Biomarkers in Parkinson Disease: Studies on Clinical, Radiological and Biological Biomarkers.

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I would like to dedicate this thesis to my grandfather, Dr. Tom Crotty, who continued to publish his research with such enthusiasm and motivation until his death at 90 years old. He is such an inspiration to us all and is dearly missed.

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and resources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

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Abstract of thesis

Parkinson disease is the second most common neurodegenerative disorder after Alzheimer disease. It affects 2 to 3 percent of those over 65 years with an age-dependent prevalence. Currently, the diagnosis of PD is hampered by the limited sensitivity and specificity of the available investigations. The diagnosis is usually made based on the clinical presentation which has a number of significant limitations. First of all, the disease has been present for decades before motor symptoms develop. Secondly, using clinical exam alone, the misdiagnosis rate remains high with both over- and under-diagnosis common. It is important to make an expeditious and correct diagnosis of PD, especially in this era of increasing interest in neuroprotective strategies for PD and other neurodegenerative conditions. Delaying the diagnosis until motor symptoms develop is suboptimal as more than 40% of dopaminergic neurons have been destroyed at this stage. We also need to ensure that true cases of PD are being enrolled in PD trials and that these trials are not being confounded by the inclusion of individuals with other causes of parkinsonism. To accomplish these goals, there is a need for PD biomarkers that are both sensitive and specific.

The objective of this thesis was to investigate, using a case-control study design, a number of potential biomarkers for PD. These biomarkers included clinical, biological and radiological markers.

In the first study, we investigated the role of autonomic neuropathy as a clinical biomarker for PD. Using thermal threshold testing, nerve conduction testing and questionnaires, the PD group demonstrated a higher prevalence of autonomic neuropathy. Other outcome measures, including the presence of non-motor symptoms, pain, depressive symptoms and electrophysiological evidence of large fiber neuropathy were also found to be more prevalent in the PD group.

In the second and third studies, we explored the potential role of CSF biological biomarkers in PD. In the second study, we evaluated CSF cytokine levels with the aim of identifying a unique cytokine pattern in the CSF of PD subjects. We failed to detect a cytokine pattern and found no difference in cytokine levels between PD and control groups. However, within a cohort of the PD group, we identified an association between

IL-2 levels and disease severity, with higher concentrations of IL-2 seen in those with more severe disease.

In the third study, we measured GDF5 protein levels in the CSF and found lower concentrations of GDF5 in the PD group compared to controls. GDF5 levels were lower in the female PD subjects compared to males. There was no association between GDF5 concentrations and PD characteristics, age or cognition.

In the final study, we assessed the utility of SPECT imaging of dopamine transporters in the striatal region of the brain (DaTSCAN) as a radiological biomarker for PD in our healthcare system. Following a review of scans over a five-year period, 69% of scans showed evidence of dopaminergic deficit, supporting a diagnosis of PD. Review of request forms for DaTSCAN, demonstrated inappropriate referrals in 13% of cases. Chart review in a subgroup of scans documented a change in patient management in 65% of cases, based on the result of the scan.

In this thesis, we sought to identify potential biomarkers for PD. We found significant differences between the subjects with PD and controls using clinical and biological tests. We also demonstrated findings that support the utility of a radiological biomarker in clinical practice. Our studies showed promising results and require further research. In the future, we envision studies investigating a multimodal biomarker approach in large cohorts of PD subjects.

Abbreviations

AAN	American Academy of Neurology
AD	Alzheimer's disease
APD	Atypical parkinsonian disorders
BBB	Blood brain barrier
BDI	Beck Depression Inventory
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BPI	Brief Pain Inventory
CBD	Corticobasal degeneration
DIP	Drug-induced parkinsonism
DLB	Dementia with Lewy bodies
ELISA	Enzyme-linked immunosorbent assay
ET	Essential tremor
GBA	Glucocerebrosidase
GDF5	Growth differentiation factor 5
GDNF	Glial cell-line derived neurotrophic factor
GWAS	Genome-wide association study
HAAS	Honolulu- Asia Aging study
H&Y	Hoehn & Yahr scale
IEFND	Intraepidermal nerve fiber density
LEDD	Levodopa-equivalent daily dosage
LRRK2	Leucine-rich repeat kinase 2
mDA	midbrain dopaminergic
MDS	Movement Disorder Society
MIBG	123 I-Meta-IodoBenzylGuanidine
MMSE	Mini-mental state examination
MOCA	Montreal Cognitive Assessment
MRI	Magnetic Resonance Imaging
MSA	Multiple system atrophy
NCS	Nerve conduction studies
NfL	Neurofilament light chain
NMS	Nonmotor symptoms
NTN	Neurturin
OR	Odds Ratio
PD	Parkinson disease
PET	Positron Emission Tomography
PIGD	Postural instability with gait disturbance
PPV	Positive predictive value
PSP	Progressive supranuclear palsy
PSPN	Persephin
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted

RBD	REM sleep behavior disorder
RR	Relative risk
SN	Substantia nigra
SNCA	Synuclein, alpha gene
SPECT	Single Photon Emission Computed Tomography
SPSS	Statistical Package for the Social Sciences
TCS	Transcranial Ultrasound
TGF	Transforming growth factor
TTT	Temperature threshold testing
UKPDSBB	United Kingdom Parkinson Disease Society Brain Bank
UPDRS	United Parkinson Disease Rating Scale
VMAT2	Vesicular Monoamine Transporter type 2

Chapter 1: Introduction

1.1 Introduction

Parkinson disease (PD) was first described in 1817 by James Parkinson in his seminal piece ‘An Essay on the Shaking Palsy’ in which he described six people, three of whom he had personally examined, and the other three whom he had observed on the streets of London (1). The ‘shaking palsy’ was defined as a combination of rest tremor, lessened muscular power, abnormal truncal posture and a festinant, propulsive gait (1). PD is the second most common neurodegenerative disorder after Alzheimer disease (AD). It is characterized clinically by bradykinesia and other cardinal motor features, and pathologically by neuronal loss in the substantia nigra (SN) and widespread accumulation of intracellular α-synuclein protein, also known as Lewy bodies (2). PD affects 2 to 3% of people over 65 years of age and is twice as common in men ((3,4)). This age-dependent prevalence is of particular importance as populations worldwide continue to age. As of yet, there are no curative or preventative strategies for PD or any of the other age-related neurodegenerative disorders. To compound this problem, the number of people affected by PD is expected to double between 2005 and 2030 due to the ageing population (5).

The most commonly recognized symptoms of PD are often remembered using the acronym ‘TRAP” and consist of tremor, rigidity, akinesia or bradykinesia, and postural instability. However, not all patients have these motor symptoms and instead their quality of life is affected by profound non-motor symptoms (NMS) including autonomic dysfunction, sleep disorder, psychiatric, cognitive and sensory abnormalities. Currently, there is no single diagnostic test for PD and it is diagnosed using The United Kingdom Parkinson’s Disease Society Brain Bank Clinical Diagnostic (UKPDSBB) criteria (2) or, more recently, with the Movement Disorder Society (MDS) clinical diagnostic criteria (6) (table 1). The diagnosis is often delayed as it is made after multiple serial examinations identify its cardinal motor deficits; show disease progression; document responsiveness to dopaminergic therapies and exclude atypical signs. The ultimate diagnosis of PD is made late in the neurodegenerative process as motor symptoms only manifest when 40 to 60% of dopaminergic cells and 80% of synaptic function are lost (7). This premotor pre-diagnostic period is estimated to last up

to seven years (8) and is thought to be the result of compensatory mechanisms and plasticity within the cortical-basal ganglia-thalamocortical system (9).

Table 1: MDS clinical diagnostic criteria (6)

1. Parkinsonism: must have bradykinesia and one of the following rest tremor or rigidity.
2. Clinically established PD: parkinsonism; no absolute exclusion criteria; 2 or more supportive criteria and no red flags.
3. Clinically probable PD: parkinsonism; no absolute exclusion criteria; red flags are cancelled out by supportive features.

Absolute exclusion criteria:

- Exam- cerebellar abnormalities, cortical sensory loss or vertical supranuclear gaze palsy;
- Diagnosis of dementia: behavioural variant FTD (bvFTD) or primary progressive aphasia (PPA) within 5 years of disease;
- Restricted lower limb parkinsonism for more than 3 years;
- Treatment with dopamine receptor blocker medication in a dose or time-course consistent with drug-induced parkinsonism;
- Absence of dopaminergic therapy response;
- Neuroimaging showing normal presynaptic dopaminergic system on neuroimaging;
- Documentation of alternative condition.

Supportive criteria: dopaminergic therapy response; rest tremor of a limb; presence levodopa-induced dyskinesia; presence of cardiac sympathetic denervation on MIBG scintigraphy or olfactory loss

Red flags: wheelchair bound within 5 years of onset; no progression of signs or symptoms over 5 years; early bulbar dysfunction; severe autonomic failure with first 5 years; inspiratory respiratory dysfunction; recurrent falls within 3 years; disproportionate anterocollis or contractures; absence of non-motor symptoms despite 5 years of disease; pyramidal tract signs; bilateral symmetric parkinsonism

Despite the use of the UKPDSBB criteria and a 3-step procedure (table 2) the diagnostic accuracy for PD at initial visit is only 80%, when compared against the gold standard neuropathological examination (10). The highest diagnostic accuracy occurs when symptoms have been present for more than 5 years (11). The positive predictive

value (PPV) for probable PD, classified as having 2 of 3 cardinal motor symptoms and responsive to dopaminergic medications is only 53% if symptoms have been present for less than 5 years compared to 88% in patients in whom symptoms have been present for more than 5 years (12). The accuracy of clinical diagnosis is also dependent on the expertise of the physician. Movement disorder specialists misdiagnose early PD 10% of the time, whereas misdiagnosis may reach 50% in primary care (13). Movement disorder specialists had a sensitivity of 81.3% and a specificity of 83.5% compared to non-specialists who were slightly more sensitive at 89.7% but much less specific at 49.2% (10).

Table 2: Three-step procedure in diagnosing PD (10)

1. Presence of parkinsonism
2. Assess exclusion criteria
3. Identify supportive features

The core cardinal features of PD, namely, bradykinesia, tremor and rigidity can also be seen in other neurodegenerative diseases. Misdiagnosis has been shown to occur even in specialized centers where post-mortem findings of presumed PD patients resulted in a change in diagnosis in 25% of patients to Multiple System Atrophy (MSA), Progressive Supranuclear Palsy (PSP), Corticobasal degeneration (CBD), Essential Tremor (ET), drug-induced parkinsonism (DIP) and vascular parkinsonism (14). Assessment of responsiveness to dopaminergic medication can support PD diagnosis and is often used in uncertain cases. In one study of 82 patients with parkinsonism, diagnosis of PD by acute levodopa challenge showed a sensitivity of 70.9%, specificity of 81.4% and PPV of 88.6% (15).

The current gold standard for diagnosis of PD is pathological confirmation of the core pathological features: substantia nigral degeneration and Lewy bodies. However, this examination is not possible during life by biopsy and furthermore, not all PD

subjects have these characteristic pathological findings. Some PD subjects with LRRK2 and Parkin mutations, genetic forms of PD do not demonstrate Lewy bodies on post-mortem examination (16).

Improving diagnostic accuracy and identifying diagnostic markers for PD is key for improving clinical care and advancing research. In an era where there is increased focus on the development of neuroprotective strategies, there is an increased need to identify suitable biomarkers for PD diagnosis, prognosis and progression. During this review, I will focus primarily on diagnostic clinical, radiological, genetic and biological biomarkers.

1.2 Biomarkers

Biomarkers, as defined in 2001 by the Biomarkers Definitions Working Group, are 'objectively measured characteristics that are indicators of normal biological processes, pathogenic processes, or responses to interventions' (17). Biomarkers can be broadly classified according to the type of information they provide and can be clinically-based; imaging-based or biologically-based. Biological biomarkers can be further subcategorized into biochemical, genetic or proteomic markers. Biomarkers can also be categorized according to their role in the disorder and can be described as 'trait, state or rate' biomarkers. A 'trait' biomarker indicates susceptibility to a disease; a 'state' biomarker diagnoses the disease and a 'rate' biomarker is used for prognostication. At present, there are no approved biomarkers for diagnosing PD.

In 2001, the Working Group of the German Society of Experimental and Clinical Neurotherapeutics (GESENT) assessed the biomarkers available for neurodegenerative conditions and proposed that an ideal biomarker should be 'linked to fundamental features of PD neuropathology and mechanisms underlying neurodegeneration in PD; correlated to disease progression; able to monitor the actual disease status; pre-clinically validated and confirmed by at least two independent studies' (18).

The ideal biomarker for PD would be sensitive and specific, and therefore able to identify almost all cases of PD in its premotor stages, as well as being able to discriminate between PD and other neurodegenerative diseases. It should be reliable due to its consistent performance and validity. It should also be inexpensive, easy to

use and non-invasive. It is also important to be aware of the lead time of the biomarker i.e. when it will be positive in relation to motor symptom development (18).

The International Parkinson and Movement Disorder Society (MDS) task force recently described three stages of early PD (table 3) and designated research criteria for probable prodromal PD (19). Their criteria included both motor and non-motor features, as well as estimates of risk, based on age, gender, and other known PD risk factors. The MDS suggested that, in order to calculate the final post-test probability for prodromal PD with greater than 80% certainty, the prior probability of prodromal PD should be combined with the likelihood ratios of individual markers (19).

Table 3: MDS research criteria for prodromal PD (19)

1. Preclinical PD- no symptoms or signs but neurodegeneration has begun.
2. Prodromal PD- symptoms and signs present but not meeting diagnostic criteria
3. Clinical PD- meets diagnostic criteria for PD as per MDS criteria

1.2.1 Current studies on biomarkers in PD

There are multiple ongoing and completed studies investigating biomarkers in PD (see review by (20)). I have briefly summarised them by study design below.

Population-based cohorts include the Honolulu-Asia Aging Study (HAAS) (21) and The Prospective evaluation of Risk factors for Idiopathic Parkinson's Syndromes (PRIPS) study (22).

The HAAS began in 1991 as a continuation of the Honolulu Heart Program (21). It is a prospective, longitudinal, population-based study in 8,006 Japanese-American men who were born between 1900 to 1919. Their endpoints are the diagnosis of PD; identification of premotor features of PD; and autopsy review for Lewy bodies. They have identified 137 people with PD over 30 years of follow-up and reported associations between PD and presence of hyposmia, constipation and excessive daytime somnolence (23).

The PRIPS study is a European, prospective, longitudinal, population-based study. They recruited 1,847 PD-free subjects who were fifty years or older. Their endpoints are the diagnosis of PD; evaluation of risk by age and gender; and identifying premotor symptoms in relation to SN echogenicity. Eleven of their subjects developed PD over 3 years of follow-up. They found an increased risk of developing PD with SN hyperechogenicity, increased age greater than 60 years, hyposmia and United Parkinson Disease Rating Scale (UPDRS) greater than 3. Notably, some of these risk factors had low PPV given their presence in the general elderly population too (22).

Enriched- risk cohorts include the Parkinson's Associated Risk Study (PARS) (24); the Tuebinger evaluation of Risk factors for Early detection of NeuroDegeneration (TREND) study (25); and the Progression Markers in the Premotor Phase (PMPP) (26).

The PARS study is a prospective, longitudinal, enriched-risk study, with two phases. The initial phase enrolled 4,900 people, consisting of both PD relatives and controls. Each subject completed the University of Pennsylvania Smell Identification test (UPSIT) and questionnaires by mail. In phase two, 303 subjects, divided into those with and without hyposmia, underwent further evaluations in-house of other clinical, radiological and biological biomarkers (24).

The TREND study is a prospective, longitudinal, enriched-risk study of 1,179 subjects, all of whom are older than 50 years and have hyposmia and/or depression and/or REM sleep behavior disorder (RBD). Subjects undergo biannual assessments of clinical and radiological biomarkers (25,27).

The PMPP study is a prospective, longitudinal, enriched-risk study of individuals at high risk for developing PD (HRPD), early stage PD subjects and controls. The HRPD subjects have SN hyperechogenicity and one PD motor sign; two premotor or slight motor symptoms; or a combination of early motor symptom and a positive family history of PD (20,26). All groups undergo assessments of clinical, biological and radiological biomarkers.

Other research investigating biomarkers include the Longitudinal And Biomarker Study in PD (LABS-PD) (28); the Parkinson Progression Marker Initiative (PPMI) (29); and the Parkinson's Disease Biomarker Program (PDBP) (30).

The LABS-PD is a prospective, longitudinal study conducted by the Parkinson Study Group which recruits subjects from previously completed clinical trials and follows them long-term. Their first PD cohort, PostCEPT consisted of subjects enrolled in the PRECEPT trial. Subjects are then followed serially with validated assessments of motor and non-motor symptoms along with biomarker sampling of biological fluids and radiological imaging (28).

The PPMI is sponsored by the Michael J. Fox Foundation for PD research. It is a multicenter study which recruits de novo PD subjects, subjects without evidence of dopaminergic deficit (SWEDD), genetic PD, prodromal PD subjects and controls. The ultimate goal of the PPMI is to identify PD biomarkers to assist with developing disease-modifying therapies. Enrolled subjects are followed serially with data collected for potential clinical, imaging and biospecimen biomarkers (29).

The Parkinson's Disease Biomarker Program (PDBP) is run by the National Institute of Neurological Disorders and Stroke (NINDS). It uses a consortium design to provide a repository of biospecimens including RNA, DNA, serum, plasma and CSF for further biomarker investigations (30).

1.3. Clinical biomarkers

This area can be divided into those in the premotor or preclinical phase and those in the motor or clinical phase.

Premotor PD was brought to our attention with Braak's neuropathological staging. Braak et al. differentiated six histopathological stages of PD. During the premotor stages 1 and 2, Lewy body pathology is confined to the olfactory bulb, the lower brainstem in the medulla oblongata, the pons, the dorsal motor nucleus of vagus and the myenteric plexus in the peripheral nervous system. In stages 3 and 4, the Lewy bodies spread rostrally with Lewy bodies visualised within the SN, resulting in the motor symptoms of PD. In advanced disease, pathologic stages 5 and 6, Lewy bodies are present in the neocortex and may explain the dementia often seen in advanced PD

(31,32). However, this staging is imperfect as not all people with PD follow this staging sequence. Some people with PD have no Lewy bodies and others have earlier nigral or cortical involvement before spreading to the brainstem (33).

1.3.1. Motor symptoms

As previously discussed above, PD is a clinical diagnosis based on the presence of core motor features (6). Bradykinesia correlates best with dopaminergic loss in PD (34,35). Subtle motor abnormalities including reduced facial and voice expression can be detected up to a decade before overt parkinsonism (36). Berg et al. studied a general population cohort and reported that a UPDRS score greater than zero was associated with a relative risk (RR) of developing PD of at least 5.65 and for UPDRS scores greater than four the RR increased to 16.54 (27). In another study by PRIPS, they found that a UPDRS score greater than one was associated with a 1.9% risk of developing PD over 3 years and a UPDRS score greater than four was associated with a 7.8% risk of developing PD over 3 years (20). In a study of subjects with idiopathic REM sleep behavior disorder (RBD), an 'at-risk' population, elevated UPDRS scores were seen 4.5 years before diagnosis of PD and a UPDRS greater than 3 had a specificity of 94.4% when assessed 3 years before diagnosis with parkinsonism (36).

However, there are several disadvantages with the UPDRS or more recent MDS-UPDRS scale. First, it is subjective with known inter-rater variability (37). Secondly, assessing change over short time periods is hard as PD often progresses slowly, with average two-point increases per year recorded on the MDS-UPDRS III motor scale (38,39). Thirdly, the UPDRS is non-specific with research showing elevated UPDRS with mean scores of 12.5 in the general population, especially in the elderly, females and those with comorbidities including diabetes, ET and arthritis (40).

