The Role of Corneal Confocal Microscopy in Parkinson's Disease and Atypical Parkinsonism

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List of Abbreviations

[^{18}F] fluorodeoxyglucose	FDG
[^{18}F] fluorodopa	F-dopa
^{123}I-metaiodobenzylguanidine	MIBG
Addenbrooke's Cognitive Examination-Revised	ACE-R
Area under the receiver operating characteristic curve	AUC
Cerebrospinal fluid	CSF
Charged couple device camera	CCD
Corneal confocal microscopy	CCM
Corneal nerve branch ensity	CNBD
Corneal nerve fibre area	CNFA
Corneal nerve fibre density	CNFD
Corneal nerve fibre length	CNFL
Corneal nerve fibre width	CNFW
Corneal total nerve branch density	CTBD
Dopamine active transporter	DAT
Eccrine glands	EG
Heidelberg Retina Tomograph Rostock III Corneal Module laser scanning confocal microscope	HRT III CCM

Intraclass correlation coefficients	ICC
Intraepidermal nerve fibre density	IENFD
Magnetic resonance imaging	MRI
Montreal Cognitive Assessment	MoCA
Movement Disorder Society Unified Parkinson's Disease Rating Scale	MDS UPDRS
Multiple system atrophy	MSA
Neuropathy Disability Score	NDS
Parkinson's disease	PD
Parkinson's Disease Questionnaire -39	PDQ-39
PD-related metabolic pattern	PDRP
Pilosebaceous units	PSU
Positron emission tomography	PET
Postural instability/gait difficulty	PIGD
Progressive supranuclear palsy	PSP
Protein misfolding cyclic amplification	PMCA
Rapid eye movement	REM
Real-time quaking-induced conversion	RT-QuIC
Receiver operating characteristic curve	ROC
Single-photon emission computerized tomography	SPECT
Slit-scanning confocal microscope	SSCM
Spinous cell layer	SCL
Standard deviation	SD
Standard error of the mean	SEM

Survey of Autonomic Symptoms	SAS
Tandem scanning confocal microscope	TSCM
Technology based objective markers	TOMs
Tremor dominant	TD
Unified Parkinson's Disease Rating Scale	UPDRS

Abstract

Several neuropathological studies have demonstrated alpha synuclein deposition and small fibre denervation in skin biopsies of participants with Idiopathic Parkinson's disease (PD), suggesting the possibility of using small fibre loss as a biomarker in idiopathic PD. Whilst estimation of nerve fibre density with a skin biopsy offers an objective means of quantifying small fibre pathology, it is invasive, time-consuming, costly and not repeatable. Corneal Confocal Microscopy (CCM) has been shown to be a valid technique to detect small fibre neuropathy non-invasively and reliably.

Cross sectional studies in small cohorts of PD participants have demonstrated corneal nerve loss in PD participants compared to healthy controls. This thesis aims to further investigate the role of CCM as a biomarker in PD by using larger sample sizes, comparing manual and automated analysis techniques, assessing longitudinal change in CCM parameters and comparing corneal nerve morphology between participants with PD, progressive supranuclear palsy (PSP) and multiple system atrophy (MSA).

This thesis established that CCM using automated analysis detects corneal nerve loss in PD participants. Manual and automated analysis have good correlation. The longitudinal study did not demonstrate any significant change in CCM parameters after 12 months but found that PD participants with the most corneal nerve damage at baseline had greater motor progression after 12 months. A comparison of corneal nerve loss between PD, MSA and PSP participants showed differential nerve loss, with marked changes seen in PD and MSA and less nerve loss in PSP.

CCM may be a useful tool in identifying PD participants who are faster motor progressors to enrich clinical trials and differentiate between PD, PSP and MSA participants.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Dedication

I dedicate this thesis to my family whose humour, love and support have sustained me for as long as I can remember.

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Firstly, I would like to thank my supervisory team, Professor Monty Silverdale and Dr Christopher Kobylecki for their support and encouragement over the past three and a half years. I have learnt a great deal about research and thinking outside the box from them and I have been very lucky to have had such supportive, inspiring supervisors. I am indebted to Professor Rayaz Malik for his expertise in corneal confocal microscopy and I would also like to thank Maryam Ferdousi and Alise Kalteniece for their corneal confocal microscopy input during the course of the research.

It has also been a privilege to have met all the research volunteers who were very generous with their time. I was very impressed with their commitment and interest in Parkinson's disease research. They are a reminder of the reason we are doing research in the first place.

The research in this thesis has been made possible by grants and many people who are acknowledged in the relevant sections of each manuscript.

