., 2005). We performed NCS in additional nerves, the tibial motor and peroneal sensory nerves, anticipating that a more thorough examination would more certainly exclude the possibility of neuropathic abnormalities. In studies of treated PD patients, particularly those with levodopa infusion, decreased SNAP amplitudes and CMAP velocities have also been described (Mancini et al., 2014) suggesting that early neurodegenerative changes in PD persist throughout the disease and may be further exacerbated by treatment.

We performed additional NCS on the peroneal nerve with stimulation above the knee to allow identification of a conduction block. Three patients were identified to have significant decrease

in CMAP amplitude over this segment of nerve, thus suggesting a focal problem at the knee. It is uncertain if this contributed, as there was no motor deficit identified.

To our knowledge, our study was the first to perform CCM in a cohort of PD patients, utilizing an automated nerve counting program to eliminate observer bias. In doing so, we were able to show a significantly lower CNFL, CNFD and CNBD in PD patients than in a control cohort of patients of a similar age. These parameters are known to be decreased in a variety of systemic diseases with concomitant neuropathies, the most widely investigated being diabetic neuropathy (Tavakoli et al., 2012). One small study performed CCM in 4 PD patients and found corneal nerve density (expressed as total length of nerve fibers per unit area, and thus most similar to our CNFL measure) was not different from 5 control subjects of a similar age (Reddy et al., 2013). Interestingly, the majority of these PD patients were on levodopa therapy (length of time was not expressed) with at least one of these (25%) having severe disease when testing were performed. The difference in CNFD (as number of fibers per unit area) between cohorts was near our cut-off for significance. A likely explanation for this could be that the measure of CNFD is prone to subjectivity and error (perhaps even with automated image analysis) due to the inherent structure of corneal nerves; as daughter nerve fibers often travel through the cornea in parallel, they interconnect frequently (Marfurt, 2011) which inevitably renders selecting fibers for counting difficult. Nevertheless, CCM has been shown to be a very sensitive technique, able to detect corneal nerve regeneration following kidney and pancreatic in diabetic neuropathy (Tavakoli et al., 2013), as well as to track nerve regeneration many years after corneal refractive surgery (Cruzat et al., 2010).

Our results with CCM were unfortunately not supported by our findings in the skin biopsies, where we did not identify significant differences in ENFD between cohorts. This was unexpected, based on the high degree of correlation between ENFD and CCM parameters in previous studies (Quattrini et al., 2007, Brines et al., 2013). Although the fiber types found in these areas are primarily the same (Aδ and C fibers) there are some differences to note, that may be exploited by different neuropathic processes. Firstly, the nerve fibers innervating the skin in the lower leg are the longest axons in the body, whereas those in the eye have a relatively much shorter distance to travel from the cervical sensory ganglia. If the pathological process affecting corneal nerves is in any way related to that affecting the substantia nigra, the proximity of these structures (relative to other peripheral nerves) may in part account for the early development of abnormalities in these fibers. This seems plausible in light of reports which suggest that PD pathology may be a spreading process beginning in the brain stem ((Ulusoy et al., 2013); also in Braak's seminal paper (Braak et al., 2003)); of note, several cranial nerves emerge from the brain stem, notably the trigeminal nerve of which the ophthalmic division innervates the cornea. Another difference is such that corneal fibers are densely interconnected within Bowman's membrane whereas epidermal fibers, upon branching from their deep nerve bundles appear to follow unique pathways and may be subject to different pathology.

We asked patients if they wore contact lens (n=3; Table 2) in order to exclude possible effects this might have on corneal nerve fibers; however, current literature indicates that contact lenses diminish corneal sensitivity but do not affect nerve fiber number (reviewed in (Cruzat et al., 2010)). Our patients were not known to have corneal infections. CCM parameters have also been noted to alter significantly post cataract surgery but appear to recover within a year following the

procedure with 60% recovery after 6 months (De Cilla et al., 2014). In our study, few patients (n=5) reported having cataract surgery in their past, with 2 individuals (1CTLR and 1PD) having surgery within a year of our assessment. After removal of these patients from analysis, we found that CNFD was no longer significantly different between cohorts (p=0.056), whereas the other parameters remained significant. Retrospectively, the patients which we removed had substantially lower corneal nerve parameters than other subjects, and at could have outright been considered abnormal, even in the context of PN.

We hypothesized that blood levels of cobalamin, Hcy and MMA, pertinent metabolites to the methionine metabolic pathway, would be within normal limits in a cohort of untreated PD patients naïve to levodopa and thus comparable to healthy controls. We did not find any differences in the levels of these constituents between control subjects and our cohort of 'de novo' PD patients in which early signs of PN were detected; this suggests that cobalamin, Hcy and MMA may not be responsible for onset of PN in untreated PD patients. However, in light of previous findings of elevated levels of Hcy and MMA in treated PD patients (Postuma and Lang, 2004, Toth et al., 2010), we cannot exclude the possibility that these metabolites may also be exacerbating PN in some PD patients, and aggravating the process in those who may develop early neuropathic features. A number of patients in our study showed low-normal levels of cobalamin, a finding which itself requires further attention as it is well known that a proportion of patients, particularly the elderly with 'normal' cobalamin (200-300 ng/L) may have an underlying cellular deficiency of cobalamin (Carmel et al., 2003, Mason, 2011). In fact, since the advent of folate fortification as of 1998 which significantly normalized folate levels in the population (Dietrich et al., 2005, Pfeiffer et al., 2005), cellular cobalamin deficiency has become

a more commonly encountered clinical scenario (Carmel et al., 2003). Worse still is that the identification of cobalamin deficiency itself remains a challenge, with additional measurement of MMA remaining a top diagnostic test, with high sensitivity. Thus, in light of our findings of normal levels of MMA we are more confidently able to also exclude the possibility of functional cobalamin deficiency in our PD and control cohorts. Additionally, normal MMA levels in conjunction with the normal levels Hcy further confirm that folate levels are also normal in our patient population.

We were fortunate in this study to have a cohort of PD patients naïve to any other PD treatments (even though these would not have been excluded) that alter the pathways of methionine pathway, such as COMT inhibitors (Lamberti et al., 2005); as a result we can more reliably say that the metabolites we have measured have not been affected by confounding factors.

The measurement of metabolites within the blood is often a complex issue, as it is dependent on virtually all processes that occur within the body; the measurement of blood Hcy, MMA and folate are no different, as investigated by a German group of researchers (Herrmann et al., 2000). Herrmann and colleagues showed that blood levels of these metabolites are altered invariably with age, as well as with creatinine levels – a clinical indicator of proper kidney function. In an *ad hoc* analysis we were able to show that this was case for Hcy levels which were significantly correlated with patient age (Figure 12). Our cohorts were, however, comprised of similarly aged adult and senior individuals with few individuals in the late 7^{th} decade of life where metabolite levels may be expected to be significantly different. Additionally, two patients (one in each

cohort) had elevated creatinine levels indicative of some mild functional kidney impairment; however it is unlikely these cases contributed significantly to skew results.

In view of the absence of blood abnormalities in combination with our findings more PN in PD subjects as per CCM (and perhaps the UENS as well), we must also recognize that PN in PD may have a unique etiology that has not yet been considered. One may hypothesize that whatever mechanisms are leading to cell death in the brain, particularly in areas beyond the substantia nigra of PD patients, may also be involved in PNS degeneration (enteric nervous system involvement and extranigral degeneration in animal models reviewed in (Lebouvier et al., 2009, Knaryan et al., 2011)). It is also possible that a neurodegenerative toxin is present systemically in various levels in PD patients, thus leading to different severities of PNS abnormalities. Further research will be required in order to elucidate other potential etiological associations.

Limitations

In order to accurately interpret the results of this study, there are notable limitations to acknowledge that have not yet been examined above. First and foremost, although most comparisons of PN test results between PD and control cohorts were negative, our findings on CCM suggest neuropathy may be a part of early untreated PD. Although these signs nerve damage developing early in 'de novo' PD patients indicate strongly that treatment may not be responsible for the onset of PN in many PD patients, it may still contribute to progression of disease later on. It is possible that PN results from an interaction of PD pathogenesis and treatment effects. Second, we must be aware that the findings in this study may be a result of normal aging or competing diseases that may occur in our patients; thus, we expect that the

similarity in age between our PD and control individuals served as a valid control for this purpose. Interestingly, 18 PD patients (67%) brought along a relative (spouse) to participate as a control, thus the diet/lifestyle was likely similar (quasi controlled) between patient groups. However, our patients were inevitably be exposed to a variety of nutritional supplements, notably vitamin supplements as cobalamin, which serve as a potential confounder in the measurement of serum metabolites and development of neuropathy; these were duly noted and taken into account upon data analysis.