Objective testing with the Purdue pegboard or alternate tap test can also detect motor impairment before diagnosis of PD (36). At three years before diagnosis, the alternate-tap test was shown to predict future parkinsonism with 79.5% sensitivity and 75% specificity, and the Purdue pegboard predicted future diagnosis with 71% sensitivity and 81.8% specificity (36). Timed motor performances may be more useful as they provide objective assessments. The Purdue pegboard test, a timed motor test requires only 57 to 75% of the total patients needed for UPDRS scale, to assess

change from baseline as its endpoint (41). Other timed motor performances like functional reach, timed all walk, timed block sort take and timed dotting have also shown high reliability on repeated testing (37) and can assess those at risk for developing PD (42). Other quantitative assessments have been studied for the other cardinal motor features of PD (review by (18)). Saccadic eye movements can be affected too with observed variability in task-specific saccadic latencies due to loss of central dopaminergic pathways (43).

1.3.2. Non-motor symptoms (NMS)

More than 90% of people with PD experience NMS during the course of their disease (44). Many of these NMS appear before the motor phase and include GI dysfunction, RBD and anosmia. Braak's neuropathological staging supports their early appearance as α-synuclein is detected in the skin, olfactory bulb and GI tract before the involvement of the SN (32).

1.3.2.1 Olfactory loss

Olfactory loss is a proven prodromal marker of PD with abnormalities noted in tests of odor identification, threshold detection and discrimination (45,46). Hyposmia is seen in over 80% of patients with PD and its presence does not appear to be affected by disease severity or duration (47–49). It precedes motor symptoms by 2 to 7 years (50,51) with a RR of 3.9 to 5.2 for developing PD at 3 to 4 years, respectively (27,52). Double et al. found that 82% of patients with early PD defined as Hoehn and Yahr (H&Y) stage 1 had impaired olfaction compared to 23% of age- and sex-matched healthy controls (53). For PD, the sensitivity of olfactory dysfunction was 82%, specificity 82% and PPV of 77% (53). Siderowf et al. found in their study of early PD subjects a correlation between olfactory function and the striatal density of dopamine transporters in the whole striatum (r= 0.66), being strongest in the putamen (r= 0.74), using SPECT imaging (54). Müller et al. examined fifty subjects with parkinsonism (37 people with PD, 8 with MSA, 2 with CBD, 1 with PSP, 1 with psychogenic parkinsonism, 1 with ET) and graded their olfactory testing as normal, anosmia, severe hyposmia, moderate hyposmia and slight hyposmia. Sensitivity was 100% but specificity was low

at 69% for diagnosing parkinsonism when anosmia, severe and moderate hyposmia were categorized as positive test results. If only anosmia and severe hyposmia were classified as positive results, both the sensitivity and specificity were approximately 80% for PD. In regards to differentiating MSA from PD, severe hyposmia had a PPV of 100%, sensitivity of 78% and specificity of 100% (55).

Forty percent of people with PD are unaware of their smell loss (45). Currently available olfactory tests include University of Pennsylvania Smell Identification Test (UPSIT) (56) and Sniffin' sticks (57). Unfortunately, the use of hyposmia as a sole biomarker for PD is limited as it is seen in other neurodegenerative conditions and in 25% of the general elderly population (58). Nonetheless, it could be used to identify those 'at-risk' to develop PD or further enrich 'at-risk' populations including those with idiopathic hyposmia, idiopathic RBD, constipation and family members of PD patients. It could also be applied in combination with another biomarker to increase its sensitivity and specificity for diagnosing prodromal PD (22). Stiasny-Kolster et al. compared olfactory function between 36 subjects with idiopathic RBD and 30 healthy controls. The RBD patients had a higher olfactory threshold, lower olfactory discrimination score and lower olfactory identification score compared to controls (59). Ponsen et al. followed asymptomatic first-degree relatives of PD patients over a 2-year period. At 2 years, 10% of the individuals with idiopathic hyposmia had developed clinical PD as opposed to none of the normosmic relatives in the cohort. The average annual rate of decline in SPECT tracer uptake was also significantly higher in the hyposmic group, in both the asymptomatic hyposmic relatives and in those that developed PD (50).

1.3.2.2 Sleep disorders

REM sleep behavior disorder (RBD) is when patients act out their dreams due to the loss of the normal REM sleep atonia (60). A synucleinopathy condition like MSA, dementia with Lewy bodies (DLB) and PD develop in more than 80% of people with RBD (see review in (61)). Fifteen to 33% of people with PD have an identifiable idiopathic RBD phase (62) compared to approximately 1% of people in the general population (62,63). Eight to 9% of this RBD cohort develop a synucleinopathy per year (64). A ten-year prospective cohort of 89 subjects with idiopathic RBD had a conversion

rate to a neurodegenerative disease of 30% at 3 years and 66% at 7.5 years; or 10% per year (65). A prospective longitudinal study investigating probable PD subjects with questionnaires and polysomnography found an increase in RBD and associated hallucinations over 8 years with 11% of their cohort affected with RBD after 3 years, 29% after 6 years and 34% after 8 years (66). Definitive diagnosis of RBD requires polysomnography (PSG). However, alternative questionnaires and surveys for diagnosing RBD are under investigation. The REM Sleep Behavior Disorder Single-Question Screen (RBD1Q), has a specificity of 87% and sensitivity of 93.8% for detecting RBD by itself, when compared to PSG (67). From neuropathological studies, α-synuclein deposition was found in the brains of 94% of patients with idiopathic RBD (68). Presynaptic deficits on SPECT and olfactory dysfunction have also been seen in those with idiopathic RBD. RBD is highly specific at approximately 65%, higher than other clinical biomarkers (47).

However, there are several limitations with using RBD as a diagnostic biomarker. As previously mentioned, the definitive diagnosis of RBD requires polysomnography (PSG) with chin electromyography (EMG) showing increased tonic chin EMG activity during REM sleep (69). PSG is not readily available in most institutions and often has long waiting lists. RBD has a low sensitivity in PD with only a third of all subjects experiencing RBD and increased prevalence in older patients with longer disease durations (70). PD subjects with LRRK2 mutation do not frequently experience RBD as a prodromal symptom and if it develops it is usually later in the disease course (71,72). There is also a long lead time of up to 13 years between RBD onset and development of a neurodegenerative disease (60).

Excessive daytime sleepiness (EDS) measured using the Epsworth Sleepiness Scale (ESS) (73) is prevalent in PD ranging from 16 to 60% depending on the PD population and the criteria applied (74–77). EDS was seen in the HAAS to confer a 3-fold increased risk of developing PD (78). The US NIH-American Association of Retired Persons (AARP) cohort found that participants who napped more than 1 hour per day in 1996 to 1997 had an approximately 50% higher chance of reporting a PD diagnosis compared to non-nappers (79). Other sleep disturbances seen in PD include insomnia, nocturia, circadian rhythm disorders and restless leg syndrome (80,81).

1.3.2.3 Autonomic dysfunction

Dysautonomia is experienced in PD subjects and includes constipation, orthostasis, urinary and erectile dysfunction. Constipation is common in the PD population with 80% of patients reporting the symptom (82). Constipation is also associated with an increased RR of developing PD of 2 to 3-fold (83–86). The HAAS showed that subjects who had less than one bowel movement per day had a 4-fold increased risk of PD compared to those who had two or more bowel movements per day (83). However, like hyposmia, the prevalence of constipation in the general population is high at 15 to 20% and therefore constipation has a low specificity and predictive value as a sole biomarker for PD (see review in (61)). The lead time for constipation varies depending on the study and ranges from 2 to 20 years (85,87,88). Constipation is thought to be due to α-synuclein pathology in the myenteric plexus, as seen on human colonic biopsies (89) and in transgenic mice that overexpress α-synuclein (90). Phosphorylated α-synuclein deposition was found in 45% of prodromal PD's tissue blocks, a mean of 7 years prior to diagnosis compared to 26% of controls (91). However, this finding was not supported in another study which failed to detect a difference in α-synuclein deposition between controls and PD subjects (92).

Orthostatic hypotension has a RR of 1.37 to 3.03 for PD (87) with a lead time of up to 20 years in patients with idiopathic RBD (88). An autonomic function clinic reported that 19% of patients with orthostatic hypotension and 25% with delayed orthostatic hypotension developed a synucleinopathy within 10 years of diagnosis. This was a 10-fold increase in incidence compared to the general population (93).

Urinary symptoms have also been associated with higher risk of PD with a RR of 2.3 (94) and odds ratio (OR) of 1.9 (87). However, like constipation and hyposmia its prevalence in the general population is quite high and therefore the specificity of urinary issues for PD is low. The lead time is unknown with one study reporting the RR of developing PD was similar at 2 years and 10 years of follow-up at 2.7 and 1.92 respectively (87).

Sexual dysfunction has been evaluated as a marker for prodromal PD. Its presence has been associated with a RR of 1.17 to 3.8 (87) compared to those without

symptoms of sexual dysfunction (95). It is a non-specific symptom as common in the elderly male population. Its lead time ranges from 2 to 10 years (87,88).

1.3.2.4 Neuropsychiatric conditions

Mood disorders have also been observed in the prodromal period of PD with the presence of depression, anxious personality traits, apathy and decreased novelty-seeking increasing the RR of developing PD (96,97). Depressive symptoms prior to motor symptoms occur in up to 30% of patients (98) and confer a RR of 1.5 to 2.5 for PD (87,99–102). Shiba et al. found that depression predated diagnosis by 5 years, whereas anxiety was present up to 20 years before motor symptoms. The OR for depression is 1.9; anxiety 2.2 and both anxiety and depression is 2.4 (103). However, the sensitivity and specificity of mood disorders alone is low as they are frequently seen in controls too (104). The lead time is variable and long, ranging from 3 to 25 years (104,105).

Other prodromal markers that are actively being studied include restless legs syndrome, color vision loss and cognitive impairment ((106), see review (61)). The Rotterdam study reported that subjects who developed PD had abnormalities on several cognitive tests in the prodromal period (107). The PARS study also found that ‘at-risk’ patients, described as those with evidence of olfactory loss and dopamine transporter deficiency had reduced global cognitive function, impaired memory and executive dysfunction (108).

1.4 Radiological biomarkers

There are multiple imaging modalities for both the central and peripheral nervous system to assist with diagnosing PD (see reviews (69,109,110)). The most frequently studied modalities are Magnetic Resonance Imaging (MRI), Transcranial Ultrasound (TCUS), Positron Emission Tomography (PET), Single Photon Emission Computed Tomography (SPECT) and ^{123}I-Meta-IodoBenzylGuanidine (MIBG) myocardial scintigraphy. Functional neuroimaging is an exciting area in PD as it is less influenced by compensatory mechanisms in the cortical-basal ganglia-thalamocortical circuitry and thus could be used to make diagnosis of PD earlier, in its premotor stage.

1.4.1 Nuclear medicine imaging

Nuclear medicine imaging is performed by injecting the patient intravenously with a radiotracer and then scanning them with SPECT or PET to determine where and to what extent the radiotracer has localized in the organ or tissue of interest. There are several radioactive tracers that have been shown to be of use in PD. Each of these tracers assesses a different component of the dopaminergic presynaptic terminals: ^{18}F-fluorodopa measures aromatic acid dopa decarboxylase activity; ^{123}I- ß-CIT visualizes the synaptic membrane dopamine transporter; and ^{11}C-dihydrotetrabenazine assesses vesicular monoamine transporter type 2 (VMAT2) (see reviews (69,109–111)). Imaging of these radiotracers in PD demonstrates a loss of nigrostriatal dopaminergic function manifested by decreased uptake of the radiotracer. At the time of PD diagnosis these imaging modalities will demonstrate a 30 to 65% reduction in dopaminergic innervation within the putamen (109,112). In PD, the annual reduction in striatal tracer uptake is thought to be 4 to 13% compared to 0 to 2.5% in healthy controls (8,112–114).

1.4.1.1 Brain SPECT

The sensitivity of ^{123}I-ß-CIT SPECT for PD diagnosis is approximately 92% and specificity 100% when compared to clinical diagnosis by a Movement disorder specialist, at 6-month follow-up (115). In another multicenter study, Benamer et al. assessed ^{123}I-ß-CIT SPECT findings in patients with PD, MSA, PSP, ET and age-matched controls. They found a sensitivity of 98% and specificity of 100% for differentiating PD from ET. However, it was unable to differentiate PD from the atypical parkinsonian disorders of PSP and MSA, as they all demonstrate impaired tracer uptake (116). SPECT initial imaging results have been remarkably consistent with the clinical diagnoses made at 2-year follow-up (117). Dopamine transporter scans are currently FDA approved for certain indications (table 4) (111,118). The EMA recently endorsed DaTSCAN use in PD clinical trials (119).

Table 4: Indications for DaTSCAN as per FDA, EMA and SNM guidelines (107,113)

1. Differentiate Essential tremor from parkinsonian disorders
2. Differentiate Dementia with Lewy bodies from Alzheimer's disease
3. Distinguish drug-induced parkinsonism from parkinsonian disorder

1.4.1.2 Brain PET

Brain PET detects changes in regional metabolism in the brain (120). ^{18}F-fluorodopa PET assesses the turnover of dopamine by tagging L-dopa, a substrate for the dopa decarboxylase enzyme. In PD subjects, decreased ^{18}F-fluorodopa uptake is seen in the posterior putamen (121). ^{18}F-fluorodeoxyglucose PET imaging evaluates resting regional cerebral glucose metabolism. PD subjects demonstrate increased metabolic activity in the lentiform nucleus and thalamus, and reduced lateral frontal, paracentral, inferior parietal and parieto-occipital activity, compared to controls (122).

Brain PET and SPECT have the potential to identify subclinical PD prior to diagnosis and be used as a premotor biomarker in 'at-risk' populations. Early support for this came from studies identifying imaging changes in the pre-symptomatic twins of patients with PD, as well as observing bilaterally abnormal imaging in subjects with unilateral PD (109). In subjects with RBD, increased metabolic activity was seen on ^{18}F-fluorodeoxyglucose PET (123,124), along with reduced uptake of ^{18}F-fluorodopa, ^{123}I-ß-CIT (see review by (125)), and ^{11}C-dihydrotetrabenazine (126) compared to controls. Twenty to 40% of patients with RBD have evidence of dopaminergic denervation and this progresses over time (127–129). In the PARS study cohort, 11% of hyposmic participants showed decreased tracer uptake compared to 1% of normosmic individuals (130). Dopaminergic denervation was more likely to be seen in those patients who were male; in those with hyposmia; or those suffering from constipation with OR of 5.5, 12.4 and 4.3, respectively (130). Patients with dopaminergic reduction, defined as more than 65% reduction in tracer uptake, had a RR of 17.47 to develop PD over a 4-year period (131).

However, these PET and SPECT scans have their own unique limitations as diagnostic biomarkers. They cannot differentiate between PD and other atypical parkinsonian conditions, like MSA, PSP and DLB. This is important to be aware of as each of these conditions have a different pathogenesis, treatment and prognosis. Theses scans are also currently limited in their ability to measure clinical disease progression as demonstrated by the lack of correlation between clinical assessment and striatal uptake in the ELLDOPA cohort (110,132), although better correlation was seen in other studies (133). The lead time from abnormal imaging to diagnosis with PD has a large range of 2 to 20 years (35,114,121,134). Lastly, these tests are relatively expensive, require exposure to radiation and are only available in specialized centers.

1.4.1.3 Cardiac SPECT

Cardiac SPECT uses ^{18}F-fluorodopamine or ^{123}I-MIBG, a radio-iodinated analogue of guanethidine, an adrenergic blocking agent. Both markers use the same metabolic pathway as noradrenaline, and their uptake in the heart correlates with both the functional integrity and density of post-ganglionic presynaptic cardiac sympathetic neurons (109). In Braak's stage one, there is vagal nerve involvement which results in a loss of cardiac sympathetic innervation. On cardiac SPECT there is decreased MIBG uptake and reduced heart to mediastinum ratio in PD subjects (32,135). A meta-analysis of cardiac SPECT scans showed a pooled sensitivity of 90% and specificity of 86% for diagnosing PD. For early PD, cardiac SPECT demonstrated a sensitivity of 94% and specificity of 80% for PD (135). Another meta-analysis found that cardiac SPECT could distinguish Lewy body-related conditions (i.e. PD and DLB) from non-Lewy body pathologies (i.e. MSA and AD) (136). Cardiac sympathetic nerve involvement is also seen in incidental Lewy body pathology, a pathological precursor to PD (137) and reported in people with dysautonomia, sleep, neuropsychiatric and mood disorders, all of which are potential premotor, non-motor features of PD (138).

1.4.2 MRI Brain

Conventional MRI is useful in clinical practice to distinguish PD from secondary structural causes of parkinsonism, like strokes or masses in the basal ganglia. More advanced MRI techniques can assess microstructural changes and functional connectivity alterations in the brain. Changes in iron deposition, loss of neuromelanin and alterations in nigrosome 1 have been observed in people with PD compared to matched controls (139,140). Attenuation in neuromelanin can differentiate early PD from controls with a sensitivity and specificity of 73% and 87% in the lateral SN and 82% and 90% in the locus coeruleus (139). Two cross-sectional studies have shown a positive correlation between T2 relaxation time in the caudal putamen and disease duration, indicating a progressive loss of iron with increasing disease duration (141,142). However, another study which measured quantitative MR parameters sensitive to volume atrophy, iron deposition and microstructural damage in subcortical structures found no relation of relaxation rates to disease progression (143). Using functional MRI, differences in the functional connectivity within basal ganglia network, the default-mode network and the sensorimotor resting network have been observed in people with PD compared to controls (see review by (144)). MRI with diffusion tensor imaging (DTI) demonstrated reduced fractional anisotropy in the SN of PD subjects, compared to controls (143,145,146). DTI in RBD subjects also found changes in the brainstem areas relevant to REM sleep, compared to controls (147). MRI with diffusion kurtosis imaging (DKI) can also distinguish PD subjects from healthy controls, and was found to have a higher sensitivity and specificity than DTI (148).

1.4.3 Transcranial Ultrasound (TCUS)

TCUS evaluates SN echogenicity by applying an ultrasound beam through the temporal bone window and assessing the ultrasound echoes from the SN (149,150). Zecca et al. found a significant correlation between the echogenic area of the SN and the concentration of iron, H- and L-ferritin in post-mortem brains (151). In 1995, Becker et al. demonstrated SN hyperechogenicity in living patients with PD (152). SN hyperechogenicity is seen in 90% of patients with PD compared to 9 to 19% of community dwelling older people without PD (152–155). It is thought to act as a marker

of vulnerability, a ‘trait’ biomarker, as it does not change or correlate consistently with disease severity or disease progression (153,154,156). A prospective trial of asymptomatic subjects aged 50 years or older found that the presence of SN hyperechogenicity increased the risk of developing PD by 17- to 20-fold (20,157). Berg et al. showed that SN hyperechogenicity had sensitivity and specificity of 80% and 81% for development of PD over 3 years (22). If both hyposmia and family history of PD were present, the specificity increased to 91% (22). A study in patients with mild parkinsonism and SN hyperechogenicity showed a sensitivity of 91%, specificity of 82% and PPV of 93% for PD diagnosis after 1-year follow-up (158). In ‘at risk’ populations for PD, SN hyperechogenicity was seen in 36 to 50% of patients with idiopathic RBD (129,159,160).

TCUS has also been used to differentiate PD from other forms of parkinsonism. SN hyperechogenicity can differentiate PD from atypical parkinsonian syndrome with 95% specificity and 90% sensitivity (158). SN hyperechogenicity is seen in less than a third of patients with PSP and rarely in MSA-P (161). Hyperechogenicity of the lentiform nucleus is frequently seen in subjects with MSA and PSP but rarely in PD (161). The extent of SN hyperechogenicity does not correlate with disease severity using the H&Y stage, disease duration or degree of nigrostriatal degeneration assessed using ^{123}I-FP-CIT SPECT (162). The lead time for SN hyperechogenicity to developing PD is currently unknown as it is thought to be more a ‘trait’ biomarker, as discussed above.