Rationale for submission in journal format

The chapters in this thesis address separate areas in the development of corneal confocal microscopy as a biomarker in Parkinson's disease. Each manuscript is a stand-alone piece of work but together they form a coherent body of research addressing gaps in the literature. Submitting in journal format has facilitated prompt submission/publication of research as the work was coming through. Thus, it has been agreed with my supervisory team that submission in journal format is appropriate. The following chapters have been published/ submitted or are pending submission for publication.

Chapter 3: Automated corneal nerve analysis: A rapid and reproducible technique to quantify neurodegeneration in participants with Parkinson's disease: Submission pending

Chapter 4: Corneal confocal microscopy detects small fibre neurodegeneration in Parkinson's disease using automated analysis: Published in Scientific Reports, 2020

Chapter 5: Corneal confocal microscopy identifies a more severe disease phenotype in Parkinson's disease: Published in Movement Disorders, 2021

Chapter 6: Corneal confocal microscopy shows different degrees of nerve loss in atypical Parkinsonian disorders: Submission pending to Parkinsonism and Related Disorders

1. Introduction

1.1. The Definition and Diagnosis of Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. PD has traditionally been defined by three cardinal clinical signs: bradykinesia, resting tremor and rigidity. The identification of pathological changes and the recognition of genetic mutations has added a level of complexity to the way we define PD.

Pathologically, PD is characterised by the presence of Lewy bodies in dopaminergic cells of the substantia nigra (Gibb and Lees, 1988; Hughes *et al.*, 1992) A pathological premortem diagnosis is not commonly made in PD as it is not practical in life. Although Lewy bodies are the neuropathological hallmark of the disease, the relationship between Lewy bodies and the neurodegenerative process is not clear. Most studies that have attempted to correlate Lewy body density with disease duration, onset and severity have not demonstrated any significant correlation successfully (Schulz-Schaeffer, 2010). Schneider et al who recently reviewed the neuropathology of genetic synucleinopathies with Parkinsonism found that Lewy bodies may be present in syndromes clinically distinct from Parkinson's such as mitochondrial membrane protein associated neurodegeneration, and not in all genetic forms of Parkinson's disease. Most reported autopsies in Parkin mutations do not have Lewy body pathology (Schneider and Alcalay, 2017)

The discovery of alpha synuclein, the pathological protein aggregated in neurones, neurites, presynaptic terminals and glia of patients with PD, and the definition of PD as a synucleinopathy has helped us to better understand the non-motor manifestations and progression of PD. PD is no longer viewed as a motor disorder characterised by bradykinesia, rigidity, tremor and postural instability caused by degeneration of the dopaminergic striatonigral pathway, but a multisystem disease secondary to widespread distribution of alpha synuclein (Schulz-Schaeffer, 2010). Braak et al developed a neuroanatomically based staging scheme which postulates that Lewy body pathology begins in the medulla oblongata and

olfactory bulb. The pathology then progresses in a caudal to rostral manner, eventually extending into the cerebral cortex (Braak *et al.*, 2003). Non motor symptoms may be correlated to the presence of Lewy bodies in defined regions (Harding *et al.*, 2002; Harding, Broe and Halliday, 2002) supporting the concept that alpha-synuclein pathology spreads sequentially through anatomical structures in the brain (Schulz-Schaeffer, 2010).

The clinical and pathological manifestations of PD have shaped the way we currently diagnose PD. However, much work remains to be done to elucidate the pathogenetic processes that underlie the disease, for us to fully understand the concept of PD and refine our diagnostic methods. The search for biomarkers that can establish the presence of disease, differentiate different PD subtypes and act as a marker of its progression will also change the way we diagnose and monitor PD in the future.

1.2. Biomarkers in Parkinson's disease

1.2.1. What is a biomarker?

A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"(Robb, Mcinnes and Califf, 2016). Biomarkers can be further subdivided as markers of trait, state and rate (Fox and Growdon, 2004), although there may be overlap as to which of these roles a biomarker may fulfil. A measure of disease trait is a marker that confers susceptibility to the disease such as a genetic mutation that predicts likelihood of developing PD (Fox and Growdon, 2004). A marker of disease state is a diagnostic marker or a marker of disease subtype and/or severity (Chahine and Stern, 2017). Rate biomarkers are used to monitor disease progression, prognosticate disease and measure the effects of therapeutic intervention (Fox and Growdon, 2004).