Furthermore, PD is a chronic progressive disorder and the associated NMS, as discussed earlier, may develop at various stages of the disease. Similarly, PN may exhibit a very slow progression and may take years to manifest at the clinical level. For these reasons, our mostly negative findings on the majority of the clinical exam and ENFD do not exclude the possibility that PN may develop robustly later in PD as a result, or not, of treatment with levodopa. Thus, we have ensured that all our patients were seen by expert movement disorders physicians to assure a diagnosis of true idiopathic PD. Another limitation in our study is such that the assessing neurologist was not blinded to cohort assignment during assessment; however we believe this would not be possible in any case, due to the noticeable presence of PD in most of our patients. This may have impacted upon clinical and electrophysiological scoring, although we did not find significant differences between in NCS measures between patients and control subjects. Other assessments, namely epidermal nerve fiber counting (for ENFD determination) were performed in a blinded manner in all cases. Corneal nerve fiber assessment was performed via an automated program and thus was not subject to the same potential for bias.

It is also possible that type II errors occurred in our study due to lower than expected enrollment. We did not enroll our anticipated 'at least' 30 individuals per cohort, especially in the control cohort, and thus we may have failed to identify a difference in ENFD and in NCS measures. This was likely a large contributor to any type II error that may have occurred in our study, not allowing us to identify true differences between patient groups. As mentioned, a high prevalence of PN in our control cohort may have further contributed to this error. Of note, sample size calculations, which have nevertheless become the steadfast method for directing study recruitment, are inherently based on a single endpoint which is not always practical where many outcomes may be possible. PN is particularly such a case as it is very much a continuum of abnormality, such that a patient may have very mild subclinical signs with no symptoms, another may present with acute onset neuropathic signs and symptoms and all others will exhibit some combination of features in between. For this reason, our classification of patients as having or not having PN based on a pre-determined cutoff score on the UENS could also be considered a limitation and re-evaluated in future studies.

Finally, we would like to have studied a follow-up period in which some or all PD patients had begun levodopa therapy, to permit a paired or 'repeated measures' comparison of neuropathy measures before and after treatment initiation; this would allow for the highest degree of control for confounding factors as possible, as has not yet been done in any study of PN in PD.

Conclusion

Taken together, our results suggest an early sensory predominant neuropathy occurring in a proportion of PD patients prior to treatment, affecting both large and small nerve fibers. This does not exclude the possibility that PN may be further exacerbated by treatment in PD patients. The presence of neuropathic abnormalities in our cohort of 'de novo' PD patients, namely alterations in CNFD and CNFL suggests that PN may be a unique NMS of PD requiring individual attention and treatment to preserve the highest quality of life in PD patients. The etiology of PN in PD requires further investigation to successfully target treatments.

In order to strengthen the findings that will result from this study, peripheral nerve structure and function as well as blood levels of cobalamin, homocysteine and MMA should be measured in PD patients after beginning and remaining on treatment with levodopa for some time. This would provide added information regarding the effects of levodopa on the integrity of peripheral nerves in PD patients. These studies should be considered for future endeavours.

Tables

Table 2. Motor and non-motor signs of PD, table adapted from (Jankovic, 2008)

Motor signs	Non-motor signs
Tremor, bradykinesia, cogwheel rigidity, postural instability	Cognitive impairment, bradyphrenia, tip-of-the-tongue phenomenon
Hypomimia, dysarthria, dysphagia, drooling	Depression, apathy, anhedonia, fatigue, behavioral & psychiatric problems
Decreased arm swing, shuffling gait, difficulty rising from chair, turning in bed	Sensory symptoms (anosmia, pain, paresthesias)
Micrographia, slow activities of daily living	Dysautonomia (orthostatic hypotension, constipation, urinary dysfunction, abnormal sweating)
Glabellar reflex, dystonia, striatal deformity	Sleep disorders (REM behavior disorder, sleep fragmentation)

Table 2. Patient characteristics

	PD	**Control**	**Sig. diff. (p)***
Age (mean, range), *yrs*	63 (42-78)	63 (45-77)	0.70
Sex, *n*	11F, 16M (41% F)	14F, 8M (64% F)	0.15
PD duration (mean, range), *yrs*	0.91 (0.01-4.02)	-	-
UENS (mean, range), *score***	3 (0-10)	2 (0-12)	0.17
Score ≥ 4, *n*	10 (37%)	5 (23%)	0.36
Supplements, *n*			
B12	2 (7%)	3 (14%)	0.65
B-complex	5 (19%)	2 (9%)	0.44
Folic acid	1 (4%)	0 (0%)	-
Multivitamin	11 (41%)	6 (27%)	0.38
Contact lens users	1	2	-
Eye surgery***	0	2	-

*Fisher's exact test was used to test for significant differences between cohorts for nominal and ordinal variables. In some cases where small proportions existed (ie. only one patient was positive in a cohort, the comparison could not be performed); the students t-test was used for interval and ratio variables

**UENS score is calculated out of 42 points

***Surgery includes cataract surgery and corrective eye surgery (ie. LASIK)

Figures

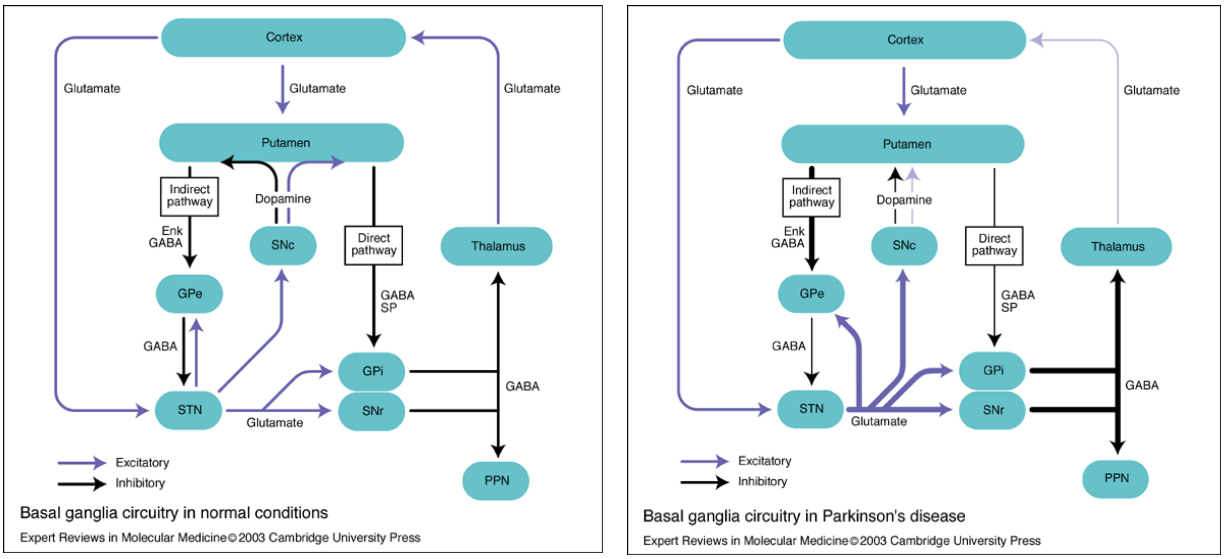

Figure 2. Basal Ganglia circuitry in a normal (left) and Parkinson's disease (right) brain. Figure taken from (Lewis et al., 2003).

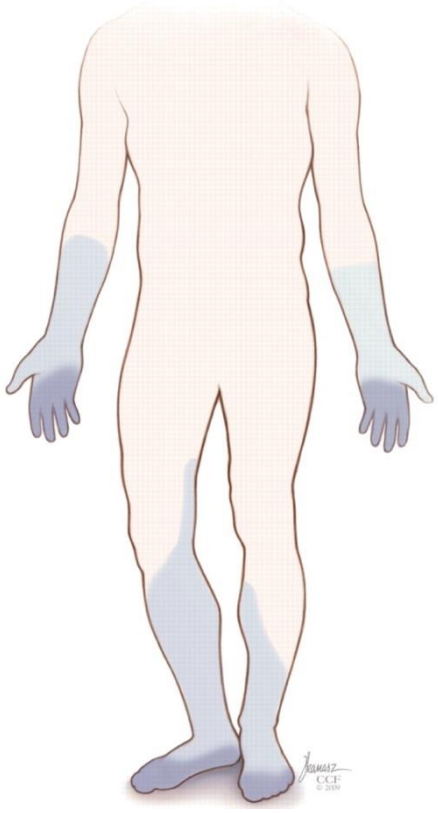

Figure 2. Stocking-glove pattern of a peripheral neuropathy. Figure taken from (Tavee and Zhou, 2009).