TCUS is a relatively inexpensive and safe test that does not expose the patient to harmful radiation. However, there are specific challenges with TCUS which include being both operator and patient-dependent; requiring both adequate temporal bone windows in the patient and the operator’s expertise to visualize the SN (12,163). It is present in approximately 10% of the general population (22). Interestingly, it has been suggested that asymptomatic SN hyperechogenicity may indicate incidental Lewy body disease in these people who may or may not convert to PD in the future (163). Further research will be needed to investigate this hypothesis further. Nevertheless, TCUS currently holds a level A recommendation for supporting PD diagnosis and distinguishing it from other forms of parkinsonism (164).

Other imaging-based studies in PD include [11C](R)-PK11195 PET scan which assesses markers of neuroinflammation in the brain (165–167) along with a recently developed radioligand that binds to α-synuclein fibrils in post-mortem PD brains (168).

1.5 Genetic biomarkers

Over the past two decades there has been increased research and interest in studying and understanding genetics in PD. In the past, genetics were not considered to influence PD due to its late onset and seemingly sporadic nature. However, this assumption is now known not to be the case with genetic forms of PD accounting for at least 5 to 10% of all cases of PD (169). Furthermore, having a family history of PD increases your odds of developing PD by 3- to 4-fold (170) and approximately 16% of people with PD will report a first-degree relative with PD too (171). Genetic information can be objectively measurable and therefore could be a suitable biomarker. However, as this information does not change over lifetime, these genetic variants or mutations are best considered as 'trait' biomarkers, a marker of risk for developing the disease. In 1997, the first genetic mutation was identified in the Contursi kindred, a missense mutation in α-synuclein encoded by SNCA on the long arm of chromosome 4 (172). Since then there has been a growing list of new mutations and genes associated with parkinsonism, assigned the PARK loci (173). In table 5 I have outlined features of the more common mutations, (adapted from table in review, see (173)). Common genetic mutations include the autosomal dominant mutations affecting the SNCA gene with missense mutations, duplications and triplications; the Leucine-rich repeat kinase 2 (LRRK2) gene and the Vacuolar protein sorting-associated protein 35 (VPS35) gene along with the autosomal recessive mutations in the Parkin, PTEN-induced kinase 1 (PINK1) and DJ-1 gene (174,175). Many of the genetic mutations leading to familial forms of PD have the same clinical phenotype as idiopathic PD. However, often non-motor symptoms like psychiatric and cognitive impairment play a more prominent role, like in PARK7 and PARK 4, respectively (174). Although the inheritance pattern is known for most mutations, the penetrance is often incomplete, and the age of onset remains highly variable for many genetic variants.

LRRK2 mutations are the commonest known genetic cause of PD with the G2019S mutation occurring in 4% of hereditary and 1% of sporadic PD (176). LRRK2 mutation has incomplete, age-dependent penetrance for PD with 28% of carriers affected at 59 years and 74% affected at 79 years (176). LRRK2 mutation carriers show subclinical dopaminergic abnormalities (177) along with impaired trunk acceleration, smoothness of sway and gait variability during challenging tasks (178). LRRK2 PD subjects have higher rates of non-motor symptoms including more depressive symptoms and worse color discrimination compared to idiopathic PD (179).

The most important risk factor for PD is heterozygous mutations in the glucocerebrosidase (GBA) gene (180). Heterozygous mutations are found in 1 to 3% of normal population and 3 to 15% of PD patients (181). GBA mutation is more common in particular ancestries with 15% of Ashkenazi Jewish patients having the mutation compared to 3% of non-Ashkenazi Jewish patients (181). GBA mutation has a high penetrance and is associated with an OR for developing PD of 5.4 (181). In GBA mutation carriers it is estimated that 13.7% will develop PD at 60 years of age and 29.7% at 80 years of age (182). GBA-related PD has a similar phenotype to idiopathic PD. However, GBA positive PD subjects have more non-motor symptoms with more impaired olfaction, cognition (180,183) as well as a greater prevalence of RBD, depression, bradykinesia, rigidity and resting tremor at presentation (181).

Population-based genome-wide association studies (GWAS) have also helped to identify genetic risk loci in the human genome. No medium to high-risk common risk alleles have been identified in PD, unlike in AD with discovery of the APOE e4 allele (174). However, some low-risk susceptibility variants have been discovered in PD. The first loci to be detected by GWAS were at the genome encoding MAPT, on chromosome 17; α-synuclein, on chromosome 4 along with risk loci on LRRK2, chromosome 12 (184,185). Although these single variant risk alleles have ORs from 0.7 to 1.8 (186), they account for additional hereditability and are responsible in a small but additive manner towards the risk for developing PD (187,188).

The utility of genetic testing as a diagnostic biomarker is still undefined with genetic information conferring risk of disease rather than diagnosis. Currently, genetic testing is not part of routine clinical practice unless there is suspicion for familial PD due

to young age of onset; presence of dystonia or atypical features. Nonetheless, the recognition of genetic forms of PD is important as many of the products of the mutated genes have been linked to PD pathogenesis including oxidative stress and mitochondrial dysfunction (174).

Regarding genetic biomarkers, an exciting area of research is studying the manifesting and non-manifesting carriers of LRRK2 and GBA mutations. This research may help to identify potential biomarkers for PD; understand the underlying pathogenesis and create drug therapies. Genetic biomarkers would also ensure the recruitment of homogeneous populations into trials, as seen in recent GBA and LRRK2 studies (189,190). The PPMI study's prodromal arm, the P-PPMI is assessing subjects with a mutation (LRRK2, GBA or SNCA+), RBD or anosmia (191). The Oxford Parkinson's Disease Centre (OPDC) also follows subjects with a first-degree relative of PD (192).

Table 5: Genetic forms of PD (173)

Locus symbol	Gene	Chromosome	Inheritance	Clinical clues
PARK 1	SNCA missense	4q22.1	AD	Classic PD phenotype
PARK 2	PARK2 encoding parkin	6q26	AR	Slowly progressive disease; Lower limb dystonia
PARK 4	SNCA duplication or triplication	4q22.1	AD	Prominent dementia
PARK 6	PINK1	1p36.12	AR	Slowly progressive disease
PARK 7	DJ1	1p36.23	AR	Onset in 20s; psychiatric features
PARK 8	LRRK2	12q12	AD	Slow progression; more dystonia
PARK 9	ATP13A2	1p36.13	AR	Onset in teens; complex phenotype
PARK 17	VPS35	16q11.2	AD	Classic PD phenotype

1.6 Biological biomarkers

Both biological fluids and tissues have been analyzed as potential diagnostic biomarkers for PD. In this section, I will focus on some of the more researched markers in the blood, CSF and tissue.

1.6.1 α-synuclein

α-synuclein is the most well-studied protein marker in PD due its presence in Lewy bodies along with its association with the pathogenesis of PD after the discovery of point mutations and multiplications in the SNCA gene. It is secreted by cells and found in the CSF, serum, plasma, skin and nerves. It is a protein expressed in neuronal synapses and is thought to be involved in the control of synaptic plasticity and neural differentiation (193). Studies have shown increased oligomeric α-synuclein and higher phosphorylated α-synuclein in the plasma of PD subjects compared to controls with specificity of 85% and sensitivity of 53% (194). Higher levels of nitrosylated α-synuclein have also been found in peripheral blood mononuclear cells (PBMCs) of PD subjects compared to controls (195). A CSF cohort study in subjects with PD, MSA, DLB, PSP, NPH and other neurological disorders, found low CSF α-synuclein (<1.6 pg/mL) to have a sensitivity of approximately 71% and specificity of 53% for diagnosing PD and a PPV of 91% for diagnosing a synucleinopathy (196). A meta-analysis of CSF reported α-synuclein levels to be lower in PD subjects compared to controls and higher in PD subjects compared to those with MSA (197). Another meta-analysis showed that CSF α-synuclein had a sensitivity and specificity of 88% and 40%, respectively for the diagnosis of PD (198). CSF α-synuclein has also been studied as a prognostic biomarker in PD. One study found higher levels of CSF α-synuclein associated with more rapid motor and cognitive decline over two years (199). Lastly, antibodies to α-synuclein have also been identified and titres were shown to decrease with disease progression (200). A recent meta-analysis reviewed results from 17 papers. Some papers suggested antibodies in those with early disease, others did not (201). Clinical heterogeneity, small sample sizes and assay variability were potential causes for inconclusive results. Attempts to reproduce α-synuclein results in the CSF and blood

have been difficult with discordant results often reported. These inconsistencies are most likely due to differences in the α-synuclein isoforms measured and methodologies of assays (192). Sample processing is also important, and given the abundance of α-synuclein in RBCs, any hemolysis will affect the results (202).

α-synuclein has been observed in the GI tract, skin and salivary glands of PD subjects. As mentioned in our non-motor symptom section, phosphorylated α-synuclein was found in colonic biopsies of 45% of those with prodromal PD, a means of 7 years prior to diagnosis compared to 26% of controls and 48% of those with clinical PD (91). In contrast, another study found no difference in α-synuclein deposition between the PD and control groups (92). Higher densities of α-synuclein have been recorded in more rostral structures of the gut, however this again has not been seen consistently in all studies (91). Differences in these results are felt to be secondary to variable tissue preparations, staining techniques and antibodies applied (203).

In PD subjects with loss of PINK1 function, their skin fibroblasts showed increased expression of α-synuclein compared to controls (204). Increased aggregation of α-synuclein and fiber loss in autonomic sudomotor and pilomotor fibers has been seen in PD subjects (205,206). Gibbons et al. studied skin biopsies and found higher levels of cutaneous α-synuclein deposition in PD subjects compared to controls. They reported α-synuclein deposition at all stages of disease and extrapolated these findings to premotor PD subjects too. They found the α-synuclein ratio to be more sensitive and specific than other α-synuclein isoforms and had a sensitivity and specificity of over 85% (206).

α-synuclein has also been observed in the submandibular glands of both post-mortem and living PD subjects. Beach et al. demonstrated high sensitivity and specificity for α-synuclein in submandibular glands. It was found in 89% of PD subjects and no controls (207,208). Adler et al. carried out needle core biopsies of the submandibular gland in early PD subjects and saw α-synuclein in 74% of PD subjects compared to 22% of control subjects (209). Adler et al. also showed α-synuclein in 75% of advanced PD patients (210). A recent post-mortem study, found α-synuclein in the

retina of autopsied PD subjects and those with incidental Lewy body disease, but not in control subjects (211).

α-synuclein in 'at-risk' RBD populations have been studied too (212,213). A cross-sectional study of submandibular glands found α-synuclein deposits in 89% of RBD subjects, 67% of PD subjects and no controls (212). Like measurements in the CSF and blood, there are inconsistent results in tissue samples. Nonetheless, we feel that this area holds great promise given its apparent high sensitivity and specificity for PD. We anticipate ongoing research in α-synuclein and expect more standardized collection and processing techniques to be developed soon.

1.6.2 DJ-1

DJ-1 is a homodimeric protein and was linked to PD after familial PD cases were discovered in the setting of mutations in the gene coding for DJ-1, PARK7 mutation, an autosomal recessive form of PD (214). DJ-1 is part of the cellular defense against oxidative stress and is thought to work as a chaperone, a transcriptional regulator, a regulator of protein degradation pathways and an antioxidant protein (215). It has been studied in the CSF, blood and saliva. Decreased levels of DJ-1 have been found in the CSF of PD subjects with a sensitivity of 90% and specificity of 70% for diagnosing PD (216). Levels of DJ-1 also correlated with those of α-synuclein, with both being lower in PD subjects compared to controls (216).

Unfortunately, like α-synuclein, current literature on DJ-1 is inconsistent with both increased and decreased levels reported in both the blood and CSF of PD subjects (217–219). In one study comparing PD, MSA and control subjects higher CSF DJ-1 levels were found in MSA, followed by PD and then control subjects. DJ-1 levels were able to discriminate between MSA and PD subjects with a sensitivity and specificity of 81% and 52%, respectively (220). Specific antibodies for oxidized DJ-1 were developed after elevated levels of oxidized DJ-1 were found in the brains of PD patients (221). Increased DJ-1 and reduced α-synuclein levels have been recorded in the saliva of PD patients (222). Analysis of saliva may be useful for future biomarker discovery as it is non-invasive and typically free of blood contamination, thereby avoiding hemolysis, which can confound the results.

1.6.3 Neurofilament light chain (NfL)

Neurofilament light chain (NfL) is a subunit of neurofilament, one of the major axonal cytoskeleton proteins. The presence of neurofilament suggests axonal injury and axonal transport defects (223). Increased NfL is seen when there is degeneration of the large myelinated axons (224). Consequently, elevated levels of NfL is non-specific with high blood and CSF levels observed in multiple sclerosis (225), traumatic brain injury (226), stroke (227), spinal cord injury (228) and other neurodegenerative diseases including FTD (229), ALS (230), synucleinopathies, tauopathies and AD (231). Nevertheless, studies of elevated NfL levels have been able to differentiate PD from atypical parkinsonian syndromes (APS), as much higher NfL levels are seen in APS than in PD (232,233). This is postulated to be due to less severe or less diffuse axonal neurodegeneration in PD compared to APS (232).

1.6.4 Metabolomics

Metabolomics is the study of metabolic pathways by measuring small-molecular weight (<1.5 kD) metabolites involved in the polyamine, purine, pyruvate pathway and redox markers (234). A metabolomic analysis on serum reported lower concentrations of caffeine, bilirubin, ergothioneine and tryptophan along with higher concentrations of biliverdin and levodopa metabolites in PD subjects compared to controls (235). Bogdanov et al. carried out a metabolomic analysis on plasma and found reduced uric acid and increased glutathione levels in PD subjects compared to controls (236). Increased plasma pyruvate has also been observed in drug-naive PD subjects compared to controls (237). A metabolomic analysis on CSF found elevated 3-hydroxykynurenine, reduced oxidized glutathione and alterations in levels of n-acetylated amino acids in the PD subjects (238). Metabolomics may assist with progression. Lewitt et al. demonstrated a correlation between plasma xanthine structures and medium- or long-chain fatty acids and UPDRS scores (239).

1.6.5 Oxidative stress markers

Oxidative stress is known to play a role in the pathogenesis of PD. In PD, both an increase in free radicals and a reduction in defense mechanisms against oxidative

stress are seen (69,193). Elevated levels of hydroxyl radicals (240), F(2)-isoprostanes, 5-hydroxyeicosatetraenoic acid products and cholesterol oxidation products have been recorded in the plasma of PD subjects (241). Higher levels of malondialdehyde have been seen in both the CSF and blood of PD patients (242). Increased levels of 8-Hydroxy-2'-deoxyguanosine (8-OHdG) have been observed in the urine and blood of PD subjects (236,241,243,244). Higher levels of reactive oxygen species (ROS) have also been demonstrated in the peripheral blood cells from both drug-naive and treated PD patients (245,246). There is a decreased redox ratio in the blood and increased percentage of oxidized to total CoQ10 in PD subjects (247,248).

Urate, the main antioxidant in the plasma and a potent radical scavenger has consistently been shown to be reduced in PD subjects compared to controls (see review in (249)). Low levels of urate is a risk factor for developing PD and associated with disease progression (249–251). Glutathione, another endogenous antioxidant is found to oxidized in the SN and peripheral blood cells of PD subjects (252). Other studies have investigated glutamate uptake in platelets from PD patients (253), and protective enzymes systems such as glutathione reductase or copper and zinc superoxide dismutase (242).

1.6.6 Inflammatory markers

Neuroinflammation is present in neurodegeneration through microglial activation, astrogliosis and lymphocytic infiltration (254,255). Activated microglial cells have been found within the SN in autopsied PD patients and increased levels of pro-inflammatory cytokines have been recorded in SN and spinal fluid (256). A recent meta-analysis of blood cytokines found increased levels of TNF-α, IL-6, IL-1β, IL-10, IL-2, CRP and RANTES in PD subjects compared to controls (257). Monocyte-associated inflammatory markers and IL-8 were found in PD subjects (258). Studies have also shown an association between cytokine levels and the risk of developing PD. Using data from the Health Professionals Follow-up Study, plasma IL-6 levels were positively associated with developing PD with an OR of 3.5 for those subjects in the highest quintile compared to the lowest quintile (259). Inflammatory gene cytokine polymorphisms of

TNF-α and IL-1β have also been associated with an increased risk of developing PD (260).

1.7 Challenges of biomarkers

There are numerous roadblocks and challenges in the discovery of biomarkers, which are both inherent to PD and in general. First of all, the diagnosis of PD is fraught with difficulties due to our current clinical diagnostic criteria which results in both delayed diagnoses and a significant rate of misdiagnoses. As neuropathological examination remains the gold standard we cannot confirm diagnosis until post-mortem assessment. Therefore, enrolling PD subjects into biomarker studies and clinical trials is often confounded by including other causes of parkinsonism, which have different pathogenesis and prognosis. Secondly, PD is a heterogenous disease and people with PD are often affected differently. Their prodromal period, non-motor and motor symptoms vary between individuals. Thus, a single diagnostic or prognostic biomarker may not be applicable or sufficient for all PD subjects. Last but not least, PD is a slowly progressive neurodegenerative condition and changes in clinical, radiological and biochemical parameters may take years to develop. Hence sensitive markers with short lead times are warranted so as to prevent long duration clinical trials, which would result in significant expense and delay results.

Nevertheless, there has been significant effort and strides made in identifying biomarkers in PD. As discussed in this review, there are a myriad of study groups and biobanks working collectively to recruit subjects with PD; genetic forms of PD; prodromal PD and 'at-risk' groups for developing PD. This present review has summarized the more commonly studied clinical, radiological, genetic and biological biomarkers for diagnosing PD. As we enter an era of trials on neuroprotective strategies in PD it is important that we understand better its underlying pathogenesis and diagnose individuals correctly before enrolling them into trials. Most likely a multimodal approach will be necessary with biomarkers from several domains used in the diagnosis. This is an exciting time but there remain many challenges ahead.

Chapter 2: Autonomic neuropathy in PD

2.1 Abstract

Introduction: Autonomic dysfunction is common in PD and can be a risk factor for developing PD. Lewy bodies and Lewy neurites are found in the autonomic regulatory centers in Braak stage 1 of PD and in the peripheral autonomic nerves in presumed incidental Lewy body disease. In this study we investigated for evidence of autonomic neuropathy in PD subjects compared to controls. We also evaluated for the presence and severity of non-motor symptoms, depression, pain and dysautonomia.

Methods and materials: We studied 36 subjects with PD and 23 age-matched controls. Subjects completed validated questionnaires (SCOPA-AUT, Beck Depression inventory, Brief Pain Inventory and NMS-QUEST) for dysautonomia, depression, pain and non-motor symptoms. They also underwent nerve conduction studies (NCS) and temperature threshold testing (TTT).

Results: Autonomic neuropathy was more prevalent in the PD group compared to controls ($p<0.05$). The PD group had more dysautonomia, pain and depressive symptoms on questionnaires ($p<0.01$). In the PD group, abnormal NCS were associated with higher levodopa-equivalent daily dosages ($p<0.05$).

Conclusion: We identified increased autonomic neuropathy in our PD group compared to controls, using this multimodal approach of SCOPA-AUT questionnaire and TTT to diagnose a small fiber neuropathy.

2.2 Introduction

Parkinson disease (PD) is the second most common neurodegenerative condition worldwide (261). Using clinical criteria alone, misdiagnosis occurs in up to 24% of cases when compared to the gold standard neuropathological assessment (see review (262)). The identification of biomarkers to help diagnose PD, including at a 'premotor stage' is likely to be of increasing importance as research focuses on developing neuroprotective therapies (263,264).

Autonomic dysfunction is more prevalent in PD subjects compared to controls and its presence is often related to disease duration, disease severity and the use of antiparkinsonian drugs (see review by (82)). Symptoms of dysautonomia include abnormal blood pressure, gastrointestinal, urinary, and sexual function, along with impaired temperature regulation (82). Dysautonomia in PD is thought to be both central and peripheral in origin. In Braak's neuropathological staging, Lewy bodies are found in the brainstem autonomic regulatory centers in stage one, the 'premotor stage' (265). On skin biopsy, PD subjects show accumulation of phosphorylated α-synuclein in small nerve fibers and loss of small nerve fibers (266,267). α-synuclein aggregates have also been seen in epicardial peripheral autonomic system in participants with presumed incidental Lewy body disease (268) and on colon biopsies, in the years prior to diagnosis (89). Constipation, a symptom of dysautonomia is a recognised risk factor for PD and confers a 2.7 to 4.1-fold increased risk of developing PD (83).