Theoretically a single biomarker may act as a measure of state, trait and rate. However, it is unlikely that a single biomarker will act as a predictive marker, a diagnostic marker and a marker of progression. It is more likely that separate biomarkers or integration of several biomarkers will be necessary to fulfil the three separate roles (Fox and Growdon, 2004).

1.2.2. Why do we need biomarkers in Parkinson's disease?

1.2.2.1. Biomarkers to monitor disease progression

The major unmet need in the treatment of PD is the development of a drug that will alter the course of the disease. There are several obstacles in developing a neuroprotective agent. One of the major problems involve the scales/measures that are employed in trials to monitor the effects of therapy on disease progression.

Clinical Scales

Clinical trials often employ clinical scales to monitor disease progression and as measures of primary endpoints. There have been several unsatisfactory outcomes from clinical trials designed to look for disease modifying effects. Motor scores could not satisfactorily differentiate the symptomatic effects of treatment from the rate of disease progression (Kempster Peter, 2016). The Unified Parkinson's Disease Rating Scale (UPDRS) is the most widely used scale in clinical trials. However, there are concerns about the sensitivity of UPDRS in monitoring participants with mild disease. UPDRS is not ideally configured to assess mild disease (Movement Disorder Society Task Force on Rating Scales for Parkinson's Disease, 2003). There are also issues with inter- and intra-rater reliability. Post et al demonstrated that inter-rater differences in the assigned UPDRS score can be as large as 16 points (Post *et al.*, 2005). Some clinical trials are designed to assess whether administration of a trial drug changes the UPDRS score significantly compared to placebo over a period of time. The Pioglitazone in early PD: phase 2 trial is an example of such a study. The null hypothesis was that Pioglitazone

reduces the mean UPDRS decline over 44 weeks by 3 points or more compared with placebo (NINDS Exploratory Trials in Parkinson Disease (NET-PD) FS-ZONE Investigators, 2015). If inter-rater variability can be as large as 16 points, UPDRS is not the ideal scale for measuring small changes such as 3 points. In an attempt to address this issue, the Movement Disorder Society developed a revised version of the UPDRS called the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) to try to better capture subtle differences in motor features and non-motor features (Goetz *et al.*, 2008) . In addition, Horvath et al tried to establish a threshold for the smallest change of MDS-UPDRS motor scores that would be clinically meaningful, termed minimal clinically important difference (MCID). They suggested that -3.25 points was the cut-off for detecting minimal but clinically meaningful improvement and 4.63 points was the cut-off for detecting minimal but clinically meaningful worsening (Horváth *et al.*, 2015). Some authors argue that non motor features and the activities of daily living subsection of the UPDRS may better reflect disease progression than the motor sub scores (Harrison *et al.*, 2009).

The frustrations of clinical scales as a measure of disease progression have led to a recognised consensus that there is a need to identify reliable biomarkers to track disease progression in order to make a breakthrough in drug development. Michael J Fox Foundation initiated the Parkinson's Progression Marker Initiative (Parkinson Progression Marker Initiative, 2011) aimed at developing biomarkers that can more accurately assess progression of disease. Michael J Fox Foundation also have an 'Improved Biomarkers and Clinical Outcome Measures Program' to support research that will develop biomarker tools, which funded the research in this thesis.

Imaging

Radiotracer imaging of nigrostriatal dopaminergic function is a technique that has received attention as a potential biomarker to monitor disease progression, but this method is also fraught with issues. A variety of different positron emission tomography (PET) and single-photon emission computerized tomography (SPECT) ligands have been used. These include ligands that measure cerebral glucose metabolism ([^{18}F]fluorodeoxyglucose (FDG)), cerebral blood flow (N-isopropyl-P [123I]-iodoamphetamine), pre-synaptic dopaminergic function related to fluorodopamine synthesis and storage ([^{18}F]fluorodopa (F-dopa)) or dopamine active transporter (DaT) function (McGhee *et al.*, 2013).

Several clinical trials of putative neuroprotective treatments in PD have used F-dopa PET and DaT SPECT as primary or secondary endpoints (Parkinson Study Group, 2002; Whone *et al.*, 2003). Unfortunately, interpretation of imaging data is complicated because pharmacological treatment can affect the targets of ligands. Dopamine agonists for example have significant pharmacologic effects on DaT regulation and changes in imaging findings may be separate from the effects of disease progression (Ahlskog, 2003).

FDG PET has been used to identify metabolic patterns as a marker of response to treatment (Feigin *et al.*, 2007). However, most studies have been small in sample size and conclusions are hard to draw (McGhee *et al.*, 2013; Chahine and Stern, 2017). Radiotracer imaging is also expensive and not widely available.