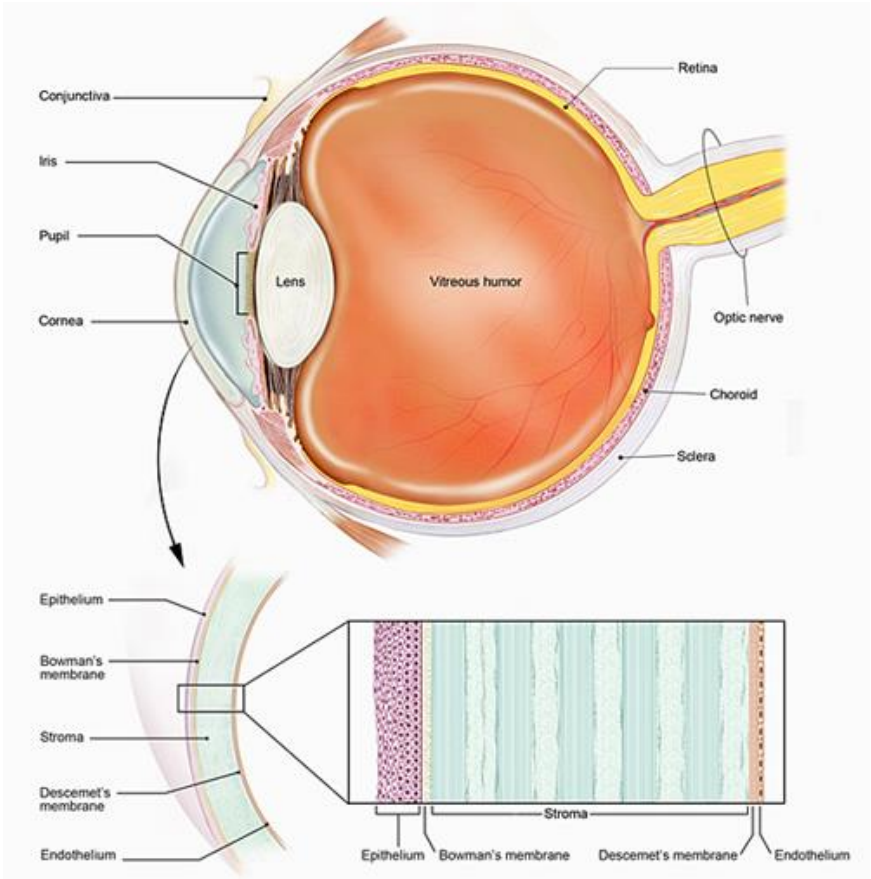

Figure 3. Layers of the cornea. Bowman's membrane is a layer near the front of the eye, which contains the main nerve plexus supplying the corneal epithelium. Figure taken from the National Eye Institute, NIH website (2013)

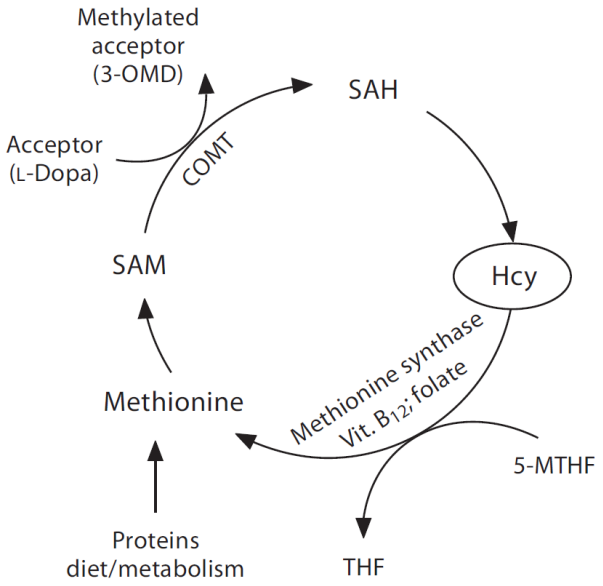

Figure 4. Pathway of Levodopa (L-DOPA) metabolism. Figure adapted from (Levin et al., 2010).

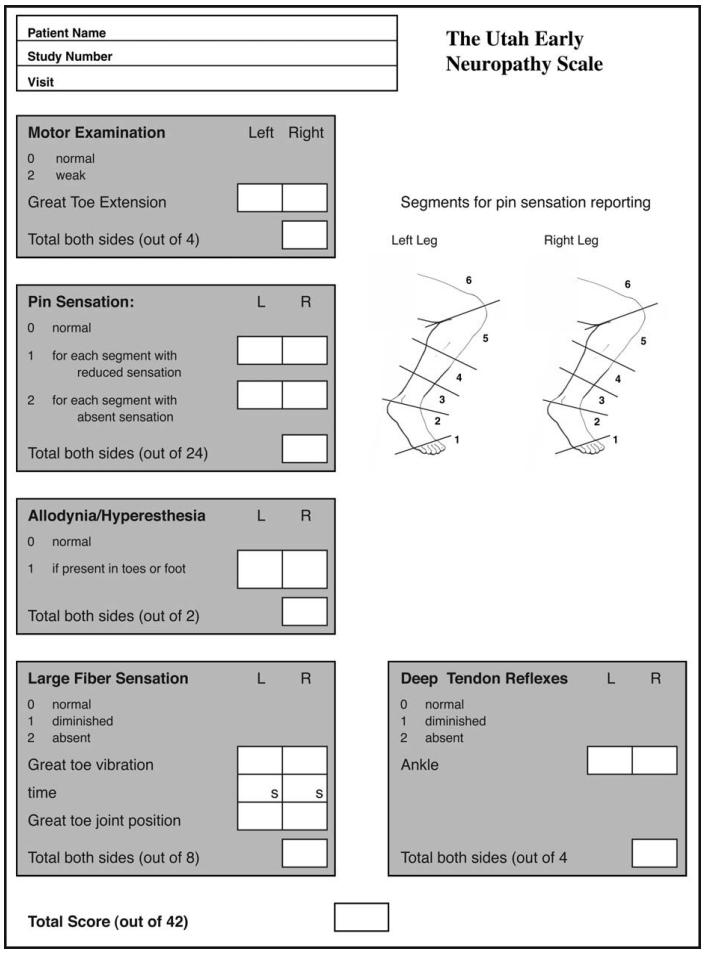

Figure 5. Utah Early Neuropathy Scale (Singleton et al., 2008). The scale is comprised of 5 sections, each scored individually for each limb. Reduced pin sensation is scored higher, as it affects more proximal portions of the leg.

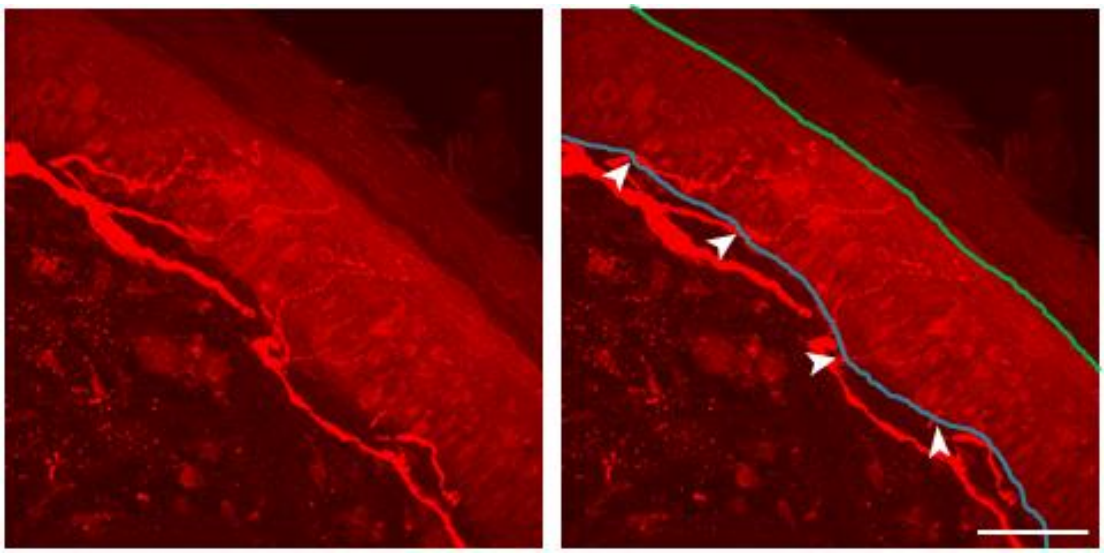

Figure 6. Determination of ENFD as per EFNS guidelines. Left image is an original unmarked image obtained by fluorescent confocal microscopy. Right image contains markings which indicate the pertinent features that are used for ENFD determination. Green line indicate the superficial aspect of the epidermis used to determine epidermal length; Blue line indicates the basement membrane, the junction between dermis and epidermis; arrowheads indicate nerve fiber crossings that would be counted towards ENFD determination. Scale bar 50µm.

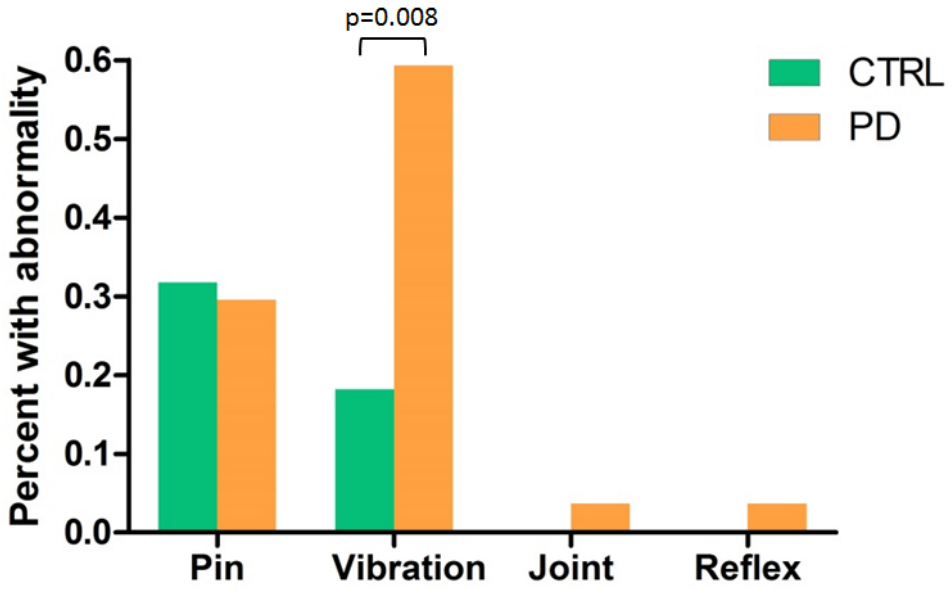

Figure 7. UENS scores per section. UENS sections where no abnormalities were found in either cohort are not shown (see Figure 5 for the list of all UENS sections). Bars indicate the percent of individuals within each cohort (PD or control) with abnormality in the respective section of the UENS (≥1 point). P value indicated from Fischer's exact test.