Identification of autonomic nerve involvement prior to motor symptoms may have a role as a biomarker in PD and assist with earlier diagnosis (269). Objective testing for autonomic nerve involvement is often complex and specialized, with previous studies using cardiovascular reflex testing and sympathetic skin responses (269). Intraepidermal nerve fiber density skin biopsies have shown reduced dermal sympathetic nerve density including decreased autonomic innervations of the blood vessels, sweat glands, and erector pili muscles in PD subjects compared to controls (267). Unfortunately, these tests are minimally invasive and not widely available.

Autonomic neuropathy, a form of small fiber neuropathy is diagnosed in the presence of two of the following: clinical symptoms or signs of small fiber damage; intraepidermal nerve fiber density (IENFD) reduction; or temperature threshold testing

(TTT) abnormalities (270,271). In our study we hypothesised that autonomic neuropathy is more prevalent in PD subjects compared to controls. We investigated for evidence of autonomic neuropathy using a novel approach of SCOPA-AUT questionnaire and TTT. We defined autonomic neuropathy as present if there was both symptoms of dysautonomia on the Scales for Outcomes in Parkinson's Disease- Autonomic (SCOPA-AUT) questionnaire and at least one abnormal TTT. We also evaluated for the presence and severity of dysautonomia, non-motor symptoms, pain and depression in our PD and control groups.

2.3 Methods and materials:

Study design: A case-control study carried out in the Department of Neurology and Neurophysiology in Cork University Hospital, Cork, Ireland. Ethical approval was received from the Clinical Research Ethics Committee of the Cork Teaching Hospitals (CREC) (appendix).

Subjects: Control subjects were relatives or friends of people with PD or volunteers. All PD subjects fulfilled the Queen Square Brain Bank criteria for PD (2). Disease phenotype (tremor-dominant or postural instability with gait disturbance (PIGD)), disease severity according to Hoehn & Yahr scale (H&Y) and disease duration from symptom onset were recorded. Dopamine medications with levodopa- equivalent daily dosage (LEDD) was documented on the day of testing. Initial screening included a neurological exam with complete sensory testing. All subjects completed standardised questionnaires and underwent both NCS and TTT. Exclusion criteria were conditions that could affect pain sensitivity (e.g. formally diagnosed neuropathy, other known neurological and psychiatric disorders) and common risk factors for peripheral nerve dysfunction (e.g. alcoholism, diabetes, vitamin B12 deficiency, chemotherapy). Blood tests: HbA1c or fasting blood sugar, thyroid stimulating hormone, ESR, vitamin B12 and CBC were reviewed. Those with abnormal results were excluded from analysis.

Questionnaires: All participants completed validated questionnaires assessing depression with the Beck Depression Inventory (BDI) (272), autonomic dysfunction with

the SCOPA-AUT (273), pain with Brief Pain Inventory (BPI) (274) and non-motor symptoms with NMS-Quest (275).

SCOPA-AUT questionnaire is a 25-item self-administered questionnaire exploring symptoms related to autonomic dysfunction of the gastrointestinal (7 symptoms), urinary (6 symptoms), cardiovascular (3 symptoms), thermoregulatory and pupillomotor (5 symptoms), and sexual domains (2 symptoms for men and 2 symptoms for women). Each symptom is scored based on severity from 0 (never experiencing symptom) to 3 (often experiencing symptom). The total score ranges from 0 (no symptoms) to 69 (all symptoms occurring often) (273).

Apparatus and Procedure: I performed the NCS and TTT. If I was unable to get the NCS on a participant, I received assistance from the neurophysiology technician. Testing was carried out in a temperature-controlled room (23°C). NCS' recording was performed using a standard electrodiagnostic device Nihon Kohden NeuroPack. We evaluated the sural nerve and peroneal nerve on the right lower limb of controls or least affected side in the PD group. Outcomes were based on our lab's own normative data.

'Thermal thresholds' (cold sense, warm sense, cold pain and heat pain) were evaluated using a thermal sensory analyser (Medoc, TSA-2001, Ramat Yishai Israel) with a Peltier-based contact thermode measuring 3 cm x 3 cm. Cut-off temperatures were 0 and 50°C. The baseline temperature was 32°C (center of neutral range) and alterations in temperature were administered with ramped stimuli at 1°C per second. 'Methods of limits' was used where the participant pushes a button when a change in temperature or pain is sensed. The mean of the 4 trials was inputted for further analysis (276,277). Using normative data available (278), subjects were matched for gender and age and we were able to identify abnormal TTT values (supplemental material).

Statistical analysis: All continuous data was presented with mean (SD) or mean (10^{th}, 90^{th} percentile) for highly skewed variables to summarise the spread. Statistical analysis was performed using Chi-squared test, Fisher's Exact test, two-sample independent t-test, Pearson's or Spearman's correlation coefficients, where appropriate. For highly skewed data (SCOPA-AUT, NMS-Quest and BDI) where a suitable normalization transformation could not be found, means were compared using the bootstrap

independent samples test and based on 5000 stratified bootstrap samples. The 25-items of the SCOPA-AUT scale were measured on an ordinal scale as per prior research (273). Statistical significance was set at $p<0.05$ for the primary outcome variable in the study groups' comparison, patient characteristics and for additional exploratory statistical analysis of the data. For all other secondary outcome variables listed above the significance level was adjusted and set at $p<0.01$ for between study group comparisons, in order to reduce the risk of type 1 errors when testing for multiple simultaneous hypotheses (279). All tests were 2-sided. The primary outcome of autonomic neuropathy, the SCOPA-AUT total mean score and the presence of abnormal TTT were controlled for the effects of depression (BDS) and pain (BPI), potential confounder variables. Statistical analysis was performed using SPSS 24.0.1.

2.4 Results:

Fifty-nine subjects met the inclusion criteria (Figure 1). Table 1 shows the characteristics of the PD (36 subjects) and control (23 subjects) groups. The PD group had more males than the control groups ($p<0.01$). 86.1% of PD subjects were early H&Y stage of disease (H&Y 1 and 2) and the mean disease duration was 4.9 years.

Figure 1: Subject recruitment consort diagram

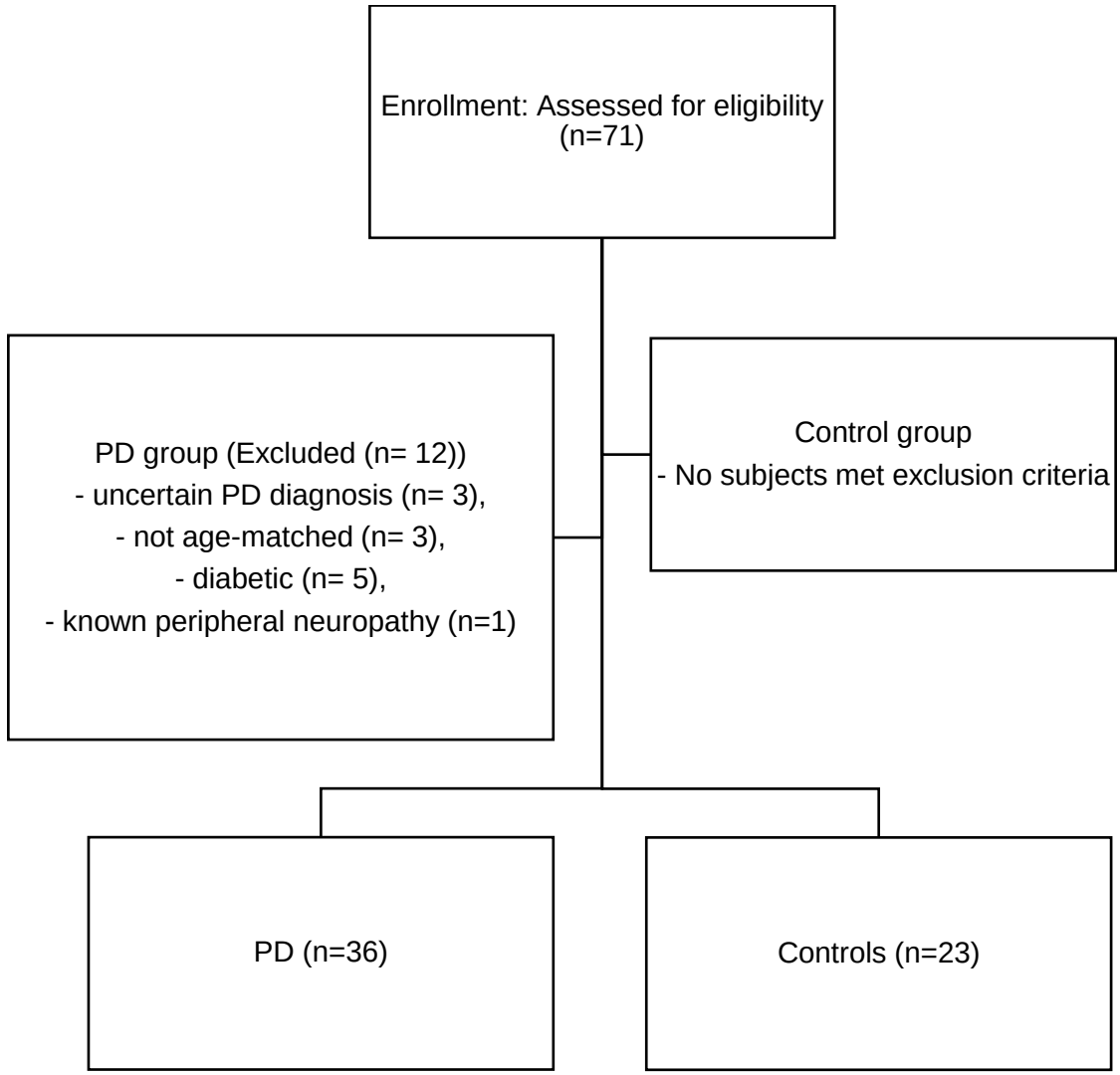

Table 1: Subject Characteristics in Parkinson and Control groups

Study groups (number)	Parkinson disease (N=36)	Controls (N=23)	Test Statistic	P value 2-sided
Gender (M: F)	27: 9	9: 14	$X^2(1) = 7.591$	0.006
Age (mean; SD)	64.1; 7.9	60.0, 7.8	$t(57) = 1.936$	0.058
Disease duration, years (median; mean; (10^{th}, 90^{th} percentile))[a]	2.0; 4.9; (0.31, 15.3)	N/A	N/A	N/A
H&Y stage- (N (%)) 1^{st} 2^{nd} 3^{rd} and 4^{th}	12 (33.3%) 19 (52.8%) 5 (13.9%)	N/A	N/A	N/A
Phenotype (Tremor dominant: Akinetic rigid)	28: 8	N/A	N/A	N/A
Levodopa- equivalent daily dose (LEDD, mg/day) Mean (10^{th} and 90^{th} percentile)	417.7 (105.4; 867.4)	N/A	N/A	N/A
Nerve conduction studies Number (normal: abnormal)	N=33 20: 13	N=22 18: 4	$X^2(1) = 2.781$	0.095

a: Shapiro-Wilk's test of normality ($p<0.0001$)

Autonomic neuropathy

Evidence of autonomic neuropathy, a subtype of small fiber neuropathy, based on symptoms of small fiber dysfunction on the SCOPA-AUT questionnaire and one abnormal TTT (Table 2), was more common in PD subjects compared to controls ($X^2(1) = 7.721$; $p=0.005$). 24 of the PD subjects (66.7%) met criteria compared to 6 controls subjects (28.6%). Autonomic neuropathy was not associated with mean scores on SCOPA-AUT, NMS-Quest, BDI or presence of pain on questionnaires ($p>0.05$).

Nerve conduction studies (NCS)

There was no significant difference in the proportion of subjects with abnormal NCS between the two groups ($p>0.05$) (Table 1). Abnormal NCS was not associated with age or gender. In the PD group, abnormal NCS was associated with higher LEDD (mg/day) (mg/day) (586.23± 399.14 mg/day) and spread compared to normal NCS (299.63± 191.76 mg/day) ($p<0.029$) (Figure 2).

Questionnaires

We found significant differences between the PD and control group on the questionnaires- SCOPA-AUT, NMS-Quest and BPI (Table 2). The mean SCOPA-AUT score for PD subjects was on average 5.64 (95% CI 2.57 to 8.71) higher than control subjects, after controlling for the effects of depression and pain ($p=0.01$). Within the SCOPA-AUT sub-sections, there was also a significant difference in both the absence and presence of symptoms (Table 3) and their individual severity on SCOPA-AUT (Table 4) with more severe gastrointestinal (GI), thermoregulatory, bladder and blood pressure symptoms in the PD group (all p values<0.05). Cronbach's alpha for the scale domains (alpha=0.701) showed good internal consistency.

Depression

The mean total score on BDI was also higher in PD group compared to controls but did not reach statistical significance for secondary outcomes ($p=0.02$) (Table 2).

Brief Pain Inventory

Presence of pain was more frequently reported in the PD group compared to controls (p=0.001) (Table 2). The odds of having pain for a subject with PD was 9.33 times (95% CI 2.34 to 37.196) that of a control patient.

Table 2: Questionnaires for PD and control groups*

Study groups	Parkinson disease (PD) Mean (SD), Q1, Q3	Controls (C) Mean (SD), Q1, Q3	Bootstrap independent sample test[a]		
			P – C Mean Difference (Std. Error)	95% confidence interval Lower, Upper	P value 2-tailed
SCOPA-AUT[b]	N=36 11.1 (6.4) 6, 15	N=23 3.6 (3.6) 0, 7.0	7.8 (1.4)	5.2, 9.8	p<0.001
NMS-Quest[b]	N=34 8.2 (6.4) 3.0, 12.5	N=21 2.3 (4.0) 0, 3.5	5.8 (1.4)	3.1, 8.5	p<0.001
BDI[b]	N=30 8.3 (7.3) 3.00, 13.3	N=22 4.3 (4.4) 0, 6.5	3.95 (1.6)	0.9, 6.95	p=0.020
BPI Number (pain present: absent)	N= 36 21:15	N=23 3:20	Test statistic	$X^2(1) = 11.929$	p=0.001

*: Questionnaire scores, not controlled for BDI or BPI

a: Unless otherwise noted, bootstrap results are based on 5000 stratified bootstrap samples

b: Levene's test for equality of variance $p < 0.05$

Figure 2: LEDD (mg/day) in PD group with normal and abnormal NCS

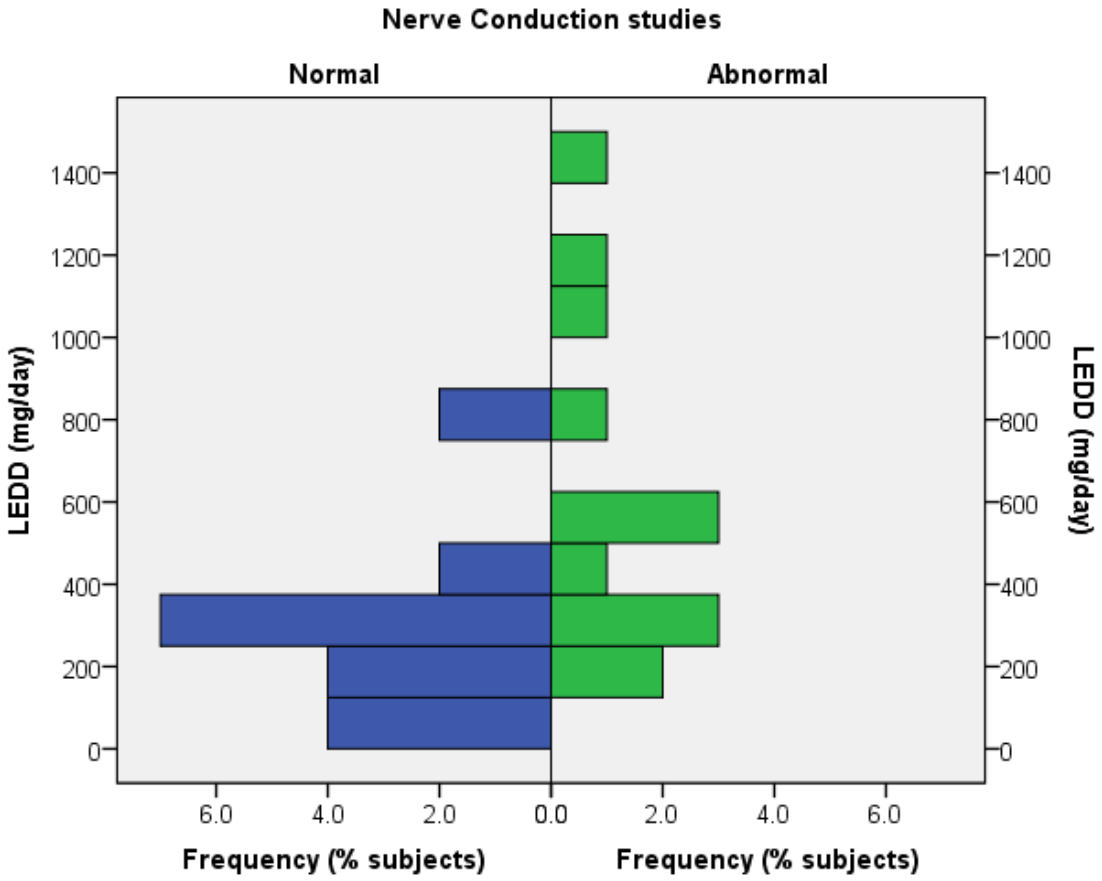

Table 3: Presence or absence of symptoms on SCOPA-AUT subscales for PD and control groups

Symptoms	Parkinson disease (N=36)	Controls (N=22)	Test Statistic	P value 2- sided
Bladder (Yes: No) % present	 33:3 91.7%	 13:9 59.1%	 Fisher's exact	 0.006
Gastrointestinal (Yes: No) % present	 31:5 86.1%	 9:13 40.9%	 X2 (1) = 13.036	 <0.001
Thermoregulation (Yes: No) % present	 24:12 66.7%	 5:17 22.7%	 X2 (1) = 10.545	 0.001
Sexual function (Yes: No) % present	 14:22 38.9%	 9:13 40.9%	 X2 (1) = .023	 0.879
Cardiovascular (Yes: No) % present	 14:22 38.9%	 3:19 13.6%	 X2 (1) = 4.203	 0.040

Table 4: Severity of symptoms on SCOPA-AUT subscales for PD and control groups

Study groups	Parkinson disease (PD) N=36 Mean (SD), Q1, Q3	Controls (C) N=22 Mean (SD), Q1, Q3	Bootstrap independent sample test[a]		
			P – C Mean Difference (Std. Error)	95% confidence interval Lower, Upper	P value 2-tailed
Bladder[b,c]	4.3 (2.7) 2, 6	1.4 (1.7) 0, 2.25	2.9 (0.6)	1.3, 3.0	< 0.001
Gastrointestinal[b,c]	2.9 (2.3) 1.0, 4.0	0.8 (1.1) 0, 1.3	2.1 (0.4)	1.8, 3.97	< 0.001
Thermoregulation[b,c]	2.1 (2.3) 0, 3.75	0.4 (0.9) 0, 0.3	1.7 (0.4)	0.8, 2.5	0.002
Sexual function[b,c]	1.1 (1.6) 0, 2.0	0.6 (0.9) 0, 1.0	0.5 (0.3)	-0.1, 1.1	0.162
Cardiovascular[b,c]	0.8 (1.1) 0, 1.75	0.1 (0.4) 0, 0	0.6 (0.2)	0.3, 1.0	0.008

a: Unless otherwise noted, bootstrap results are based on 5000 stratified bootstrap samples
b: Shapiro-Wilk's test of normality (p< or = 0.001)
c: Levene's test for equality of variance p < 0.05

Questionnaire Scores by Study Group within Gender

When analysed within gender, there remained significant difference in both the male and female subgroups with higher mean scores on SCOPA-AUT, NMS-Quest and BDI questionnaire ($p<0.05$) in the PD group compared to controls. In both groups, there was no association between questionnaire scores and age or gender.