Different magnetic resonance imaging (MRI) techniques have been studied to measure disease progression. Using diffusion MRI and a computational bitensor model, a pattern of elevated free water in the substantia nigra of PD patients that progresses over time has been shown (Ofori *et al.*, 2015). In a multi-site cohort study using data from the Parkinson's Progression Marker Initiative study, Burciu et al reported an increase in free water in the posterior substantia

nigra of PD participants and no change in controls over a one-year period. The authors also found an increase of free water in PD participants over four years (Burciu *et al.*, 2017). In addition to studies using diffusion MRI, longitudinal studies using functional MRI have demonstrated a decline in functional activity in PD participants compared to controls over time (Burciu *et al.*, 2016). The results from MR-based PD biomarkers require further validation and replication (Chahine and Stern, 2017). Many research based MRI techniques are not available in clinical practice, so the logistics of dissemination will also need to be considered.

Biofluids

Markers from biofluids have been explored as potential rate biomarkers. Cerebrospinal fluid (CSF) alpha synuclein has been studied as a biomarker of disease progression (Majbour *et al.*, 2016). However, the data is limited, and a recent study reported no correlation with longitudinal MDS-UPDRS scores or DaT scans (Mollenhauer, Caspell-Garcia, *et al.*, 2019).

Uric acid has been studied as a biomarker based on its antioxidant properties and decreased levels of uric acid in the substantia nigra have been demonstrated in PD (Church and Ward, 1994). Schwarzschild et al studied serum urate as a predictor of clinical and radiographic progression in PD. This study demonstrated that there is an inverse relation between uricemia and PD progression in a longitudinal study. They also found that the rate of progression in PD declined with increasing levels of baseline serum urate (Schwarzschild, 2008). Whilst these findings may have modest clinical utility in predicting the rate of progression in an individual newly diagnosed with PD and may aid design of future neuroprotective drugs targeting oxidative stress pathways (Schwarzschild, 2008), urate levels on their own are unlikely to be a useful biomarker for monitoring the effects of drug treatment in clinical trials as there are many confounding factors that can affect its levels (male sex, obesity, hypertension).

Neurofilament light chain (NFL) has also gained interest as a potential rate biomarker in PD. Neurofilaments are highly phosphorylated cytoskeletal proteins of neurons and are released into extracellular fluid in response to axonal damage (Gaiottino *et al.*, 2013). Higher NFL concentrations in CSF and blood have been associated with greater severity of motor symptoms and shorter survival in PD (Bäckström *et al.*, 2020; Ye *et al.*, 2021). In addition, longitudinal increase in serum NFL has been shown to significantly correlate with MDS UPDRS total scores and some cognitive measures, suggesting a potential role for neurofilament light chains to be a biomarker of disease progression (Mollenhauer *et al.*, 2020).

Technology based objective markers

Technology based objective markers (TOMs) are potentially attractive rate markers. There is a wide array of technologies available for objective motor testing including 'wearable' sensors, mobile communications and cloud computing. Current 'wearable' technologies are used to capture frequency and amplitude of movements and the fluctuations of balance and gait impairments with varying degrees of success. The challenges in utilizing TOMs to monitor disease progression in clinical research include distilling and translating the large volumes of data collected into clinically meaningful outcomes (Espay *et al.*, 2016).

1.2.2.2. Biomarkers to detect disease presence

Diagnosing PD correctly is not only important for patients' clinical management and prognostication but also for the progress of science via clinical, epidemiological and pharmacological studies. Currently, PD is predominantly a clinical diagnosis and misdiagnosis is common. A systematic review and meta-analysis of the accuracy of clinical diagnosis of PD performed by Rizzo et al demonstrated that the accuracy of clinical diagnosis by non-specialists was 73.8% (95% credible interval (CrI) 67.8%- 79.6%) and accuracy of clinical diagnosis by movement disorder specialists was 79.6% (95% CrI 46%-95.1%) at initial assessment (Rizzo

et al., 2016). The advent of biomarkers that can reliably differentiate idiopathic PD from its mimics will avoid improper labelling and management.

Failure to develop neuroprotective therapies in PD is due to multiple factors. Identification of the right study population is crucial (Stocchi and Olanow, 2013). Most studies recruit patients who have been diagnosed with PD. One of the possible causes of failure to develop neuroprotective agents is due to the fact that by the time PD is diagnosed, irreversible and extensive neuronal loss has already occurred. Pathological and imaging studies indicate that neuronal loss occurs five to ten years before the clinical manifestations of PD (Marek and Jennings, 2009). Kordower et al demonstrated a 50-90% loss of neurones from the earliest time points of clinical diagnosis and by 4 to 5 years post diagnosis, there was almost total loss of dopamine markers in the putamen (Kordower *et al.*, 2013). Therefore, the development of biomarkers that can identify the pre-diagnostic phases of PD is crucial for trialling interventions that may alter the course of the disease.