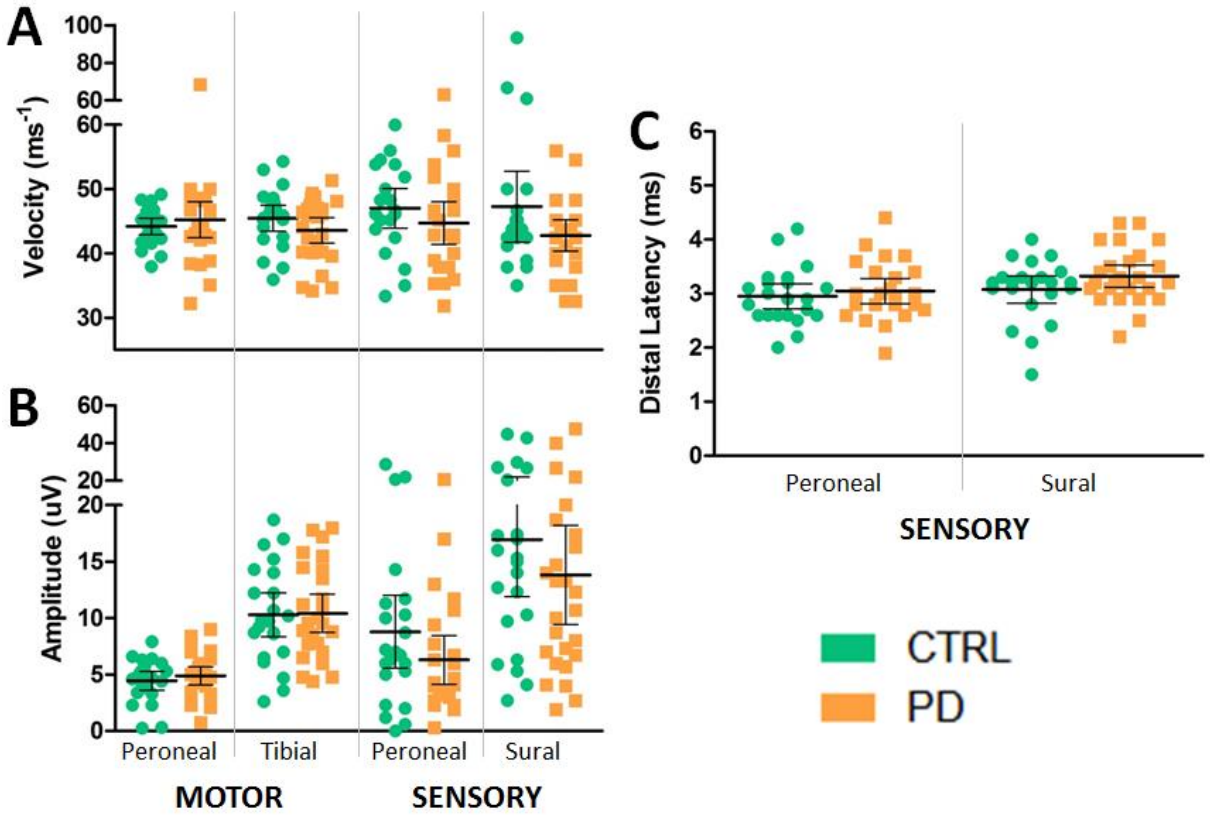

Figure 8. **Nerve conduction studies for control and PD patients.** A. Velocities for motor and sensory nerves examined. B. Motor and sensory amplitudes. C. Distal latencies for the peroneal and sural nerves. Horizontal bars indicate mean; error bars indicate 95% CI.

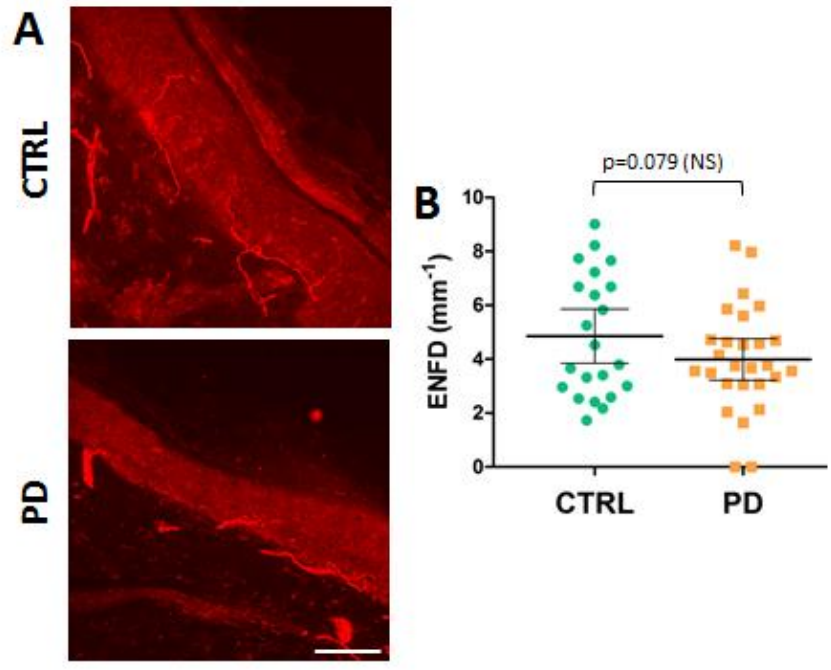

Figure 9. **Confocal stack images from epidermal sections and ENFD.** A. Image assembled from a stack of confocal images spaced 1um apart. Red=anti-UCHL1 antibody. Scale bar represents 50µm B. Horizontal bars indicate mean; error bars indicate 95% CI. CTRL n=22 and PD n=27.

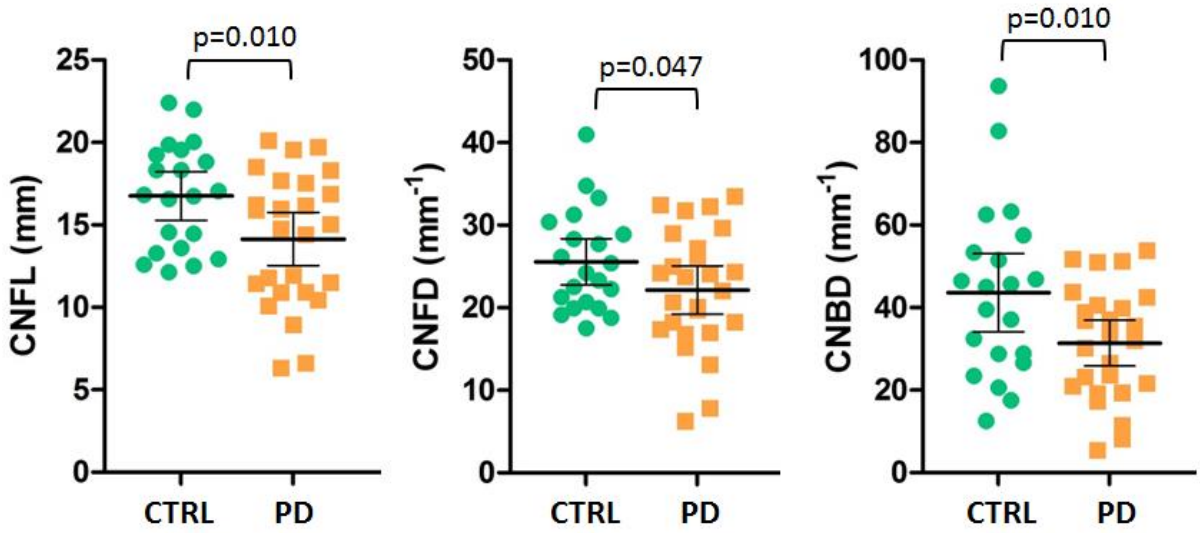

Figure 10. Corneal nerve fiber parameters from images obtained by CCM in control and PD patients. Horizontal bars indicate means; error bars indicate 95% CI. CTLR n=21 and PD n=26.