Temperature threshold testing (TTT) abnormalities:

In Table 5 we compared the percentages of our subjects in the PD and controls groups with TTT abnormalities. Hand cold sense and warm sense detected the highest number of abnormalities with higher proportion of abnormal warm sense thresholds in the PD group (p=0.041). The odds of having an abnormal TTT for a PD subject was 4.795 (95% CI: 1.092 to 21.065) times the odds of a control subject having an abnormal TTT, when adjusted for the presence of depression and pain (p=0.038). The presence of an abnormal TTT was not associated with mean scores on SCOPA-AUT, NMS, BDI questionnaire; or the presence of pain on the BPI questionnaire (p>0.05).

Table 5: TTT in PD and Control Groups, derived from normative data ((278) explained in supplemental data)

Thermal thresholds	Parkinson disease N=36	Controls N=21	Test	P value 2-sided
Cold sense				
- hand (Abnormal N (%))	13 (36.1)	5 (23.8)	X(1)= 0.929	0.335
- foot (Abnormal N (%))	3 (8.3)	0 (0)	Fisher's	0.289
Warm sense				
- hand (Abnormal N (%))	10 (27.8)	1 (4.8)	Fisher's	0.041
- foot (Abnormal N (%))	3 (8.3)	3 (14.3)	Fisher's	0.659
Cold pain				
- hand (Abnormal N (%))	7 (19.4)	2 (9.5)	Fisher's	0.461
- foot (Abnormal N (%))	1 (2.8)	1 (4.8)	Fisher's	1.00
Hot pain				
- hand (Abnormal N (%))	5 (13.9)	3 (14.3)	Fisher's	1.00
- foot (Abnormal N (%))	2 (5.6)	0 (0)	Fisher's	0.526

2.5 Discussion

We found a significantly higher prevalence of autonomic neuropathy in our PD subjects compared to controls, using a novel diagnostic approach of SCOPA-AUT questionnaire and TTT. Small fiber dysfunction has been reported in 96.4% of subjects with PD, defined by one abnormal test when evaluated by three modalities: TTT, contact heat evoked potentials and skin biopsy (280). We focused on SCOPA-AUT questionnaire and TTT as they are both non-invasive, readily accessible tests in most neurology departments. We felt that TTT alone was insufficient to diagnose autonomic neuropathy (281) and therefore, we required both symptoms of dysautonomia and abnormal TTT.

Regarding the TTT, we only found a difference in one TTT. The PD group had higher hand warm sense thresholds which has been reported in some prior studies (280,282), but not all (276,277). Despite the inconsistency in the literature, increased sense thresholds seem appropriate, as peripheral autonomic neuropathy is associated with less C sensory nerve fibers, which function to detect temperature. Although following on from this rationale, you would also expect that cold sensation in hands and feet, and warm sensation in feet would be affected too, which we and others have not found (283). Literature on TTT in PD is inconclusive and limited. The differences in TTT results are most likely secondary to variability in methodology and subject characteristics including thermode placement sites, sample sizes and heterogenous PD groups (278). The majority of TTT literature in PD has also focused on those with pain (276,277) and dyskinesia (280).

Prior research has demonstrated correlations between TTTs and H&Y scale (280,282). Our inability to demonstrate a relationship may be due to the large percentage of our PD subjects (86%) having early stage PD, H&Y stage 1 and 2. This lack of relationship between abnormal TTT and our early stage PD subjects suggests that TTT may not be a useful diagnostic biomarker in early PD or in prodromal PD cohorts.

We were also surprised that SCOPA-AUT scores were not associated with the TTT result. We expected to find higher SCOPA-AUT scores in subjects with abnormal TTT, as both results suggest autonomic dysfunction. As we are the first group to look at SCOPA-AUT and TTT, we have no prior data to compare our data with. Although

SCOPA-AUT surveys only autonomic domains, some of these symptoms could be seen with other medical conditions, including depression and pain. Therefore, we included questionnaires on pain and depression so that we could assess for these potential confounders (284). Our PD group had higher SCOPA-AUT scores, if pain was present ($p=0.042$). There was no association between pain and TTT result ($p>0.05$). We also found no relationship between depression and TTT or SCOPA-AUT result ($p>0.05$). However, our analysis for depression was limited by our small sample size with only 6 participants meeting criteria for depression. Ultimately, we thought that the lack of correlation between SCOPA-AUT and TTT was due to the poor sensitivity and specificity of these tests. Future studies comparing our results with more detailed autonomic function tests or skin biopsy, are warranted.

Interestingly, although none of our subjects had symptoms or signs of a large fiber neuropathy on exam, there was electrophysiologic evidence suggestive of it in 40% of our PD group and 18% of our controls, corresponding to an odds ratio of 2.93 in our PD group. Unlike small fiber neuropathy which is thought to be intrinsic to PD, large fiber neuropathy is thought to be related to dopamine therapy and has been reported in up to 55% of PD subjects compared to 8% of controls (285,286). Our results supported this as we found higher LEDD in those subjects with abnormal NCS.

Regarding our secondary outcomes, we found significantly more non-motor symptoms, dysautonomia and pain in our PD group compared to controls ($p<0.01$). Dysautonomia, pain and depressive symptoms are known to be more prevalent in people with PD and are important to investigate for and treat as they impact quality of life (173,287). In regards to symptoms of dysautonomia in PD, the gastrointestinal and urinary domains were more frequently and severely affected, which is consistent with prior literature on non-motor symptoms in PD (82).

Finally, there are some limitations of the present study. First of all, our sample size was small and therefore caution is needed in interpreting the results. A larger study is needed to confirm our results. Secondly, our groups were not gender-matched with the PD group being predominantly male in comparison to the female control group. This gender discrepancy has been seen in other similar studies (276,277). Fortunately, we did not see a difference in TTT or SCOPA-AUT results between gender and using

normative TTT data we could gender-match the data. Thirdly, both our TTT and SCOPA-AUT assessments were subjective in nature, with wide ranges in the normative TTT data and large variability in the severity of autonomic symptoms between individual persons. Although subjective, we chose them as they were non-invasive, accessible and novel. Another limitation to our protocol was that we used 'methods of limits' rather than 'methods of levels' on TTT. 'Methods of limits' is reaction time-dependent and could have introduced inaccuracies in the PD group due to their slower response times (277). Lastly, we were surprised by the electrophysiologic evidence of large fiber dysfunction in our asymptomatic PD and control groups. We did not diagnose these subjects with a large fiber neuropathy as based on AAN parameters, electrodiagnostic studies should not be used alone to make a diagnosis of neuropathy as their sensitivity and specificity is imperfect (288). Further NCS at different sites will be required in these subjects.

To our knowledge, this is the first study to use SCOPA-AUT questionnaire and TTT to diagnose a small fiber neuropathy. Using this multimodal approach, we identified a higher prevalence of autonomic neuropathy in our PD group compared to controls. Future work comparing our approach to more objective tests, in particular autonomic function tests and skin biopsy are warranted. Despite our limitations, our data adds to the current literature available on small fiber neuropathies in PD.

2.7 Supplemental material

Instructions for TTT (modified from (269))

1. Instructions for testing of cold sense threshold

“Please press the button as soon as you feel the slightest change of temperature to ´cold´. This procedure will be repeated a total of 4 times.”

2. Instructions for testing of warm sense threshold

“Please press the button as soon as you feel the slightest change of temperature to ´warm´. This procedure will be repeated a total of 4 times.”

3. Instructions for testing of cold pain threshold (CPT).

“The temperature of the skin will decrease to ´cold´. Eventually a painful component will be added to the sensation of ´cold’, please press the button immediately at the first painful sensation. This procedure will be repeated a total of 4 times”

4. Instructions for testing of heat pain threshold (HPT).

“The temperature of the skin will increase to ´warm´ and a few moments later to ´hot´. Eventually a painful component will be added to the sensation of ´hot’, please press the stop-button immediately at the first painful sensation. This procedure will be repeated a total of 4 times.”

TTT- adjusting our labs values to normative values (modified from (278))

Abnormality in the TTT measurements was determined by utilising the 95% confidence intervals (original unit) for males and females aged 40 or above, published in (269).

1. Hand cold sense- the number of degrees below 32 ^{0}C was recorded as a negative value. Abnormal results were temperature recording more than 4.2^{0}C below the base for women and more than 4.3^{0}C for men.
2. Foot cold sense- the number of degrees below 32 ^{0}C was recorded as a negative value. Abnormal results were temperature recording more than 8.8^{0}C below the base for women and more than 13.6^{0}C for men.
3. Hand warm sense- the number of degrees above 32 ^{0}C was recorded as a positive value. Abnormal results were temperature recordings more than 5.2^{0}C above the base for women and more than 6.1^{0}C for men.

4. Foot warm sense- the number of degrees above 32 ^{0}C was recorded as a positive value. Abnormal results were temperature recordings more than 11.1^{0}C above the base for women and more than 16.7^{0}C for men.
5. Hand cold pain- abnormal, if temperature above 27 ^{0}C in women or above 22.1^{0}C in men.
6. Foot cold pain- abnormal, if temperature above 29.4 ^{0}C in women or above 25.3^{0}C in men.
7. Hand heat pain- abnormal, if temperature less than 37.5 ^{0}C in women or less than 40.1^{0}C in men.
8. Foot heat pain- abnormal, if temperature less than 39.8 ^{0}C in women or less than 42.8^{0}C in men.

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Chapter 3: Cytokine levels in PD CSF samples

3.1 Abstract

Introduction: Currently, there is no diagnostic laboratory test for PD. As the immune system and inflammation play a vital role in the pathogenesis and progression of PD, we investigated cytokine levels in the CSF of PD and control subjects. Our aim was to identify a unique cytokine pattern in PD subjects. We also examined for relationships between cytokine levels and PD characteristics.

Methods and Materials: We analysed the CSF of 18 PD subjects and 18 age-matched controls. Using an ELISA, we quantitatively determined the concentration of nine cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 and TNF-α.

Results: There was no difference in the concentration of each cytokine examined, and no difference in the presence or absence of individual cytokines between the PD and control groups. In a cohort of the PD group, IL-2 levels positively correlated with H&Y scale ($r=0.923$, $n=7$, $p<0.01$).

Conclusion: We did not detect a specific CSF cytokine pattern in our PD subjects. Low levels of cytokines were found in both groups. Higher IL-2 levels were associated with more advanced PD, in a cohort of the PD samples. Future studies investigating cytokine levels should focus on other tissues including blood and brain in PD.

3.2 Introduction

Parkinson disease (PD) is the second most common neurodegenerative disease worldwide. Despite disease pathology being present for a decade before diagnosis (7), the diagnosis of Parkinson disease is often delayed. There is therefore increased interest and effort in identifying biomarkers to diagnose the condition earlier in its course and to understand its underlying pathogenesis. The immune system is thought to play a key role in the pathogenesis and progression of PD (see review in (289–291)). A number of studies have reported circulating antibodies against dopaminergic neurons (292,293), elevated levels of complement proteins (294), increased cytokines and major histocompatibility complex two antigens in people with PD (295). One hypothesis for dopaminergic degeneration in PD is apoptosis due to increased concentrations of cytokines. There are multiple domains of evidence supporting this neuroinflammatory hypothesis from basic science, epidemiological studies and translational research (see review by (291,296–298)).

However, to date there are no inflammatory biomarkers associated with PD. Additionally, the utility of measuring cytokines or in identifying a unique cytokine pattern in people with PD is unknown. A better understanding of the role of cytokines in PD is warranted as they could become a potential diagnostic or prognostic biomarker for PD and be applied in prodromal PD studies or therapeutic trials.

Cytokines are immunological messenger proteins which enable communication between the immune system and the brain; adjust immune responses and monitor immune cell interactions (299). In PD, activated microglial release cytokines which attract both T cells and monocytes to the site and they in turn release more cytokines and induce COX-2 and iNOS expression (300). Post-mortem studies in PD have shown increased density of glial cells expressing IFN-γ, TNF-α, IL-6 and IL-1β in the substantia nigra (SN) of PD subjects compared to controls (301–303).

Cytokine concentrations have been measured in the serum, plasma and CSF of people with PD. However, available data is both limited and inconsistent. A recent meta-analysis of plasma and serum cytokines in PD found increased levels of TNF-α, IL-6, IL-1β, IL-10 and IL-2 in some PD subjects (257). However, other studies reported no difference or even decreased levels in the blood of PD subjects compared to control

groups. Chen et al. observed increased levels of IL-6 but not of TNF-α or other inflammatory markers (259). Dufek et al. saw a decrease in serum mannan-binding lectin and elevated TNF-α in only a subgroup of their PD patients. They found no difference in the levels of serum IL-6, the acute phase reactants or factors of the complement system (304). Hasegawa et al. showed decreased levels of TNF-α, IL-1a, IL-1B and IL-6 by monocyte/macrophages and peripheral blood mononuclear cells in PD (305). A recent study by Choi et al. carried out a multiplex analysis of 22 cytokines and chemokines in the serum and CSF of subjects with PD, AD and controls. They identified no difference in serum cytokine concentrations between any of the study groups (306).

There is even less data available on CSF cytokines in PD, despite CSF felt to more reliably represent the internal milieu of the brain. Furthermore, the majority of CSF data available is from studies in the late 1990s. Blum-Degen et al. found higher levels of IL-1β and IL-6 in the CSF but not in the paired plasma sample (307). Mogi et al. detected elevated concentrations of IL-2 and IL-6 in the ventricular CSF of PD subjects but undetectable levels in the lumbar CSF (308). Nagatsu et al. observed increased levels of TNF-α, IL-1β, IL-2, IL-4 and IL-6 in both the ventricular and lumbar CSF (256). However, Wilms et al. identified no difference in CSF cytokine concentrations in PD subjects compared to controls or other neurological illnesses and inability to induce glial cell activation using CSF from PD subjects (309). Lindqvist et al. recently studied CSF cytokines in subjects with PD and controls but found no difference in their levels except for C-reactive protein which was elevated in subjects with non-motor symptoms and PD-dementia (PDD) (310). Hall et al. evaluated CSF cytokines in controls, subjects with PD, PDD, MSA and PSP and found higher IL-8 concentrations in PD subjects compared to controls ($p<0.05$) (311). Additionally, they reported correlations between inflammatory markers, motor scales, cognitive impairment and neuropsychiatric manifestations (311). Choi et al. measured CSF in their multiplex analysis and could only detect MCP-1, one of the 21 cytokines in the array, and there was no difference in its levels between the groups (306).

Although, current results on cytokine levels in PD are disappointing and inconsistent, we think that ongoing research on cytokines in PD pathogenesis is

important, given the evidence for its role in PD. In human genetic studies, DNA polymorphisms have been identified in inflammatory cytokines and these polymorphisms may modify both the risk of developing PD and the age of onset of PD (297). The Health Professionals Follow-up Study, found that men with the highest quintile of serum IL-6 had 3.4 times higher odds of developing PD compared to men in the lowest quintile, over a mean of 4 years (259).

In our study, we measured cytokines in the CSF of both PD and control subjects. Our aim was to identify a specific cytokine pattern in PD subjects, which could then be used as a diagnostic biomarker. We hypothesised that the concentration of cytokines in the CSF would be higher in PD compared to controls. We also investigated for relationships between PD characteristics and individual cytokine levels.

3.3 Methods and materials

Study design: Participants in this study were recruited from three sites: Cork University Hospital, Cork, Ireland; Santry Orthopaedic Clinic, Dublin, Ireland and Queen Square Hospital, University College London, England. All subjects provided written informed consent for spinal fluid collection. The study was performed in accordance with the provisions of the Helsinki declaration, and the research protocol was approved by the institutional clinical research ethics committee (appendix).

Participants: Both people with PD and age-matched controls were recruited. Our control subjects included both elective and emergency orthopaedic patients undergoing hip or knee surgeries in CUH and Santry Orthopedic Clinic, Dublin. They provided CSF at the time of spinal anaesthesia. They had no known neurological disorders and were not documented to be receiving dopaminergic or anti-inflammatory medications (NSAIDs) at the time of lumbar puncture. Our PD group consisted of subjects with idiopathic PD, diagnosed according to the United Kingdom Parkinson's Disease Society Brain Bank (2), from CUH and Queen Square Hospital. Subjects from CUH were inpatients having a lumbar puncture as part of their diagnostic workup. They gave additional consent for a research sample to be taken. Subjects from Queen Square Hospital were recruited from their Movement disorder clinic and had CSF collected primarily for research and

biobanking. We collected data on disease duration, defined from symptom onset and staging according to Hoehn & Yahr scale (H&Y) (312). All patients were receiving dopaminergic therapy, either levodopa alone or in combination with dopamine agonists. Both groups of participants underwent cognitive testing using MOCA or MMSE. Those who had MMSE completed were converted to MOCA equivalent scores, using a recognised conversion scale (313).

CSF collection, processing and storage: I collected, processed and stored CSF for our CUH patients. Standard operation procedures for CSF were reviewed between the three centers and were similar, if not the same. Lumbar puncture was performed in the morning after an overnight fast. CSF was collected in serial sterile polypropylene tubes and centrifuged at 4000rpm for 10 min at 4°C. The supernatants were aliquoted in 0.5mL samples and frozen at -80°C within 1 hour of collection.

ELISA analysis: I performed initial pilot ELISA analysis with assistance of lab technician. Analysis of cytokine protein levels was performed using MSD multi-spot assay system, V-PLEX Pro-inflammatory Panel 1 (human) kits, catalogue number K15049D which measured the cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 and TNF-α. We followed the manufacturer's instructions and thawed, undiluted CSF specimens were assessed in duplicate. All samples were coded so that the operator was blinded to patient data. Standard range, the lower limit of detection, the intra- and inter-assay coefficients of variation were reviewed for each cytokine and are available on www.mesoscale.com. The lower level of detection (pg/mL) were: IFN-γ=0.05, IL-1β=0.01; IL-2=0.01; IL-4=0.01; IL-6=0.01; IL-8=0.01; IL-10=0.01; IL-12=0.02 and TNF-α=0.01.

Statistical evaluation: Chi-square test was used for comparing categorical outcomes between PD and controls. Continuous outcome measures were summarised using mean (SD), and the comparison of the means between PD and controls was conducted through independent samples t-test. We calculated the Spearman's correlation coefficients to examine the interrelations among these outcomes. All statistical tests

were two-sided, with $p<0.05$ considered statistically significant. Adjustment of Type-1 error for multiple tests was not considered as the primary comparison was cytokine levels between groups. Statistical analyses were performed using IBM SPSS Statistics version 20.

3.4 Results

We consented 21 subjects with PD and 19 healthy controls. We removed three participants in the PD group and one control subject as they had undetectable levels for all cytokines. In Table 1, we presented the characteristics of the remaining PD (18 subjects) and control (18 subjects) groups. There were more men in the control group than in the PD group ($p<0.05$). Mean age and cognition were similar between the two groups ($p>0.05$). The mean disease duration of the PD group was less than ten years with the majority classified as having early disease (H&Y scale 1 and 2).

Table 1: Characteristics of the study participants

Characteristics	PD (N=18)	Controls (N=18)	Statistic	P value
Gender (M: F)	12:6	17:1	Fisher's exact probability	0.088 OR: 8.5 CI: 0.903-80.025
Age (mean ± SD)	63.72 ± 8.74	65.62 ± 11.4	t(34)=0.561	0.578
Cognition on MOCA (mean ± SD)	26.19 ± 2.91	25.67 ± 1.65	t(26.852)=0.670	0.509
Disease duration, yrs (mean ± SD); (10^{th} and 90^{th} percentile)	9.47 ± 6.08 (1.5, 18.2)	N/A	N/A	N/A
Hoehn & Yahr (H&Y) Stage (N (%))	1^{st}= 3 (16.7%) 2^{nd}= 10 (55.6%) 3^{rd}= 2 (11.1%) 4^{th}= 3 (16.7%)	N/A	N/A	N/A

In Table 2, we presented the data on cytokine concentrations in the two groups. We found no difference in the level of each cytokine measured between the PD and control group. The spread of outcome measures was equal in both groups for the majority of cytokines, as seen by insignificant p values for Levene's test. IL-8 had the highest concentrations recorded in both groups. IL-6, IL-8 and IL-10 were detectable in more than 80% of samples. IL-2, IL-4 and IL-12 were detectable in less than 50% of samples. We found no difference between the two groups regarding the presence or absence of each cytokine ($p>0.05$).