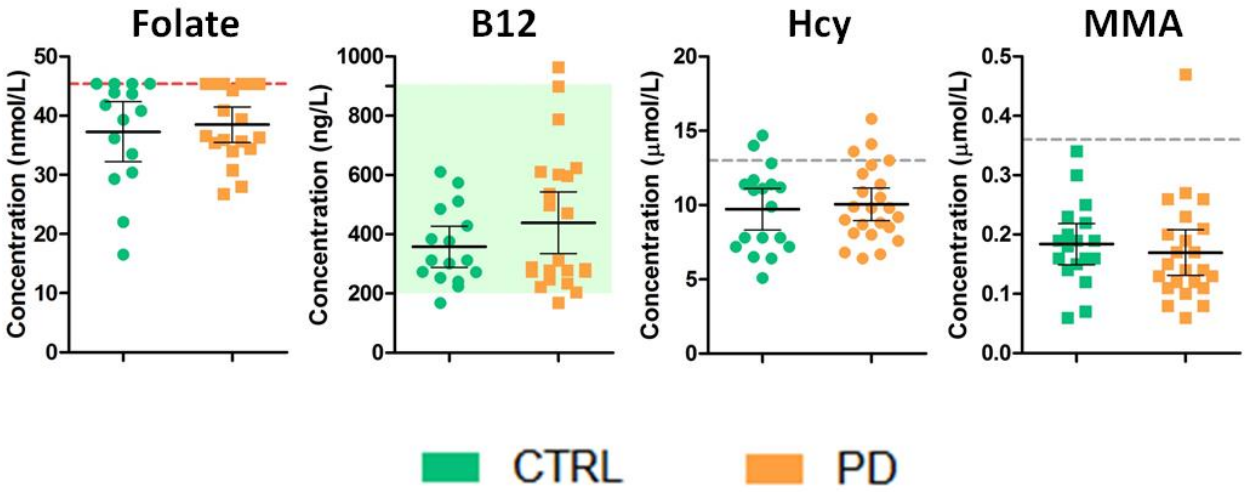

Figure 11. Pertinent blood analysis results showing levels of folate, cobalamin, Hcy and MMA. Horizontal bars indicate means; error bars indicate 95% CI. Dashed red line indicates limit of detection. Green shading (cobalamin graph) shows normal range of values. Dotted horizontal grey lines show cutoffs for normal values.

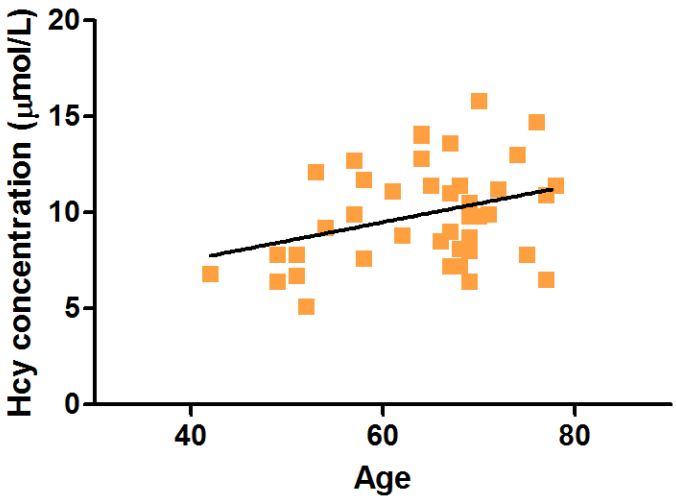

Figure 12. Linear regression analysis for patient age and blood Hcy levels. Solid line indicates linear regression line with p=0.037 significance.

References

(2013) Facts about the cornea and Corneal Disease. National Eye Institute, National Institutes of Health.

Abbruzzese G, Pigullo S, Schenone A, Bellone E, Marchese R, Di Maria E, Benedetti L, Ciotti P, Nobbio L, Bonifati V, Ajmar F, Mandich P (2004) Does parkin play a role in the peripheral nervous system? A family report. Mov Disord 19:978-981.

Alport AR, Sander HW (2012) Clinical approach to peripheral neuropathy: anatomic localization and diagnostic testing. Continuum (Minneap Minn) 18:13-38.

Antonini A, Isaias IU, Canesi M, Zibetti M, Mancini F, Manfredi L, Dal Fante M, Lopiano L, Pezzoli G (2007) Duodenal levodopa infusion for advanced Parkinson's disease: 12-month treatment outcome. Mov Disord 22:1145-1149.

Antonini A, Odin P, Opiano L, Tomantschger V, Pacchetti C, Pickut B, Gasser UE, Calandrella D, Mancini F, Zibetti M, Minafra B, Bertaina I, De Deyn P, Cras C, Wolf E, Spielberger S, Poewe W (2013) Effect and safety of duodenal levodopa infusion in advanced Parkinson's disease: a retrospective multicenter outcome assessment in patient routine care. J Neural Transm 120:1553-1558.

Barbosa PR, Stabler SP, Machado AL, Braga RC, Hirata RD, Hirata MH, Sampaio-Neto LF, Allen RH, Guerra-Shinohara EM (2008) Association between decreased vitamin levels and MTHFR, MTR and MTRR gene polymorphisms as determinants for elevated total homocysteine concentrations in pregnant women. Eur J Clin Nutr 62:1010-1021.

Beiske AG, Loge JH, Ronningen A, Svensson E (2009) Pain in Parkinson's disease: Prevalence and characteristics. Pain 141:173-177.

Berg D, Postuma RB, Bloem B, Chan P, Dubois B, Gasser T, Goetz CG, Halliday GM, Hardy J, Lang AE, Litvan I, Marek K, Obeso J, Oertel W, Olanow CW, Poewe W, Stern M, Deuschl G (2014) Time to redefine PD? Introductory statement of the MDS Task Force on the definition of Parkinson's disease. Mov Disord.

Biaggioni I (2007) Parkinson's disease: autonomic neuronopathy with impaired cardiovascular regulation. Hypertension 49:21-22.

Blandini F, Fancellu R, Martignoni E, Mangiagalli A, Pacchetti C, Samuele A, Nappi G (2001) Plasma homocysteine and l-dopa metabolism in patients with Parkinson disease. Clin Chem 47:1102-1104.

Blom HJ, Smulders Y (2011) Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. J Inherit Metab Dis 34:75-81.

Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E (2003) Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 24:197-211.

Brannagan TH, 3rd (2012) Current issues in peripheral neuropathy. J Peripher Nerv Syst 17 Suppl 2:1-3.

Brines M, Swartjes M, Tannemaat MR, Dunne A, van Velzen M, Proto P, Hoitsma E, Petropoulos IN, Chen X, Niesters M, Dahan A, Malik RA, Cerami A (2013) Corneal nerve quantification predicts the severity of symptoms in sarcoidosis patients with painful neuropathy. Technology 1:20-26.

Bromberg MB (2013) An electrodiagnostic approach to the evaluation of peripheral neuropathies. Phys Med Rehabil Clin N Am 24:153-168.

Calne DB, Snow BJ, Lee C (1992) Criteria for diagnosing Parkinson's disease. Ann Neurol 32 Suppl:S125-127.

Carmel R, Green R, Rosenblatt DS, Watkins D (2003) Update on cobalamin, folate, and homocysteine. Hematology Am Soc Hematol Educ Program 62-81.

Ceravolo R, Cossu G, Bandettini di Poggio M, Santoro L, Barone P, Zibetti M, Frosini D, Nicoletti V, Manganelli F, Iodice R, Picillo M, Merola A, Lopiano L, Paribello A, Manca D, Melis M, Marchese R, Borelli P, Mereu A, Contu P, Abbruzzese G, Bonuccelli U (2013) Neuropathy and levodopa in Parkinson's disease: evidence from a multicenter study. Mov Disord 28:1391-1397.

Cersosimo MG, Benarroch EE (2012) Autonomic involvement in Parkinson's disease: pathology, pathophysiology, clinical features and possible peripheral biomarkers. J Neurol Sci 313:57-63.
Chan SW, Dunlop RA, Rowe A, Double KL, Rodgers KJ (2012) L-DOPA is incorporated into brain proteins of patients treated for Parkinson's disease, inducing toxicity in human neuroblastoma cells in vitro. Exp Neurol 238:29-37.
Cortese C, Motti C (2001) MTHFR gene polymorphism, homocysteine and cardiovascular disease. Public Health Nutr 4:493-497.
Cruzat A, Pavan-Langston D, Hamrah P (2010) In vivo confocal microscopy of corneal nerves: analysis and clinical correlation. Semin Ophthalmol 25:171-177.
Dabbah MA, Graham J, Petropoulos IN, Tavakoli M, Malik RA (2011) Automatic analysis of diabetic peripheral neuropathy using multi-scale quantitative morphology of nerve fibres in corneal confocal microscopy imaging. Med Image Anal 15:738-747.
Dacci P, Dina G, Cerri F, Previtali SC, Lopez ID, Lauria G, Feltri ML, Bolino A, Comi G, Wrabetz L, Quattrini A (2010) Foot pad skin biopsy in mouse models of hereditary neuropathy. Glia 58:2005-2016.
Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889-909.
Davies M, Brophy S, Williams R, Taylor A (2006) The prevalence, severity, and impact of painful diabetic peripheral neuropathy in type 2 diabetes. Diabetes Care 29:1518-1522.
De Cilla S, Fogagnolo P, Sacchi M, Orzalesi N, Carini E, Ceresara G, Rossetti L (2014) Corneal involvement in uneventful cataract surgery: an in vivo confocal microscopy study. Ophthalmologica 231:103-110.
de Lau LM, Breteler MM (2006) Epidemiology of Parkinson's disease. Lancet Neurol 5:525-535.
Deacon R, Purkiss P, Green R, Lumb M, Perry J, Chanarin I (1986) Vitamin B12 neuropathy is not due to failure to methylate myelin basic protein. J Neurol Sci 72:113-117.
Devos D (2009) Patient profile, indications, efficacy and safety of duodenal levodopa infusion in advanced Parkinson's disease. Mov Disord 24:993-1000.
Dietrich M, Brown CJ, Block G (2005) The effect of folate fortification of cereal-grain products on blood folate status, dietary folate intake, and dietary folate sources among adult non-supplement users in the United States. J Am Coll Nutr 24:266-274.
Dirnberger G, Jahanshahi M (2013) Executive dysfunction in Parkinson's disease: a review. J Neuropsychol 7:193-224.
Donadio V, Incensi A, Leta V, Giannoccaro MP, Scaglione C, Martinelli P, Capellari S, Avoni P, Baruzzi A, Liguori R (2014) Skin nerve alpha-synuclein deposits: A biomarker for idiopathic Parkinson disease. Neurology.
England JD, Asbury AK (2004) Peripheral neuropathy. Lancet 363:2151-2161.
England JD, Gronseth GS, Franklin G, Miller RG, Asbury AK, Carter GT, Cohen JA, Fisher MA, Howard JF, Kinsella LJ, Latov N, Lewis RA, Low PA, Sumner AJ (2005) Distal symmetric polyneuropathy: a definition for clinical research: report of the American Academy of Neurology, the American Association of Electrodiagnostic Medicine, and the American Academy of Physical Medicine and Rehabilitation. Neurology 64:199-207.
Fahn S (2008) The history of dopamine and levodopa in the treatment of Parkinson's disease. Mov Disord 23 Suppl 3:S497-508.
Fenton WA, Gravel RA, Rosenblatt DS (2001) Disorders of Propionate and Methylmalonate Metabolism. In: The Online Metabolic and Molecular Bases of Inherited Disease(Valle, D., ed), pp 2165-2193 New York: McGraw-Hill.
Fredriksen A, Meyer K, Ueland PM, Vollset SE, Grotmol T, Schneede J (2007) Large-scale population-based metabolic phenotyping of thirteen genetic polymorphisms related to one-carbon metabolism. Hum Mutat 28:856-865.

Gazewood JD, Richards DR, Clebak K (2013) Parkinson disease: an update. Am Fam Physician 87:267-273.
Gilman S (2002) Joint position sense and vibration sense: anatomical organisation and assessment. J Neurol Neurosurg Psychiatry 73:473-477.
Goetz CG (2011) The history of Parkinson's disease: early clinical descriptions and neurological therapies. Cold Spring Harb Perspect Med 1:a008862.
Goetz CG, Tilley BC, Shaftman SR, Stebbins GT, Fahn S, Martinez-Martin P, Poewe W, Sampaio C, Stern MB, Dodel R, Dubois B, Holloway R, Jankovic J, Kulisevsky J, Lang AE, Lees A, Leurgans S, LeWitt PA, Nyenhuis D, Olanow CW, Rascol O, Schrag A, Teresi JA, van Hilten JJ, LaPelle N (2008) Movement Disorder Society-sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS): scale presentation and clinimetric testing results. Mov Disord 23:2129-2170.
Goldstein DS, Holmes C, Li ST, Bruce S, Metman LV, Cannon RO, 3rd (2000) Cardiac sympathetic denervation in Parkinson disease. Ann Intern Med 133:338-347.
Gondim Fde A, de Oliveira GR, Peixoto AA, Jr., Horta WG (2010) A case series of peripheral neuropathy in patients with Parkinson's disease. Ann Neurol 68:973-975; author reply 975.
Greenamyre JT, Hastings TG (2004) Biomedicine. Parkinson's--divergent causes, convergent mechanisms. Science 304:1120-1122.
Hattori N, Shimura H, Kubo S, Kitada T, Wang M, Asakawa S, Minashima S, Shimizu N, Suzuki T, Tanaka K, Mizuno Y (2000) Autosomal recessive juvenile parkinsonism: a key to understanding nigral degeneration in sporadic Parkinson's disease. Neuropathology 20 Suppl:S85-90.
Herrmann DN (2008) Noninvasive and minimally invasive detection and monitoring of peripheral neuropathies. Expert Rev Neurother 8:1807-1816.
Herrmann W, Schorr H, Bodis M, Knapp JP, Muller A, Stein G, Geisel J (2000) Role of homocysteine, cystathionine and methylmalonic acid measurement for diagnosis of vitamin deficiency in high-aged subjects. Eur J Clin Invest 30:1083-1089.
Hertz P, Bril V, Orszag A, Ahmed A, Ng E, Nwe P, Ngo M, Perkins BA (2011) Reproducibility of in vivo corneal confocal microscopy as a novel screening test for early diabetic sensorimotor polyneuropathy. Diabet Med 28:1253-1260.
Hoffman E, Staff N, Kabat B, Robb J, St. Sauver J, Klein C (2013) A population Based Assessment of Polyneuropathy and Associated Disability. In: American Academy of Neurology San Diego, CA.
Hornykiewicz O (2010) A brief history of levodopa. J Neurol 257:S249-252.
Houlden H, Singleton AB (2012) The genetics and neuropathology of Parkinson's disease. Acta Neuropathol 124:325-338.
Hoy SM, Keating GM (2012) Rasagiline: a review of its use in the treatment of idiopathic Parkinson's disease. Drugs 72:643-669.
Hsiao IT, Weng YH, Hsieh CJ, Lin WY, Wey SP, Kung MP, Yen TC, Lu CS, Lin KJ (2014) Correlation of Parkinson Disease Severity and 18F-DTBZ Positron Emission Tomography. JAMA Neurol 71:758-766.
Inzelberg R, Ramirez JA, Nisipeanu P, Ophir A (2004) Retinal nerve fiber layer thinning in Parkinson disease. Vision Res 44:2793-2797.
Jankovic J (2008) Parkinson's disease: clinical features and diagnosis. J Neurol Neurosurg Psychiatry 79:368-376.
Jellinger KA (2011) Synuclein deposition and non-motor symptoms in Parkinson disease. J Neurol Sci 310:107-111.
Jenner P, Olanow CW (2006) The pathogenesis of cell death in Parkinson's disease. Neurology 66:S24-36.
Johnson MS, Ryals JM, Wright DE (2008) Early loss of peptidergic intraepidermal nerve fibers in an STZ-induced mouse model of insensate diabetic neuropathy. Pain 140:35-47.

Kandil MR, Darwish ES, Khedr EM, Sabry MM, Abdulah MA (2012) A community-based epidemiological study of peripheral neuropathies in Assiut, Egypt. Neurol Res 34:960-966.
Kennedy WR, Wendelschafer-Crabb G (1993) The innervation of human epidermis. J Neurol Sci 115:184-190.
Kieburtz K, Wunderle KB (2013) Parkinson's disease: evidence for environmental risk factors. Mov Disord 28:8-13.
Kim HJ, Park SY, Cho YJ, Hong KS, Cho JY, Seo SY, Lee DH, Jeon BS (2009) Nonmotor symptoms in de novo Parkinson disease before and after dopaminergic treatment. J Neurol Sci 287:200-204.
Kimber T, Blumbergs P, Thompson P (2013) Severe ataxic polyneuropathy associated with chronic levodopa use in Parkinson's disease. Parkinsonism Relat Disord 19:847-849.
Klostermann F, Jugel C, Muller T, Marzinzik F (2012) Malnutritional neuropathy under intestinal levodopa infusion. J Neural Transm 119:369-372.
Knaryan VH, Samantaray S, Le Gal C, Ray SK, Banik NL (2011) Tracking extranigral degeneration in animal models of Parkinson's disease: quest for effective therapeutic strategies. J Neurochem 118:326-338.
Kyaw WT, Nagai M, Kaneta M, Kubo M, Nishikawa N, Tsujii T, Iwaki H, Nomoto M (2013) Effect of nicotine on the pharmacokinetics of levodopa. Clin Neuropharmacol 36:46-51.
Lamberti P, Zoccolella S, Iliceto G, Armenise E, Fraddosio A, de Mari M, Livrea P (2005) Effects of levodopa and COMT inhibitors on plasma homocysteine in Parkinson's disease patients. Mov Disord 20:69-72.
Lauria G, Bakkers M, Schmitz C, Lombardi R, Penza P, Devigili G, Smith AG, Hsieh ST, Mellgren SI, Umapathi T, Ziegler D, Faber CG, Merkies ISJ (2010a) Intraepidermal nerve fiber density at the distal leg: a worldwide normative reference study. Journal of the Peripheral Nervous System 15:202-207.
Lauria G, Cornblath DR, Johansson O, McArthur JC, Mellgren SI, Nolano M, Rosenberg N, Sommer C (2005) EFNS guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathy. Eur J Neurol 12:747-758.
Lauria G, Hsieh ST, Johansson O, Kennedy WR, Leger JM, Mellgren SI, Nolano M, Merkies IS, Polydefkis M, Smith AG, Sommer C, Valls-Sole J (2010b) European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society. Eur J Neurol 17:903-912, e944-909.
Lauria G, Lombardi R, Camozzi F, Devigili G (2009) Skin biopsy for the diagnosis of peripheral neuropathy. Histopathology 54:273-285.
Lauria G, Morbin M, Lombardi R, Borgna M, Mazzoleni G, Sghirlanzoni A, Pareyson D (2003) Axonal swellings predict the degeneration of epidermal nerve fibers in painful neuropathies. Neurology 61:631-636.
Lebouvier T, Chaumette T, Paillusson S, Duyckaerts C, Bruley des Varannes S, Neunlist M, Derkinderen P (2009) The second brain and Parkinson's disease. Eur J Neurosci 30:735-741.
Lee ES, Chen H, King J, Charlton C (2008) The role of 3-O-methyldopa in the side effects of L-dopa. Neurochem Res 33:401-411.
Levin J, Botzel K, Giese A, Vogeser M, Lorenzl S (2010) Elevated levels of methylmalonate and homocysteine in Parkinson's disease, progressive supranuclear palsy and amyotrophic lateral sclerosis. Dement Geriatr Cogn Disord 29:553-559.
Lewis SJ, Caldwell MA, Barker RA (2003) Modern therapeutic approaches in Parkinson's disease. Expert Rev Mol Med 5:1-20.
Lin CH, Wu RM, Chang HY, Chiang YT, Lin HH (2013) Preceding pain symptoms and Parkinson's disease: a nationwide population-based cohort study. Eur J Neurol 20:1398-1404.