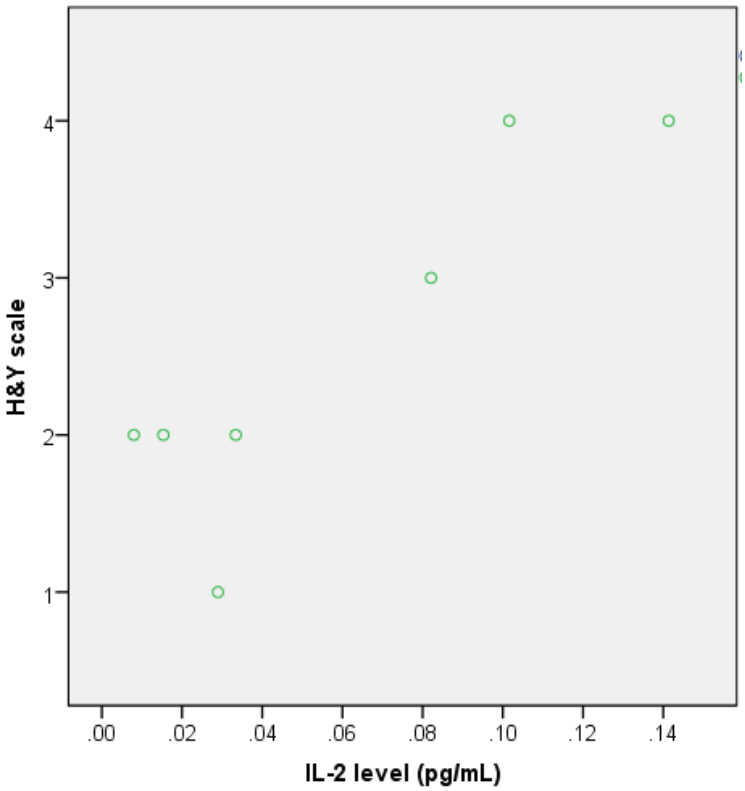
Table 2: Cytokine concentrations (pg/mL) in the PD and control groups

Study groups	Parkinson disease (PD) No. of subjects Mean (SD)	Controls (C) No. of subjects Mean (SD)	Statistic	P value
TNF-α	N=12 0.04 (0.03)	N=16 0.05 (0.04)	t(26)=1.014	0.32*
IL-8	N=17 80.00 (3.10)	N=17 80.32 (3.22)	t(32)=0.295	0.77*
IL-6	N=15 0.92 (0.31)	N=15 1.27 (1.69)	t(28)=0.777	0.45*
IL-10	N=15 0.05 (0.02)	N=15 0.09 (0.14)	t(14.848)=1.117	0.28
IFN-γ	N=11 0.20 (0.12)	N=10 0.31 (0.24)	t(19)=1.239	0.23*
IL-4	N=2 0.01 (0.003)	N=4 0.06 (0.07)	t(4)=1.016	0.37*
IL-2	N=7 0.06 (0.05)	N=4 0.07 (0.09)	t(9)=0.380	0.71*
IL-1β	N=8 0.01 (0.01)	N=11 0.01 (0.01)	t(15.767)=1.516	0.15
IL-12	N=3 21.79 (7.77)	N=6 18.36 (24.3)	t(7)=0.232	0.82*

*= Levene's test for equality of variance was >0.05

To determine whether any variable influenced the detection of cytokines, we assessed whether any of the demographic variables in each group were associated with detection of this cytokine. IL-2 concentration in the PD group correlated strongly with disease severity on the H&Y scale ($r=0.923$, $n=7$, $p<0.01$) (Figure 1). Increased IL-2 levels were seen in more severe disease. Otherwise, we identified no relationship in either group with respect to gender, age, cognition or regarding disease duration in the PD patient ($p>0.05$).

Figure 1: Correlation of IL-2 (pg/mL) and H&Y scale in the PD group



3.5 Discussion

In our study, we were unable to identify a CSF cytokine pattern for PD subjects as there was no difference in the concentration of individual CSF cytokines, and no difference in the presence or absence of individual cytokines between the PD and control groups. The most interesting finding in this study was that IL-2 levels positively correlated with disease severity in the PD group.

Our results add to the current dearth of literature available on CSF cytokine levels in PD. As previously discussed in the introduction, the results on CSF cytokines in PD are inconsistent. In recent months, a meta-analysis of CSF cytokine levels in PD, reported higher concentrations of TGF-β, IL-6 and IL-1B in subjects with PD compared to controls (314). However, this meta-analysis was limited in data, reviewing only three to six, often historic studies for each of the cytokines. The discordant results in the literature and our inability to find elevated levels could be due to differences in our study population or study methodology, including the type of CSF collected and the ELISA used.

Blum-Degen et al. investigated untreated de novo PD subjects, who had a mean age of 61 years and symptoms duration of 0.5 to 3 years (307). Our subjects with PD were on average older, with longer disease duration and receiving dopaminergic therapy. Mogi et al. recorded increased levels of IL-2 and IL-6 in ventricular CSF, but not in the lumbar CSF of their PD subjects. In fact, similar to our results, they were unable to demonstrate a difference in cytokines in the lumbar CSF, with all cytokines except for TNF-α being less than the lower limits of sensitivity of their ELISA (308,315). This difference between ventricular and lumbar CSF is explained by the known rostral-caudal gradient in CSF proteins and neurotransmitters with higher levels detected more rostrally in the ventricular CSF, at the site of protein synthesis compared to distally, in the lumbar CSF (308,316). We tested only lumbar CSF as ventricular CSF would require instrumentation and not be appropriate for our study.

In addition to the levels of individual cytokines, we were also interested in whether there was a difference in the presence or absence of individual cytokines as that difference could also be used as a PD biomarker. We tested for both pro- and anti-inflammatory cytokines as Sawada et al. showed that cytokines produced in the SN by

activated microglia are initially neuroprotective, and become neurotoxic as the disease progresses (317). As most of our PD subjects had early PD, we expected higher concentrations of the pro-inflammatory cytokines. However, we did not detect a significant difference between the PD and control groups and found low cytokine levels in both groups. Our results suggest that CSF cytokines are not a useful biomarker in PD given their low levels in both PD and control subjects, along with their inability to differentiate between the groups.

The only statistically significant finding in our study was the positive association between IL-2 concentration and disease severity on the H&Y scale. IL-2 is a Th1 cytokine produced by activated T cells and is shown to both stimulate and inhibit the immune system (318). Prior studies have shown positive associations between other inflammatory markers: CRP and TNF-α with rates of motor decline (317,319); RANTES associated with H&Y scale and disease duration (320); and IL-1β and IL-2 with rates of cognitive decline (319). As our study was cross-sectional, we were unable to assess progression over time. Our positive association between IL-2 and H&Y scale, although consistent with some of the available literature should be interpreted with caution due to the small sample size (n=7) with undetectable IL-2 in both early and advanced PD subjects. Additionally, in contrast to above, other studies have detected a relationship between lower levels of cytokines and disease progression (305). Müller et al. demonstrated that IL-6 levels inversely correlated with disease severity in PD (321).

Relationships between individual cytokines and disease progression support the neuroinflammatory hypothesis and suggest that anti-inflammatory medications may have a role in slowing disease progression. However, to date, this therapeutic strategy has not been explored, with a recent Cochrane review finding no studies on these medications in PD disease modification (322). Prior to starting an anti-inflammatory clinical trial in PD, it is imperative to have a sensitive and specific marker for the therapeutic agent. Our negative CSF cytokine results, in addition to prior inconsistent data on CSF cytokines in PD suggest that these inflammatory markers, with their current limitations are not useful. However, ongoing research on neuroinflammation is important and should continue, with future research focused on other modalities of

inflammation including genetic polymorphisms of cytokines (297) and [11C](R)-PK11195-PET brain imaging (165–167).

Finally, we would like to acknowledge some limitations in our study. First of all, our sample size was small, although with a roughly equivalent number of participants compared to prior CSF studies on cytokines in PD. Secondly, cytokines were low or undetectable in some patients, despite the use of a sensitive ELISA and following a recommended CSF collection protocol (323). Nonetheless, this impaired detection of cytokines in addition to our sample size could have introduced type 1 or type 2 errors into our results. Perhaps we should have measured serum rather than CSF, as cytokines freely diffuse past the BBB and ELISAs are primarily validated for serum rather than CSF. We intentionally studied CSF as current CSF data is limited and CSF is thought to reflect the internal milieu of the brain better. Finally, although no subjects were documented to be taking anti-inflammatory medications, we did not specifically ask for them as CSF from our collaborator sites was initially collected for other purposes. It is therefore possible that our subjects were taking these medications, on an as needed basis and their use was not documented at the time of enrollment, ultimately affecting cytokine levels (324).

To conclude, neuroinflammation via microglial activation and raised cytokines, is thought to play a crucial role in PD pathogenesis and the ongoing neurodegeneration. Despite our inability to identify a cytokine pattern in our PD subjects, we found that higher IL-2 levels were associated with more severe PD. Our study contributes to the ongoing research investigating neuroinflammation and identifying potential diagnostic and prognostic biomarkers in PD.

Chapter 4: GDF5 levels in PD CSF samples

4.1 Abstract

Introduction: Growth differentiation factor 5 (GDF5) is a potent neurotrophic factor for midbrain dopaminergic neurons. Treatment with GDF5 has been shown to promote survival and neurite growth of these cells *in vitro* and to protect them against neurotoxic insults *in vitro* and *in vivo.* Currently, there is no data on GDF5 levels in humans with Parkinson disease. In this study, we used ELISA to measure levels of GDF5 protein in the CSF of subjects with PD and controls.

Methods and materials: We analysed CSF samples from 21 PD subjects and 22 age-matched controls. Using ELISA, we determined the concentration of GDF5 protein in each sample.

Results: CSF GDF5 concentrations were significantly lower ($p<0.0001$) in the PD group (25.96± 0.11 pg/mL) than in the control group (26.76± 0.72 pg/mL). There was a trend towards more undetectable GDF5 protein in the PD group compared to controls ($p=0.069$). GDF5 levels correlated with gender ($rs(N=14) =0.59$, $p=0.03$), with lower levels measured in female PD subjects than in males ($p=0.027$).

Conclusion: Our study was the first to investigate GDF5 protein in humans with PD. We found lower CSF GDF5 protein levels in subjects with PD compared to controls, providing further evidence for reduced neurotrophic support contributing to neurodegeneration.

4.2 Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that affects 1 to 2 percent of the population over the age of 65 years (3,4). The symptoms of PD are caused by the progressive degeneration of midbrain dopaminergic (mDA) neurons and their axons projecting to the striatum, and the accumulation of α-synuclein in Lewy bodies and neurites that spread throughout the nervous system (264). The loss of this nigrostriatal pathway leads to a reduction in striatal dopamine levels and progressive motor impairments upon which the clinical diagnosis of PD is based (2,7). However, misdiagnosis occurs in up to 24% of cases (262). There has been an intensive research effort aimed at understanding the molecular mechanisms of this degeneration, on developing biomarkers for diagnosis and monitoring of disease progression, and at developing neuroprotective strategies (reviews (17,18)). One group of proteins that have been extensively studied in this regard are neurotrophic factors of the transforming growth factor (TGF)-β superfamily (325,326). Neurotrophic factors are involved in the growth, development, function and regulation of neurons.

One such neurotrophic factor is growth differentiation factor 5 (GDF5), which is a member of the BMP subgroup (bone morphogenetic protein (BMP)-14) of the TGF-β superfamily (327). Treatment with recombinant human (rh)GDF5 has been shown to increase mDA neuron survival and neurite growth *in vitro* (328–332). Moreover, intracerebral administration of rhGDF-5 protects against neurotoxic insults *in vivo* (333–335) and can improve the survival of transplanted fetal mDA neuronal grafts (336,337), in animal models of PD. These data show that GDF5 is a potent neurotrophic factor for mDA neurons. To our knowledge, there have been no studies to date that have examined GDF5 expression in the human brain, or investigated whether the expression of GDF5 is altered in PD.

In our study, we carried out a case-control study to examine the levels of GDF5 protein in CSF samples obtained from PD patients and matched controls. We used CSF rather than serum samples, as CSF is in direct contact with the brain and therefore may more accurately reflect pathologic processes in the brain. Given that GDF5 is a potent neurotrophic factor for mDA neurons, we hypothesised that there would be lower levels of GDF5 protein in the PD group than in age-matched controls.

4.3 Methods and materials

Study design: Participants were recruited to this study from three sites: Cork University Hospital, Cork, Ireland; Santry Orthopaedic Clinic, Dublin, Ireland and Queen Square Hospital, University College London, England. All subjects provided written informed consent for spinal fluid collection. The study was performed in accordance with the provisions of the Helsinki declaration and the research protocol was approved by the institutional clinical research ethics committee (appendix).

Participants: Participants were people with PD and age-matched controls. Our controls were healthy subjects who provided CSF at the time of spinal anaesthesia. They had no neurological disorders and had no documented dopaminergic or anti-inflammatory medications at the time of lumbar puncture. Our PD group consisted of subjects with idiopathic PD diagnosed according to the United Kingdom Parkinson's Disease Society Brain Bank (UKPDSBB) (2). We collected data on disease duration, defined from symptom onset and staging according to Hoehn & Yahr scale (H&Y) (312). All patients were receiving dopaminergic therapy, either levodopa alone or in combination with dopamine agonists. All subjects underwent cognitive testing using MOCA or MMSE. Those who had MMSE completed were converted to MOCA equivalent scores, using a recognised conversion scale (313).

CSF collection, processing and storage: Lumbar puncture was performed in the morning after an overnight fast. CSF was inspected to ensure that it was free of visual contamination by blood. CSF was collected in serial sterile polypropylene tubes and centrifuged at 4000rpm for 10 min at 4°C. The supernatants were aliquoted in 0.5mL samples and frozen at -80°C within 1 h of collection.

ELISA analysis: Analysis of GDF5 protein levels was performed using Cusabio, catalogue number CSB-EL009349HU kit according to the manufacturer's instructions. Thawed, undiluted CSF specimens were assessed in duplicate. All samples were coded so that the operator was blinded to patient data. Standard range of GDF5 was 28 to 1800 pg/mL.

The lower limit of detection (LLOD) was 7 pg/mL. Intra- and inter-assay coefficients of variation were CV%<8 and CV%<10, respectively.

Statistical evaluation: The statistical analyses were performed using IBM SPSS Statistics version 20.4.1. As the PD group showed wider scatters of the outcome distributions (as seen by significant p values for Levene's test), we log transformed the data and carried out t-test again to confirm results. We also performed a resampling analysis by using 5000 stratified bootstrap samples. Adjustment of Type-1 error for multiple tests was not considered as the primary comparison was GDF5 levels.

4.4 Results:

Table 1 shows the characteristics of the 43 subjects included in this study. We analyzed clinical and CSF data from the PD (21 subjects) and control (22 subjects) groups. There were more women in the PD group than in the control group ($p<0.05$). 61.9% of the PD group were at early H&Y stage of disease (H&Y 1 and 2) and mean disease duration of the group was less than ten years. There were no differences in age or cognition between the two groups.

Table 1: Characteristics of the study participants

Characteristics	PD (N=21)	Controls (N=22)	Statistic	P value
Gender (M: F)	14:7	21:1	Fisher's exact probability	0.021 OR: 10.500 CI: 1.161-94.925
Age (mean ± SD)	64.6 ± 8.5	64.9 ± 11.0	t(41)=0.1	0.915
Cognition on MOCA (mean ± SD)	26.3 ± 3.1	26.0 ± 1.7	t(30.79)=-0.4	0.669
Disease duration, yrs (mean ± SD); (10^{th} and 90^{th} percentile)	9.7 ± 5.7 (2, 17.6)	N/A	N/A	N/A
Hoehn & Yahr (H&Y) Stage (N (%))	1^{st}= 3 (14%) 2^{nd}= 10 (47.6%) 3^{rd}= 4 (19%) 4^{th}= 4 (19%)	N/A	N/A	N/A

GDF5 levels in CSF samples (Table 2, Figure 1)

Figure 1 illustrates the levels of GDF5 protein in the PD and control groups. GDF5 was below the limit of detection in 7 PD subjects and 2 controls (p=0.069). In detectable samples, GDF5 levels (pg/mL) were significantly lower in the PD group (25.96± 0.11) than in the control group (26.76± 0.72) (p<0.0001). When male gender was analyzed, there continued to be a significantly lower level of GDF5 protein in the PD group (26.00± 0.08) than in the controls (26.76± 0.72), (p<0.001). When data was log transformed and bootstrapped, the GDF5 difference remained statistically significant (p<0.001). Further analysis was not done on female gender as there was only one female in the control group.

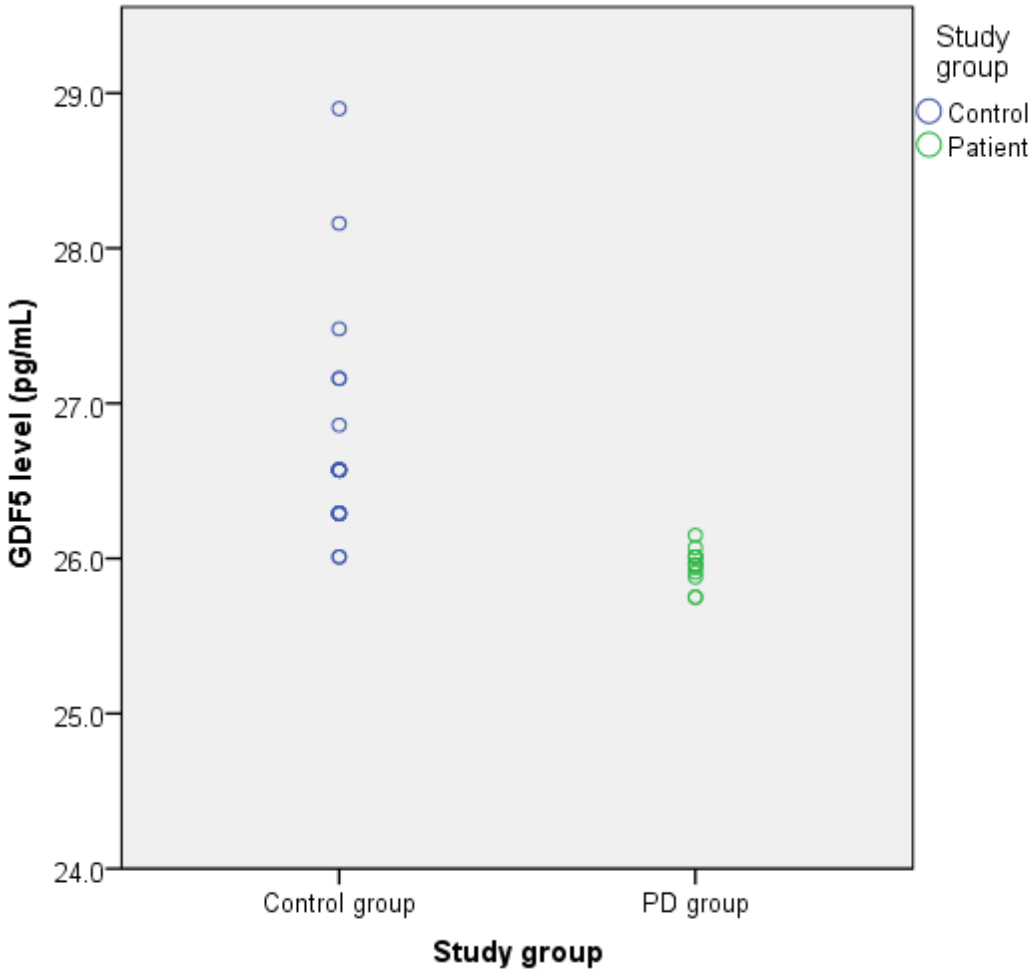
Table 2: GDF5 protein levels in CSF samples

Marker	PD Mean ± SD Median (Q1, Q3)	Controls (C) Mean ± SD Median (Q1, Q3)	Statistic	P value
GDF5[a,b]	N=14 25.95 ± 0.11 25.96 (25.9, 26.0)	N=20 26.76 ± 0.72 26.57 (26.3, 27.1)	t(20.24)= -4.91	<0.0001

a: All GDF5 measurements shown are in pg/mL.

b: Levene's test of equality of variances 0.002

Figure 1: Scatterplot of GDF5 levels (pg/mL)



Relationships amongst GDF5 level and individual variables (Table 3 and 4)

In each group, we found no relationship between GDF5 level and age or cognition ($p>0.05$). In the PD group, GDF5 level was not associated with H&Y scale or disease duration ($p>0.05$). GDF5 level was associated with gender in the PD group ($rs(N=14) =0.59$, $p=0.03$). Both males (26.00± 0.08) and females (25.87± 0.11) in the PD group had lower GDF5 levels than controls (26.76± 0.72). Within the PD group, the female subjects had significantly lower GDF5 than males ($t(12)=2.514$; $p=0.027$).