Liu XX, Wilson K, Charlton CG (2000) Effects of L-dopa treatment on methylation in mouse brain: implications for the side effects of L-dopa. Life Sci 66:2277-2288.
Lozeron P, Trocello JM, Kubis N (2013) Acquired neuropathies. J Neurol 260:2433-2440.
Maetzler W (2014) Comment: Why do nondopaminergic features in Parkinson disease matter? Neurology 82:417.
Malik RA, Kallinikos P, Abbott CA, van Schie CH, Morgan P, Efron N, Boulton AJ (2003) Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. Diabetologia 46:683-688.
Manca D, Cossu G, Murgia D, Molari A, Ferrigno P, Marcia E, Melis M (2009) Reversible encephalopathy and axonal neuropathy in Parkinson's disease during duodopa therapy. Mov Disord 24:2293-2294.
Mancini F, Comi C, Oggioni GD, Pacchetti C, Calandrella D, Coletti Moja M, Riboldazzi G, Tunesi S, Dal Fante M, Manfredi L, Lacerenza M, Cantello R, Antonini A (2014) Prevalence and features of peripheral neuropathy in Parkinson's disease patients under different therapeutic regimens. Parkinsonism Relat Disord 20:27-31.
Marciniak C (2013) Fibular (peroneal) neuropathy: electrodiagnostic features and clinical correlates. Phys Med Rehabil Clin N Am 24:121-137.
Marfurt CF (2011) Corneal nerves: Anatomy. In: Ocular periphery and disorders(Dartt, D. A. et al., eds), pp 150-158 San Diego, CA: Academic Press/Elsevier.
Marti MJ, Tolosa E (2013) Parkinson disease: New guidelines for diagnosis of Parkinson disease. Nat Rev Neurol 9:190-191.
Martignoni E, Pacchetti C, Godi L, Micieli G, Nappi G (1995) Autonomic disorders in Parkinson's disease. J Neural Transm Suppl 45:11-19.
Martyn CN, Hughes RA (1997) Epidemiology of peripheral neuropathy. J Neurol Neurosurg Psychiatry 62:310-318.
Mason JB (2011) Vitamins, trace minerals, and other micronutrients. In: Cecil Medicine(Goldman, L. and Schafer, A., eds) Philadelphia: Saunders Elsevier.
McCarthy BG, Hsieh ST, Stocks A, Hauer P, Macko C, Cornblath DR, Griffin JW, McArthur JC (1995) Cutaneous innervation in sensory neuropathies: evaluation by skin biopsy. Neurology 45:1848-1855.
Merello M, Nouzeilles MI, Arce GP, Leiguarda R (2002) Accuracy of acute levodopa challenge for clinical prediction of sustained long-term levodopa response as a major criterion for idiopathic Parkinson's disease diagnosis. Mov Disord 17:795-798.
Miller IN, Cronin-Golomb A (2010) Gender differences in Parkinson's disease: clinical characteristics and cognition. Mov Disord 25:2695-2703.
Mold JW, Vesely SK, Keyl BA, Schenk JB, Roberts M (2004) The prevalence, predictors, and consequences of peripheral sensory neuropathy in older patients. J Am Board Fam Pract 17:309-318.
Mollenhauer B, Trautmann E, Sixel-Doring F, Wicke T, Ebentheuer J, Schaumburg M, Lang E, Focke NK, Kumar KR, Lohmann K, Klein C, Schlossmacher MG, Kohnen R, Friede T, Trenkwalder C (2013) Nonmotor and diagnostic findings in subjects with de novo Parkinson disease of the DeNoPa cohort. Neurology 81:1226-1234.
Mu L, Sobotka S, Chen J, Su H, Sanders I, Adler CH, Shill HA, Caviness JN, Samanta JE, Beach TG (2013a) Alpha-synuclein pathology and axonal degeneration of the peripheral motor nerves innervating pharyngeal muscles in Parkinson disease. J Neuropathol Exp Neurol 72:119-129.
Mu L, Sobotka S, Chen J, Su H, Sanders I, Nyirenda T, Adler CH, Shill HA, Caviness JN, Samanta JE, Sue LI, Beach TG (2013b) Parkinson disease affects peripheral sensory nerves in the pharynx. J Neuropathol Exp Neurol 72:614-623.

Muller T (2010) Catechol-O-methyltransferase enzyme: cofactor S-adenosyl-L-methionine and related mechanisms. Int Rev Neurobiol 95:49-71.
Muller T, van Laar T, Cornblath DR, Odin P, Klostermann F, Grandas FJ, Ebersbach G, Urban PP, Valldeoriola F, Antonini A (2013) Peripheral neuropathy in Parkinson's disease: levodopa exposure and implications for duodenal delivery. Parkinsonism Relat Disord 19:501-507 ; discussion 501.
Nakamura T, Saionji K, Hiejima Y, Hirayama H, Tago K, Takano H, Tajiri M, Hayashi K, Kawabata M, Funamizu M, Makita Y, Hata A (2002) Methylenetetrahydrofolate reductase genotype, vitamin B12, and folate influence plasma homocysteine in hemodialysis patients. Am J Kidney Dis 39:1032-1039.
Ngwuluka N, Pillay V, Du Toit LC, Ndesendo V, Choonara Y, Modi G, Naidoo D (2010) Levodopa delivery systems: advancements in delivery of the gold standard. Expert Opin Drug Deliv 7:203-224.
Nolano M, Provitera V, Estraneo A, Selim MM, Caporaso G, Stancanelli A, Saltalamacchia AM, Lanzillo B, Santoro L (2008) Sensory deficit in Parkinson's disease: evidence of a cutaneous denervation. Brain 131:1903-1911.
Obeid R, Schadt A, Dillmann U, Kostopoulos P, Fassbender K, Herrmann W (2009) Methylation status and neurodegenerative markers in Parkinson disease. Clin Chem 55:1852-1860.
Olanow CW, Perl DP, DeMartino GN, McNaught KS (2004) Lewy-body formation is an aggresome-related process: a hypothesis. Lancet Neurol 3:496-503.
Pahwa R, Lyons KE (2009) Levodopa-related wearing-off in Parkinson's disease: identification and management. Curr Med Res Opin 25:841-849.
Park A, Stacy M (2009) Non-motor symptoms in Parkinson's disease. J Neurol 256 Suppl 3:293-298.
Petropoulos IN, Alam U, Fadavi H, Marshall A, Asghar O, Dabbah MA, Chen X, Graham J, Ponirakis G, Boulton AJ, Tavakoli M, Malik RA (2014) Rapid automated diagnosis of diabetic peripheral neuropathy with in vivo corneal confocal microscopy. Invest Ophthalmol Vis Sci 55:2071-2078.
Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ (2005) Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999-2000. Am J Clin Nutr 82:442-450.
Postuma RB, Lang AE (2004) Homocysteine and levodopa: should Parkinson disease patients receive preventative therapy? Neurology 63:886-891.
Quattrini C, Tavakoli M, Jeziorska M, Kallinikos P, Tesfaye S, Finnigan J, Marshall A, Boulton AJ, Efron N, Malik RA (2007) Surrogate markers of small fiber damage in human diabetic neuropathy. Diabetes 56:2148-2154.
Rage M, Van Acker N, Facer P, Shenoy R, Knaapen MW, Timmers M, Streffer J, Anand P, Meert T, Plaghki L (2010) The time course of CO2 laser-evoked responses and of skin nerve fibre markers after topical capsaicin in human volunteers. Clin Neurophysiol 121:1256-1266.
Rajabally YA, Martey J (2011) Neuropathy in Parkinson disease: prevalence and determinants. Neurology 77:1947-1950.
Rajabally YA, Martey J (2013) No association between neuropathy and restless legs in Parkinson's disease. Acta Neurol Scand 127:216-220.
Reddy VC, Patel SV, Hodge DO, Leavitt JA (2013) Corneal sensitivity, blink rate, and corneal nerve density in progressive supranuclear palsy and Parkinson disease. Cornea 32:631-635.
Rodgers KJ, Hume PM, Morris JG, Dean RT (2006) Evidence for L-dopa incorporation into cell proteins in patients treated with levodopa. J Neurochem 98:1061-1067.
Rossi A, Ciacci G, Federico A, Mondelli M, Rizzuto N (1986) Sensory and motor peripheral neuropathy in olivopontocerebellar atrophy. Acta Neurol Scand 73:363-371.
Sachedina S, Toth C (2013) Progression in idiopathic, diabetic, paraproteinemic, alcoholic, and B12 deficiency neuropathy. J Peripher Nerv Syst 18:247-255.

Santos-Garcia D, de la Fuente-Fernandez R, Valldeoriola F, Palasi A, Carrillo F, Grande M, Mir P, De Fabregues O, Casanova J (2012) Polyneuropathy while on duodenal levodopa infusion in Parkinson's disease patients: we must be alert. J Neurol 259:1668-1672.
Saporta MA, Shy ME (2013) Inherited peripheral neuropathies. Neurol Clin 31:597-619.
Satue M, Seral M, Otin S, Alarcia R, Herrero R, Bambo MP, Fuertes MI, Pablo LE, Garcia-Martin E (2014) Retinal thinning and correlation with functional disability in patients with Parkinson's disease. Br J Ophthalmol 98:350-355.
Schneider JS, Diamond SG, Markham CH (1987) Parkinson's disease: sensory and motor problems in arms and hands. Neurology 37:951-956.
Singleton AB, Farrer MJ, Bonifati V (2013) The genetics of Parkinson's disease: progress and therapeutic implications. Mov Disord 28:14-23.
Singleton JR, Bixby B, Russell JW, Feldman EL, Peltier A, Goldstein J, Howard J, Smith AG (2008) The Utah Early Neuropathy Scale: a sensitive clinical scale for early sensory predominant neuropathy. J Peripher Nerv Syst 13:218-227.
Smith KM, Dahodwala N (2014) Sex differences in Parkinson's disease and other movement disorders. Exp Neurol.
Spica V, Pekmezovic T, Svetel M, Kostic VS (2013) Prevalence of non-motor symptoms in young-onset versus late-onset Parkinson's disease. J Neurol 260:131-137.
Stocchi F, Barbato L, Nordera G, Berardelli A, Ruggieri S (1998) Sleep disorders in Parkinson's disease. J Neurol 245 Suppl 1:S15-18.
Sulzer D, Bogulavsky J, Larsen KE, Behr G, Karatekin E, Kleinman MH, Turro N, Krantz D, Edwards RH, Greene LA, Zecca L (2000) Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. Proc Natl Acad Sci U S A 97:11869-11874.
Sung VW, Nicholas AP (2013) Nonmotor symptoms in Parkinson's disease: expanding the view of Parkinson's disease beyond a pure motor, pure dopaminergic problem. Neurol Clin 31:S1-16.
Suresh K, Chandrashekara S (2012) Sample size estimation and power analysis for clinical research studies. J Hum Reprod Sci 5:7-13.
Taly AB, Muthane UB (1992) Involvement of peripheral nervous system in juvenile Parkinson's disease. Acta Neurol Scand 85:272-275.
Tavakoli M, Malik RA (2011) Corneal confocal microscopy: a novel non-invasive technique to quantify small fibre pathology in peripheral neuropathies. J Vis Exp.
Tavakoli M, Mitu-Pretorian M, Petropoulos IN, Fadavi H, Asghar O, Alam U, Ponirakis G, Jeziorska M, Marshall A, Efron N, Boulton AJ, Augustine T, Malik RA (2013) Corneal confocal microscopy detects early nerve regeneration in diabetic neuropathy after simultaneous pancreas and kidney transplantation. Diabetes 62:254-260.
Tavakoli M, Petropoulos IN, Malik RA (2012) Assessing corneal nerve structure and function in diabetic neuropathy. Clin Exp Optom 95:338-347.
Tavakoli M, Quattrini C, Abbott C, Kallinikos P, Marshall A, Finnigan J, Morgan P, Efron N, Boulton AJ, Malik RA (2010a) Corneal confocal microscopy: a novel noninvasive test to diagnose and stratify the severity of human diabetic neuropathy. Diabetes Care 33:1792-1797.
Tavakoli M, Quattrini C, Begum G, Fadavi H, Boulton AJ, Malik RA (2010b) Neuropad and corneal confocal microscopy: new indicators for human diabetic neuropathy. Diabetologia 53:443; A1112.
Tavee J, Zhou L (2009) Small fiber neuropathy: A burning problem. Cleve Clin J Med 76:297-305.
Tolosa E, Pont-Sunyer C (2011) Progress in defining the premotor phase of Parkinson's disease. J Neurol Sci 310:4-8.

Toth C, Breithaupt K, Ge S, Duan Y, Terris JM, Thiessen A, Wiebe S, Zochodne DW, Suchowersky O (2010) Levodopa, methylmalonic acid, and neuropathy in idiopathic Parkinson disease. Ann Neurol 68:28-36.
Toth C, Brown MS, Furtado S, Suchowersky O, Zochodne D (2008) Neuropathy as a potential complication of levodopa use in Parkinson's disease. Mov Disord 23:1850-1859.
Tranchant C, Ruh D, Warter JM (1994) [Type II Charcot-Marie-Tooth and dopa-sensitive Parkinson disease]. Rev Neurol (Paris) 150:72-74.
Ulusoy A, Rusconi R, Perez-Revuelta BI, Musgrove RE, Helwig M, Winzen-Reichert B, Di Monte DA (2013) Caudo-rostral brain spreading of alpha-synuclein through vagal connections. EMBO Mol Med 5:1051-1059.
Urban PP, Wellach I, Faiss S, Layer P, Rosenkranz T, Knop K, Weis J (2010) Subacute axonal neuropathy in Parkinson's disease with cobalamin and vitamin B6 deficiency under duodopa therapy. Mov Disord 25:1748-1752.
Vallelunga A, Pegoraro V, Pilleri M, Biundo R, De Iuliis A, Marchetti M, Facchini S, Formento Dojot P, Antonini A (2013) The MTHFR C677T polymorphism modifies age at onset in Parkinson's disease. Neurol Sci.
Wakabayashi K, Tanji K, Mori F, Takahashi H (2007) The Lewy body in Parkinson's disease: molecules implicated in the formation and degradation of alpha-synuclein aggregates. Neuropathology 27:494-506.
Wang N, Gibbons CH, Lafo J, Freeman R (2013) alpha-Synuclein in cutaneous autonomic nerves. Neurology.
Warner TT, Schapira AH (1998) The role of the alpha-synuclein gene mutation in patients with sporadic Parkinson's disease in the United Kingdom. J Neurol Neurosurg Psychiatry 65:378-379.
Weir DG, Scott JM (1999) Vitamin B12 "Cobalamin". In: Modern nutrition in health and disease(Shils, M. E., ed), pp 447-457 Baltimore: Williams & Wilkins.
Weis J, Katona I, Muller-Newen G, Sommer C, Necula G, Hendrich C, Ludolph AC, Sperfeld AD (2011) Small-fiber neuropathy in patients with ALS. Neurology 76:2024-2029.
Williams AC, Pall HS, Steventon GB, Green S, Buttrum S, Molloy H, Waring RH (1993) N-methylation of pyridines and Parkinson's disease. Adv Neurol 60:194-196.
Willis GL, Moore C, Armstrong SM (2012) Breaking away from dopamine deficiency: an essential new direction for Parkinson's disease. Rev Neurosci 23:403-428.
Wirdefeldt K, Adami HO, Cole P, Trichopoulos D, Mandel J (2011) Epidemiology and etiology of Parkinson's disease: a review of the evidence. Eur J Epidemiol 26 Suppl 1:S1-58.
Wopking S, Scherens A, Haussleiter IS, Richter H, Schuning J, Klauenberg S, Maier C (2009) Significant difference between three observers in the assessment of intraepidermal nerve fiber density in skin biopsy. BMC Neurol 9:13.
Wright BA, Waters CH (2013) Continuous dopaminergic delivery to minimize motor complications in Parkinson's disease. Expert Rev Neurother 13:719-729.
Ziegler D, Papanas N, Zhivov A, Allgeier S, Winter K, Ziegler I, Bruggemann J, Strom A, Peschel S, Kohler B, Stachs O, Guthoff RF, Roden M (2014) Early Detection of Nerve Fiber Loss by Corneal Confocal Microscopy and Skin Biopsy in Recently Diagnosed Type 2 Diabetes. Diabetes.