Table 3: Correlations in PD group between variables

	1.	2.	3.	4.	5.
1. Age	-	-.043	.717*	.652*	-.334
2. Cognition	-	-	-.055	.025	.048
3. Disease duration	-	-	-	.681*	-.296
4. H&Y scale	-	-	-	-	.156
5. GDF5	-	-	-	-	-

*= $p <0.01$

Table 4: Correlations in Control group between variables

	1.	2.	3.
1. Age	-	-.021	-.150
2. Cognition	-	-	-.213
3. GDF5	-	-	-

4.5 Discussion:

The lack of biomarkers in PD and the recognition of the role of neurotrophic growth factors in PD led us to investigate GDF5. To our knowledge this is the first study to explore GDF5 levels in human subjects. We found a significantly lower level of CSF GDF5 protein in subjects with PD than in controls. There was also a trend towards more samples in the PD group having undetectable GDF5 or levels less than the lower limit of detection. This lower level of GDF5 in our PD group seems appropriate as reduced neurotrophic support has been proposed as a cause and mediator of

neurodegeneration (338). Yet, this reduction in neurotrophic factor has not been seen with other members of the TGF-β superfamily (339).

Although limited there has been some research on other members of the TGF-β superfamily in the human brain and cerebrospinal fluid (CSF). Using real-time qPCR, increased levels of glial cell line-derived neurotrophic factor (GDNF) isoform 1 was found in the putamen of PD subjects compared to controls (340). However, other studies using less sensitive assays have not reproduced these results, finding no difference in GDNF mRNA expression in the dorsomedial prefrontal cortex (341), the mesencephalon and striatum of postmortem brains (342,343) or in the CSF of PD subjects compared to controls (339). No differences were detected in the CSF levels of persephin (PSPN). Neurturin (NTN) was undetectable in the CSF of both PD and control groups (339).

Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), members of a different family of growth factors, the neurotrophin family, have also been studied in PD. Low levels of BDNF have been recorded in the SN (344,345) and serum of PD subjects (346). BDNF levels in PD have positively correlated with disease duration, H&Y scale and motor symptoms including worsening balance (346). NGF was also found to be lower in the serum of early PD (H&Y 1 and 2) but not in later stages compared to controls (347,348).

To date, there has been no published data on GDF5 mRNA or protein levels in humans. Our research group has investigated GDF5 mRNA expression in rat models and found increased striatal expression and unchanged midbrain expression of GDF5, ten days after injury with 6-OHDA lesioning (349). However, longitudinal data past 28 days was not studied and therefore we do not know if the GDF5 levels would subsequently decrease after the acute insult (349).

We did not show a relationship between detectable GDF5 level and disease duration, H&Y scale or cognitive impairment. Some prior studies, although not all (350) have found a positive correlation between these variables and the serum level of neurotrophic factors, BDNF (346,351) and NGF (348). They postulated that lower levels of neurotrophic factors result in the disease pathogenicity and thus are expected in early disease, whereas later in the disease, higher levels of neurotrophic factors are

produced as a compensatory mechanism (346,350). Possible explanations for not observing this relationship in our study include a type 2 error due to either our small sample size or insensitive ELISA. Also, our PD group was relatively uniform in regard to disease duration and cognition, with the majority having early stage disease and no to minimal cognitive impairment.

We did, however, find an association between GDF5 levels and gender in the PD group with lower levels in the females. GDF5 levels and gender have not been investigated before. Our results are consistent with other studies showing gender-specific differences in neurotrophic factors. BDNF with or without depression (352–354), NGF and Neurotrophin-3 (NT-3) in depression (355) have been shown to be lower in females compared to their male counterparts.

Despite our results, further studies examining CSF GDF5 levels in larger cohorts are warranted given that it is the first study of CSF GDF5 protein and our GDF5 protein level (~26 pg/ml) was just below the ELISA's standard range in both groups (28 pg/ml). However, it is important to note that we are confident in our results given the narrow standard deviation in GDF5 protein levels in our PD subjects (figure 1), and the fact that the GDF5 levels that we report are approximately four times higher than the lower limit of detection.

Our study is subject to several limitations. First of all, the sample size was small and thus may have caused type one or type two errors in our results. Secondly, our groups were not gender-matched and therefore we were unable to fully analyze the gender differences in GDF5. Fortunately, when the males in each group were compared, the significant results persisted. Thirdly, our ELISA was not able to detect GDF5 protein in all samples. This may be due to physiologically low expression of GDF5 protein in the CSF or due to poor sensitivity of the ELISA. The ELISA that we used has been validated only in the serum and thus we have no CSF data to compare our results with.

Our study supports the concept of reduced neurotrophic factor support in PD pathogenesis. To the best of our knowledge, our study is the first to investigate CSF GDF5 protein levels in humans and demonstrate lower concentrations in subjects with

PD. Ultimately, this study should be replicated in larger study cohorts and across other sites to ensure its validity.

Chapter 5: DaTSCAN imaging in PD

5.1 Abstract:

Introduction: Dopamine transporter scans are FDA approved as a diagnostic biomarker in the diagnosis of clinically undefined Parkinsonism. Our aim was to assess the indications for imaging usage and its impact on future clinical management in the Irish health service.

Methods and materials: Retrospective review of scans ordered and their corresponding results over a five-year period. A chart review was carried out on a cohort of scans to assess changes in clinical management.

Results: One hundred and eighty scans (69% of total) were reported as showing evidence of dopaminergic deficit. A chart review in 81 patients showed a change in clinical management in 53 patients (65%). Scans were ordered inappropriately in 34 patients (13%).

Conclusions: ^{123}I-FP-CIT SPECT scans are being more frequently ordered and if used correctly can alter clinical management. Increased education on indications for use is required to reduce waste of resources and risk to patients.

5.2 Introduction

Parkinson disease (PD) is the second most common neurodegenerative disease and is characterised by the presence of bradykinesia plus one of rigidity, tremor, or postural instability (2). Misdiagnosis rates from 10 to 50% have been found using clinical exam alone, when compared to the gold standard pathological diagnosis (262,356,357). The most common PD mimics include tremor disorders, drug-induced Parkinsonism (DIP), vascular parkinsonism (VP) and Parkinson-plus conditions. The prognosis and management of each disorder differs significantly from PD, and from each other. Therefore, the ability to distinguish between different parkinsonian entities is of clinical importance, allowing for optimal treatment and avoiding unnecessary therapeutic trials or other tests.

In-vivo functional imaging of dopamine transporters (DAT) can improve diagnostic accuracy in atypical cases of Parkinsonism. DaTSCAN, which is the trade name for striatal presynaptic dopamine transporter imaging using ^{123}I-FP-CIT [(123)I-N-omega-fluoropropyl-2beta-carbomethoxy-3beta-nortropane] Single Photon Emission Computed Tomography [SPECT], has been licensed by the European Medicines Agency (EMA), The Society of Nuclear Medicine (SNM), and the US Food and Drug Administration (FDA) for certain indications (Table 1) (111,118). Reductions in ^{123}I-FP-CIT SPECT striatal uptake is demonstrated to have 95% sensitivity and 95% specificity with a high positive predictive value for identifying parkinsonian syndromes (PS) (111). ^{123}I-FP-CIT SPECT initial imaging results have been remarkably consistent with the clinical diagnoses made at three years follow-up (117,357). Almost 100% concordance has been found between Neuroradiologists on interpreting this imaging (357,358). The Parkinsonian syndromes (PS) which include PD, Multiple System Atrophy (MSA), Progressive Supranuclear Palsy (PSP), and Corticobasal Degeneration (CBD) show nigrostriatal degeneration on DaTSCAN neuroimaging. Other conditions, such as essential tremor (ET), DIP, vascular parkinsonism (VP), psychogenic parkinsonism, normal aging, normal pressure hydrocephalus, and dystonic tremor may demonstrate features of parkinsonism, but do not have nigrostriatal degeneration on neuroimaging (111). In the appropriate clinical setting (such as where the differential diagnoses being queried include a neurodegenerative Parkinsonism versus another mimic disorder),

^{123}I-FP-CIT SPECT can be a very useful investigation. However, as each scan costs approximately €1,200 per patient, this is a resource that should not be used routinely.

Table 1: Indications for DaTSCAN as per FDA, EMA and SNM guidelines (111,118)

4. Differentiate Essential tremor from parkinsonian disorders
5. Differentiate Dementia with Lewy bodies from Alzheimer's disease
6. Distinguish drug-induced parkinsonism from parkinsonian disorder

Our primary aim was to evaluate the current use of ^{123}I-FP-CIT SPECT in our health service as a diagnostic biomarker for parkinsonian syndromes. We reported on the indications for ordering ^{123}I-FP-CIT SPECT along with assessing trends of referral by different specialties. We investigated for correlations between dopaminergic deficit on imaging and demographics or symptomatology. We identified inappropriate referrals and assessed the impact of these scans on subsequent clinical management.

5.3 Methods and materials

Study design: A retrospective review of ^{123}I-FP-CIT SPECT request forms and their corresponding results over a five-year period from 2008 to 2013 in two tertiary care hospitals, Cork University Hospital [CUH], Cork, Ireland and University Hospital Limerick [UHL], Limerick, Ireland. Patients in two tertiary care hospitals who underwent ^{123}I-FP-CIT SPECT over this five-year period were included in the study. No additional exclusion or inclusion criteria were applied. Scans were carried out as per each institution's protocol and in accordance with international guidelines. Patients were instructed to discontinue all potential confounding medications prior to scan. All scans were read by experienced radiologists who were aware of clinical history and differential diagnoses as documented on referral. Demographics including gender, age at scan, symptoms, medications, and scan report details including indication for scan, referring specialty, and institution were manually gathered. Inappropriate referrals were defined as referral indications not approved by FDA, SNM, or EMA guidelines and included differentiating PD from other PS, dystonia, vascular parkinsonism, dementia, and unknown along with assessing progression (see Table 1 for recommended indications

for scan). We reviewed all available handwritten charts in our two hospitals in order to evaluate the utility of scans for future clinical management.

Study Ethics was received from both the Clinical Research Ethics Committee of the Cork Teaching Hospitals and the Research Ethics Committee of UHL prior to the initiation of the study (appendix).

Statistical analysis: Data was inputted into SPSS version 20.4.1. Two groups were formed for statistical analysis: those with and without dopaminergic deficits. Descriptive statistics, frequencies along with Pearson's chi-squared test were used.

5.4 Results

Patient demographics and referral sources

Two hundred and sixty-one patients underwent ^{123}I-FP-CIT SPECT over a five-year period. One hundred and forty-eight (56.7%) were male and median age was 67 years (Table 2). The number of scans ordered increased every year with the most scans completed in the final full calendar year. Scans were predominantly ordered by neurologists (54.4%), geriatricians (34.5%) and psychiatrists (6.1%). Thirteen scans were referred from other specialties including general medicine (n=6), rheumatology (n=2), respiratory (n=1), nephrology (n=1), gastroenterology (n=1) and emergency department (n=1). Fifty-five percent (55%) of scans were outside referrals, ordered by physicians working outside of our two hospitals.

Table 2: Demographics of DaTSCANs ordered

Number of patients	261
Age at scan, years	
Mean (SD)	65.6 (12.22)
Median (interquartile range)	67.0 (58.5 - 75.5)
Min, Max	24, 91
Gender, n (%)	
Female	113 (43.4%)
Male	148 (56.7%)
Mean (median) follow-up after scan in years at time of chart review	4.9 (5.0)

Referral reason

The most common reason for ordering a scan was for assessment of a parkinsonian syndrome (PS), accounting for 62.5% of referrals. Other frequent referral reasons were differentiating drug-induced Parkinsonism (DIP) from PS at 17.2% and PS vs. PD at 7.3%. Inappropriate referrals were seen in 13% of cases (Table 3).

Table 3: ^{123}I-FP-CIT SPECT indications along with results

^{123}I-FP-CIT SPECT indications	Number (% of all ^{123}I-FP-CIT SPECT scans)	% of ^{123}I-FP-CIT SPECT scans showing reduced striatal dopamine transporter signal
Parkinsonian syndrome	163 (62.5%)	71.8
Lewy body dementia	7 (2.7%)	57.1
Drug-induced vs PS	45 (17.2%)	57.8
PD vs ET	12 (4.6%)	50
PD vs dementia	2 (0.8%)	50
PS vs PD	19 (7.3%)	89.5
PD progression	2 (0.8%)	100
PD vs vascular PD	8 (3.1%)	62.5
PD vs dystonia	2 (0.8%)	50
Unclear	1 (0.4%)	100

Symptomatology

We were interested in the documentation of parkinsonism, in particular the symptoms needed for The United Kingdom Parkinson's Disease Society Brain Bank Clinical Diagnostic (UKPDSBB) criteria for diagnosing Parkinson disease (2). One hundred and thirty-three patients (51%) had a tremor, 88 patients (33.7%) had rigidity, 69 patients (26.4%) had bradykinesia and 5 patients (1.9%) had postural instability documented on their request forms for imaging.

123I-FP-CIT SPECT results and subgroup analysis

One hundred and eighty patients (69% of total) had positive scans with dopaminergic deficit qualitatively assessed. Seventy-seven (42.7%) of these scans showed correct laterality between symptom sidedness and dopaminergic deficit on imaging. Thirty-two scans (17.9%) showed bilateral dopaminergic deficits in the presence of unilateral symptoms. Twenty-one scans (11.8%) showed dopaminergic deficit on the incorrect side to unilateral symptoms. In the remaining fifty scans (27.5%), the symptom sidedness was not documented on the referral forms. When comparing the demographics and symptomatology of those with evidence of dopaminergic deficit against those with a normal scan, no statistically significant difference was found regarding age, gender, indication for scan or symptoms ($p>0.05$).

Change of management

This was assessed by review of handwritten medical notes available in our two hospitals. Eighty-one charts were available for review. Forty-three (53%) of these scans were ordered by neurologists; 25 (30.9%) ordered by geriatricians; 7 (8.6%) ordered by psychiatrists and 6 (7.4%) ordered by general medical physicians. Documentation of further management was noted in 53 of these charts (65.4%). Twenty-five patients (30.9%) had a change of diagnosis from ET or DIP to PS. Seventeen patients (21%) were started on new medications or had an increase in medication doses after confirmation of diagnosis. Eleven patients (13.5%) had either discontinued treatment or didn't start planned medication**.** Regarding specialties, changes in management were noted in 65.9% of neurology (27 patients), 94.7% of geriatrics (18 patients), 50% of psychiatry (2 patients) and 100% of general medicine (6 patients) referrals.

5.5 Discussion

Over the five-year period the number of scans ordered almost quadrupled, from 21 scans in the first year to 79 in the final year. We think this reflects the increased awareness of the utility of ^{123}I-FP-CIT SPECT in diagnosing parkinsonian syndromes (PS). Many of our patients (62.5%) were referred for this scan in cases of PS, although it was often unclear from the referral forms what other diagnoses were being considered in addition to PS. The second most common indication was for DIP versus PS (17.2%).

Prior studies report DIP accounting for 24 to 51% of cases of parkinsonism (359). DIP can present similar to PS with rest tremors or asymmetric parkinsonism (14,359). DIP is important to identify as withdrawal of the offending drug can reverse the symptoms of parkinsonism (237). ^{123}I-FP-CIT SPECT scans are ideal for these patients as neuroleptics predominantly affect the postsynaptic dopamine receptors with only a negligible affinity for the dopamine transporter (DAT) (360,361). In our study, 26 patients (57.8%) with a psychiatric diagnosis or prescribed neuroleptics were found to have evidence of dopaminergic deficit on neuroimaging, supporting a diagnosis of PS rather than DIP. Despite being susceptible for DIP, these patients were also at risk for PS given their older age, with 88.5% of them, greater than 60 years of age. The DaTSCAN results in these cases could be presymptomatic PS with subclinical SN degeneration. Research has suggested that antidopaminergic medications, like neuroleptics can unmask subclinical SN degeneration, resulting in overt parkinsonism (14,359).

Inappropriate referrals for ^{123}I-FP-CIT SPECT are important to identify and prevent as they are a waste of resources and cause an unnecessary risk to patients without benefit (111,362). We found inappropriate referrals in 34 cases (13% of total) resulting in an estimated cost of €48,000 euros to the health service. Documented reasons for ordering the scan included differentiating PS from PD; PD from dystonia; PD from Dementia with Lewy Bodies (DLB); or assessing progression in PD. Although ongoing research is investigating variant mapping techniques of ^{123}I-FP-CIT SPECT and other biomarkers for these reasons (111), they are currently not clinical indications for the scan and are not licensed by the EMA, SNM and FDA guidelines (table 1). Positive scans (i.e. evidence of qualitative dopaminergic deficit) were seen in 69% of cases. Although not evaluated in our study, there is an age-related decline of radiotracer uptake in normal patients of 3.3 to 10% per decade (363), making the interpretation of results in an older age group more difficult.

Eighty-one scans (31% of total) were normal with no evidence of dopaminergic deficit. Possible diagnoses for normal scans include ET, dystonia, dementia not related to SN degeneration, vascular parkinsonism, DIP and psychogenic parkinsonism. SWEDDs (Scans Without Evidence of Dopaminergic Deficit) is a controversial term

used to described subjects with parkinsonism and normal DaTSCANs. It is now commonly associated with dystonia or dystonic tremor but can be associated with a variety of etiologies (364).

As 55% of patients were referred from outside institutions, we were limited in our chart review. However, we were able to assess change of management in patients under the care of neurologists, geriatricians, psychiatrists, and general medical physicians. A change in management after ^{123}I-FP-CIT SPECT was clearly documented in 65% of our chart review subgroup. This was consistent with a recent multicenter, open, non-randomized study which showed change in planned management in 72% of their patients after DaTSCAN (358). Another retrospective review, reported a change in management in 63% of cases (361). Interestingly in three of our patients, the scans results were not accepted by the ordering physician suggesting some uncertainty in the scan's validity.

The overdiagnosis of PD at initial presentation occurs in 10 to 47% of patients in both community and hospital settings (11,365). This misdiagnosis of PD is more likely with non-specialists compared to Movement disorder experts (115). In our study, only three scans were requested by a recently appointed Movement disorder specialist, supporting early referral of patients with parkinsonism to specialists. Prior research had scans ordered solely by neurologists (366). However, patients with parkinsonism can present to any specialty and the feasibility and cost-effectiveness of restricting the ordering of these scans to only neurologists or Movement disorder experts is debatable (367).

Given the retrospective nature of the study, the large percentage of outside referrals to our centers for these scans and the reliance on handwritten scan request forms and chart reviews for data collection there were some limitations to this study. We were only able to assess change of management in one-third of scans due to either unclear documentation or inability to access the handwritten medical records.

Dopamine transporter scan is a diagnostic biomarker which is increasingly being used in the clinical and research setting. In fact, the EMA has recently endorsed its application in PD clinical trials (119). Given its limitations and expense, it is not feasible to use it for the clinical diagnosis in all patients with parkinsonism. However, it is a

useful biomarker in challenging cases, especially early in the disease when signs are minimal; when atypical features are present or when there are other comorbidities. In our study, we showed increased awareness and utility of dopamine transporter scans in diagnosing parkinsonian syndromes in our health service. We found that dopamine transporter imaging can assist with diagnosis and change clinical management, if used for the correct indications. We also identified a small, yet significant number of inappropriate referrals. These referrals will be important to address in the future, in order to reduce the waste of resources and prevent unnecessary radiation exposure to patients. Potential solutions include better education of the medical community or listing strict indications for the scan on the request forms.

Chapter 6: Conclusion

6.1 Summary of results

In this thesis, we completed several case-control studies investigating clinical, radiological and biological biomarkers in Parkinson disease. We identified several differences between the subjects with PD and controls.

In the first study, we detected a higher prevalence of autonomic neuropathy in PD subjects using a novel approach of SCOPA-AUT questionnaire and temperature threshold testing to diagnose autonomic neuropathy. We also found a trend towards more large fiber neuropathy in the PD group than in controls, with increased prevalence of neuropathy in those subjects on a higher levodopa-equivalent daily dosage (LEDD), a finding previously reported in the literature. Other non-motor symptoms, including depression, pain, gastrointestinal disturbances and urinary dysfunction, were more prevalent in PD subjects than in controls. Potentially, this approach of SCOPA-AUT questionnaire and temperature threshold testing could be used in clinical practice to diagnose autonomic or small fiber neuropathy and thus avoid skin biopsy or more labour-intensive autonomic function testing.

In the second study, we failed to identify a distinct cytokine pattern in the PD group. There was no difference in the concentration of each cytokine examined, and no difference in the presence or absence of individual cytokines between the PD and control groups. Interestingly, in the PD group we found a strong correlation between IL-2 levels and disease severity, with higher IL-2 levels associated with more severe disease on the H&Y scale. Our results suggest that CSF cytokine levels are not useful in diagnosing PD. However, the association of IL-2 with disease severity is intriguing and suggests a possible role for anti-inflammatory medications in hastening disease progression.

In our third study, we measured GDF5 protein levels in the CSF of both PD subjects and controls. We found a significantly lower concentration of GDF5 protein in the CSF of the PD group compared to controls. GDF5 levels in PD subjects correlated with gender, with higher levels seen in males. There was no relationship between GDF5

levels and disease duration or disease stage. Our results suggest a potential role for GDF5 protein in neuroprotective strategies, although further studies are needed to replicate this finding.

Finally, we found that DaTSCAN, a proposed radiological biomarker for diagnosing PD can be useful when applied correctly in diagnostically-challenging cases of parkinsonism. In our chart review, we observed a change in clinical management in two-thirds of patients. However, there was also evidence of inappropriate referrals in a small but significant number of cases. Increased education on the use of this biomarker in clinical practice is warranted in order to reduce waste and risk to patients.

6.2 Strengths of these studies

There are several strengths in our research studies. First of all, we reviewed a myriad of potential biomarkers in diverse domains including clinical, biological and radiological. We chose these biomarkers as the testing equipment was readily available in our department and therefore could be used for both our research and potential future research or clinical settings. Secondly, we collaborated extensively both inside and outside our institution, which increased the expertise levels used in our study. It also broadened the applicability of our results to the general population. We worked with other members of the CUH Neurology department to identify potential subjects with PD; with the Neurophysiology department in training for our NCS and TTT; and with the Anesthesiologists and Orthopedic teams for identifying potential controls for our CSF studies. We collaborated with Limerick Regional Hospital, Limerick for our DaTSCAN study and with St James Hospital, Dublin, and Queen Square Hospital, University College London, England for our CSF studies. Using the recently created Parkinson's Disease Research Cluster (PDRC) at University College Cork (UCC), Ireland, we collaborated with other PD researchers in CUH and UCC. We also participated locally in our community with the PD society in Cork city. Thirdly, we applied a novel approach to diagnosing autonomic neuropathy using the validated SCOPA-AUT questionnaire and TTT. Lastly, to the best of our knowledge, we are the first group to study GDF5 protein levels in the CSF of people.

6.3 Limitations of our studies

However, we are also aware of some limitations in our studies. Firstly, our sample size for the clinical and biological biomarkers' studies was relatively small. At the initiation of this MD, there was no established database of PD patients and no biobank of biological samples in Cork. Therefore, all subjects were freshly recruited from the community or clinics and enrolled into these studies; or attained through new collaborations with other institutions. Due to the small sample sizes, we may have missed clinically significant results or conversely, seen associations that would not be replicated in larger studies. Secondly, although collaboration is important for research and allowed us to develop relationships with other institutions and ultimately increase our sample sizes, it may have introduced confounding variables into our CSF samples. We tried to reduce sample variabilities by ensuring that the collection, processing and storage protocols were similar between institutions. Thirdly, the ELISAs applied in our CSF studies were not specifically validated for CSF. Although cytokines and GDF5 protein were detectable in most samples, they remained at very low levels. This may have been due to their low concentrations in the lumbar CSF, as seen in other studies, or due to poor sensitivity of these ELISAs for CSF, confounding our results. Other limitations are related to those that have been previously recognized in other biomarker studies, including the recruitment of non-PD subjects into the PD group; PD is a heterogeneous disorder and the different phenotypes are not equivalent; and lastly, the assessment of relatively non-specific markers which are also present in control subjects and the elderly.

6.4 Future research directions

Biomarkers and biomarker discovery are areas of increased interest and active research in PD. Identifying a sensitive and specific biomarker is essential for better understanding of the disease pathogenesis; more rapid and correct diagnosis; informing prognosis in PD; monitoring disease progression; and evaluating disease-modifying effects of new therapies in clinical trials.

In this thesis, we evaluated clinical, biological and radiological biomarkers; and identified differences in subjects with PD, in all of these individual domains. However,

there was often overlap in these markers between the two groups. In future research, we expect that all of these avenues will continue to be explored and that a multimodal approach is likely to result in the most sensitive and specific biomarker for PD.

Our clinical biomarker study on autonomic neuropathy in PD identified a higher prevalence of autonomic neuropathy in PD subjects compared to controls. However, we were surprised by the lack of association between SCOPA-AUT and TTT and thus, more research is warranted comparing the predictive value of our approach using TTT and SCOPA-AUT with that of more formalized autonomic function testing, skin biopsy, or possibly, cardiac SPECT imaging. As previously mentioned, using our approach, there was overlap between subjects with PD and controls in symptoms of dysautonomia, suggesting that dysautonomia on its own is too nonspecific as a clinical biomarker. Other clinical biomarkers that are actively being investigated are REM sleep behavior disorder, olfactory loss and technological applications for detecting subclinical motor impairments. Using the MDS prodromal PD criteria, it is now possible to calculate an individual's pretest probability for developing PD (19).

The study of biological biomarkers in PD is a minefield with an endless list of candidate markers currently being measured. Research on neuroinflammation and neurotrophic factors is critical given the clear laboratory and epidemiological evidence of both neuroinflammation and depletion of neurotrophic support in PD pathogenesis, along with the availability of potential therapies i.e. NSAIDs, immunomodulator drugs and injection of growth factors. Based on our research and others, we think that the study of cytokine levels is too inconsistent to be pursued further, at least as a sole biomarker. Multiple studies have failed to identify a reliable inflammatory marker in the serum or CSF. Other modalities, specifically PET imaging may provide a better marker for inflammation and be more suited for monitoring inflammation in PD.

In regard to neurotrophic factors, our study on CSF GDF5 protein expression is the first to examine its levels in humans and in subjects with PD. The identification of a new neurotrophic factor for further investigation in subjects with PD is intriguing, especially as GDF5 protein has been shown *in vitro* and *in vivo* to protect dopaminergic neurons. Our research group continues to investigate GDF5's role in PD. Our results will need to

be replicated in larger human cohorts. If GDF5 is consistently shown to be lower in subjects with PD, therapeutic trials could be considered in the future.

The study of biological biomarkers requires the availability of large well-characterized cohorts with standardized collection procedures, in order to reduce the inconsistent results currently seen in the literature, presumably secondary to differences in study population and methodology. We also think that unbiased screening of hundreds or thousands of markers at once will be more beneficial than the current “candidate biomarker approach” which targets a single marker due to its known involvement in the pathophysiology of PD. Fortunately, there are now several biobanks and international collaborations, making it possible to carry out this type of research, as detailed in the introductory chapter.

Lastly, the utility of DaTSCAN imaging in PD as a biomarker continues to be explored. As seen in our study, DaTSCANs are increasingly being used in the investigation of parkinsonism. The recent endorsement by the EMA for the use of DaTSCANs in clinical trials in PD is exciting for biomarker research. SURE-PD3, a phase 3 clinical trial in PD has included two DaTSCANs in its protocol (368). The first scan acts as a diagnostic biomarker, confirming dopaminergic deficit and excluding SWEDDs; and the second scan at the end of the trial investigates its use as a prognostic biomarker. Nevertheless, ongoing research on DaTSCAN imaging is warranted. The current qualitative nature of reporting DaTSCANs is too subjective and thus, both objective striatal-binding ratios and machine-algorithms are being studied (369,370). It is also an expensive test with limited specificity, being unable to differentiate idiopathic PD from other parkinsonian syndromes. This limitation is significant as these parkinsonian syndromes have different pathologies and prognosis.

The long-term aim is to have biomarkers that can recognize or corroborate the presence of pre-clinical or clinical disease, assess disease severity, and predict disease prognosis. By identifying these biomarkers, we will then be better equipped in the most important mission which is to discover effective neuroprotective therapies for PD.

Chapter 7: References

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Chapter 8: Appendices

8.1 Ethical approval for conduct of 'Autonomic neuropathy in PD study' (chapter 2)

UCC
Tel: + 353-21-490 1901
Fax: + 353-21-490 1919

COISTE EITICE UM THAIGHDE CLINICIUIL
Clinical Research Ethics Committee
Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

Our ref: ECM 4 (d) 04/09/12

9th August 2012

Dr Sean O'Sullivan
Consultant Neurologist
Department of Neurology
National Neuroscience Centre
Cork University Hospital
Wilton
Cork

Re: An assessment of the involvement of pain and autonomic symptoms in patients with parkinsonism.

Dear Dr O'Sullivan

Expedited approval is granted to carry out the above study at:

- Cork University Hospital.

The following documents have been approved:

- Application Form
- Invitation Letter
- Consent Form (Remove the word "sample" for title of consent form)
- Detailed Protocol
- The Brief Pain Inventory
- Mini-Mental State Examination
- Scopa-aut
- Beck Depression Inventory.

The co-investigator involved in this study will be:

- Dr Sean O'Dowd

Yours sincerely

Dr Michael Hyland
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

Cork University Hospital
14 AUG 2012
Neurology Department

8.1 Ethical approval for conduct of 'DaTSCAN imaging in PD' (chapter 3)

UCC

Tel: + 353-21-490 1901
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Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee

Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

Our ref: ECM 4 (iii) 09/01/13

17th December 2012

Dr Sean O'Sullivan
Consultant Neurologist
Cork University Hospital
Wilton
Cork

Re: A clinical audit of DaTSCAN use in the CUH.

Dear Dr O'Sullivan

Expedited approval is granted to carry out the above study in:

- Cork University Hospital.

The following documents have been approved:

- Application Form
- Data Collection Sheet.

We note that the co-investigators involved in this study will be:

- Oisin O'Corragain, Medical Student.

Yours sincerely

Dr Michael Hyland
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

8.1 Ethical approval for conduct of 'DaTSCAN imaging in PD' (chapter 3)

Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

HSE West,
University Hospital,
Dooradoyle,
Limerick, Ireland.

Tel: 00353 (0) 61 301111
Fax: 00353 (0) 61 301165
Website: www.hse.ie

28th April, 2014.

Dr. Grace Crotty,
Neurology Movement Disorder Research Fellow,
UCC.

Re/ Protocol Title
A Clinical Audit of DaTSCAN Use in the University Hospital Limerick.

Dear Dr. Crotty,

The Research Ethics Committee at the University Hospital Limerick has received a submission for ethical approval for the above study.

The following documents were reviewed and approved by the Research Ethics Committee:

Application to the Research Ethics Committee *Approved*

From an insurance perspective, please note that cover does not extend to those parties not employed by the Health Service Executive (HSE), or non-HSE Institutions.

Yours sincerely,

Fionnuala O'Brien,
Clinical Programmes Co-Ordinator,
(For and on behalf of the Research Ethics Committee & the Risk Management Department).

8.1 Ethical approval for conduct of 'Cytokine and GDF5 levels in PD CSF samples' (chapter 4 & 5)

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COISTE EITICE UM THAIGHDE CLINICIUIL
Clinical Research Ethics Committee
Lancaster Hall,
6 Little Hanover Street
Cork
Ireland
Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

Our ref: ECM 4 (x) 12/06/12

11th May 2012

Dr Sean O'Sullivan
Consultant Neurologist
Cork University Hospital
Wilton
Cork

Re: Potential biomarkers for Parkinson's disease: comparison of levels of neurotrophic factors in cerebrospinal fluid and serum of Parkinson's disease patients with those in healthy controls

Dear Dr O'Sullivan

Expedited approval is granted to carry out the above study in:

- Cork University Hospital
- University College Cork.

The following documents were approved:

- Signed Application Form
- Detailed Protocol
- CV for Chief Investigator
- Consent Form Version 1 dated 15th March 2012
- Participant Invitation Letter
- GP Letter

We note that the co-investigators involved in this study will be:

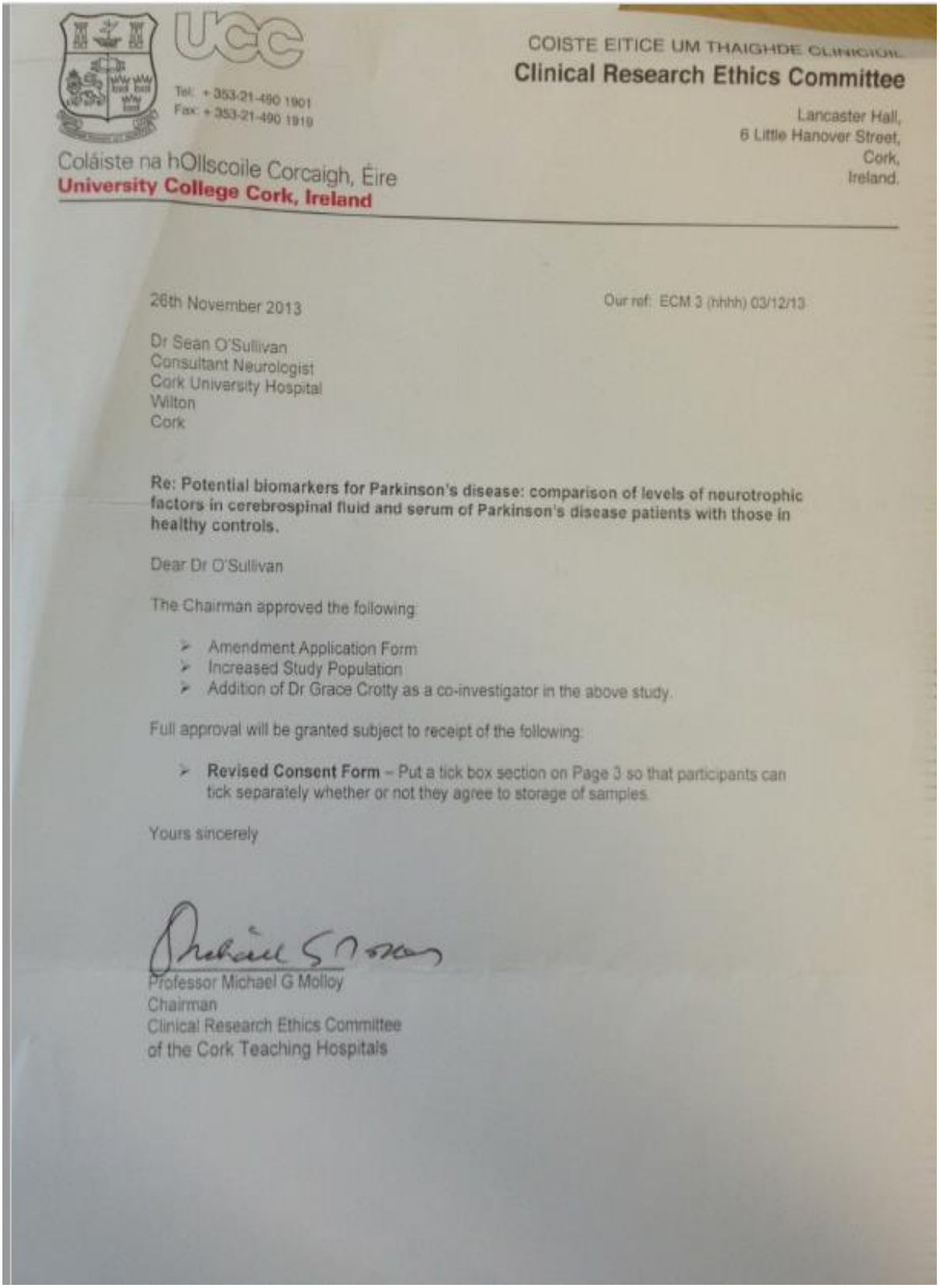
- Dr Aideen Sullivan, Dr Daniel Costello and Dr Gerard O Keefe.

Yours sincerely

Dr Michael Hyland
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

The Clinical Research Ethics Committee of the Cork Teaching Hospitals, UCC, is a recognised Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004, and is authorised by the Department of Health and Children to carry out the ethical review of clinical trials of investigational medicinal products. The Committee is fully compliant with the Guidelines as they relate to Ethics Committees and the conditions and principles of Good

8.1 Ethical approval for conduct of 'Cytokine and GDF5 levels in PD CSF samples' (chapter 4 & 5)



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University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIUIL
Clinical Research Ethics Committee
Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

26th November 2013

Our ref: ECM 3 (hhhh) 03/12/13

Dr Sean O'Sullivan
Consultant Neurologist
Cork University Hospital
Wilton
Cork

Re: Potential biomarkers for Parkinson's disease: comparison of levels of neurotrophic factors in cerebrospinal fluid and serum of Parkinson's disease patients with those in healthy controls.

Dear Dr O'Sullivan

The Chairman approved the following:

- Amendment Application Form
- Increased Study Population
- Addition of Dr Grace Crotty as a co-investigator in the above study.

Full approval will be granted subject to receipt of the following:

- **Revised Consent Form** – Put a tick box section on Page 3 so that participants can tick separately whether or not they agree to storage of samples.

Yours sincerely

Professor Michael G Molloy
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

8.2 Publication and published abstracts:

Crotty GF, O'Corragain OA, Bogue C, Crotty J, O'Sullivan SS. The Utility of Dopamine Transporter Scans for Diagnosing Parkinsonian Disorders. Ir Med J. 2018 May; 111 (5).

Published abstracts:

- Crotty G, Vaughan D, Moloney G, O'Keeffe G, O'Sullivan SS, Sullivan A. Evaluation of CSF cytokine profiles in people with Parkinson disease and age-matched controls. [abstract]. *Mov Disord.* 2017; 32 (suppl 2).
- Crotty G, O'Dowd S, McNamara B, Coleman B, O'Sullivan SS. Autonomic dysfunction and pain perception in patients with Parkinson's disease compared to healthy controls. *Mov Disord*. 2014;29 Suppl 1 :749
- Kao T, Crotty G*, O'Sullivan SS. Prevalence of non-motor symptoms amongst people with Parkinson's disease compared to controls. *Mov Disord.* 2014;29 Suppl 1 :500
- O Corragain O, Crotty GF*, O'Sullivan SS. A clinical audit of DaTSCAN use in Cork University Hospital. *Mov Disord*. 2014;29 Suppl 1 :229.

Chapters 1,3 and 4 are prepared manuscripts for submission, currently undergoing co-authours' reviews, prior to submission

Chapter 2 submitted to journal, currently awaiting journal review