Imaging

The ideal diagnostic imaging biomarker for PD should differentiate PD from healthy controls as well as other forms of Parkinsonism. Reduced binding of presynaptic DaT ligand has been widely used to detect nigrostriatal dysfunction (Saeed *et al.*, 2017). However as nigrostriatal terminal loss occurs in PD and PD mimics such as MSA and PSP, the commonly used DaT SPECT technique is unable to differentiate PD from atypical Parkinsonian disorders (Kim *et al.*, 2002).

FDG PET, which detects spatial patterns of metabolic dysfunction, on the other hand has been shown to be able to differentiate different forms of Parkinsonism (Hellwig *et al.*, 2012). A PD-related metabolic pattern (PDRP) identified using FDG PET has also been reported in participants with rapid eye movement (REM) sleep behaviour disorder, a prodromal state

(Holtbernd *et al.*, 2014). However, sample sizes have been small and replication by other groups is required (Chahine and Stern, 2017).

Another imaging marker that has been explored is the detection of nigrosome 1 using susceptibility weighted high resolution MRI (Schwarz *et al.*, 2014). Small clusters of dopaminergic cells within the substantia nigra, described based on calbindin D_{28K} negativity on immunohistochemical staining, termed nigrosomes, have been described (Damier *et al.*, 1999). The largest nigrosome, identified as nigrosome 1, positioned in the caudal and medio-lateral substantia nigra has been demonstrated using susceptibility weighted MRI at high field strengths of 7T and 3T and termed the swallow tail sign' due to the configuration of the substantia nigra (Blazejewska *et al.*, 2013; Schwarz *et al.*, 2014). The swallow tail sign has been found to be present in healthy controls and absent in PD participants (Schwarz *et al.*, 2014). 3T susceptibility weighted MRI has been used to study the discriminatory value of the swallow tail sign between PD and atypical parkinsonism. The ability of the swallow tail sign to discriminate between PD and atypical parkinsonism was marginal with AUC values of 0.56 and 0.68 (Meijer *et al.*, 2016) .

Biofluids

Different antibody assays measuring different alpha synuclein species have been developed and used on biofluids such as blood and plasma. Significantly lower CSF total alpha synuclein levels and higher CSF concentrations of oligomeric alpha synuclein and phosphorylated alpha synuclein compared to healthy controls have been reported (Eusebi *et al.*, 2017). However, the sensitivity and specificity of CSF total and oligomeric alpha synuclein have been shown to be sub-optimal which limits the utility of both alpha synuclein species as standalone diagnostic biomarkers (Wang *et al.*, 2015) . In terms of differentiating PD from other forms of atypical Parkinsonism, studies have found no significant difference in total, oligomeric or

phosphorylated alpha synuclein levels between participants with PD and other synucleinopathies or atypical Parkinsonism (Eusebi *et al.*, 2017).

More recently, two novel protein amplification assays, Protein Misfolding Cyclic Amplification (PMCA) and Real-Time Quaking-Induced Conversion (RT-QuIC) have been applied to detect 'pro aggregating' alpha synuclein in CSF. Fairfoul et al reported the first application of RT-Quic on CSF samples of patients with synucleinopathies and controls in 2016, obtaining sensitivities of 92% for dementia with Lewy bodies and 95% for PD and specificities of 100% compared to healthy controls and patients with Alzheimer's disease (Fairfoul *et al.*, 2016). Shanawaz et al showed that the alpha synuclein-PMCA assay can differentiate between samples of CSF from patients diagnosed with PD from those with MSA, with an overall sensitivity of 95.4% (Shahnawaz *et al.*, 2020).

Neurofilament light chain protein in CSF and serum have also been considered as potential biomarkers to differentiate PD from atypical PD. Several studies have found higher levels of serum and CSF neurofilament light chain in participants with atypical Parkinsonism including multiple system atrophy (MSA), progressive supranuclear palsy (PSP) and corticobasal syndrome (CBS) compared to PD and healthy controls (Hansson *et al.*, 2017; Bridel *et al.*, 2019).

Peripheral Tissue

Alpha synuclein pathology occurs outside the central nervous system in PD and may even occur prior to central nervous system involvement (Jellinger, 2011), resulting in great interest in peripheral tissue biomarkers in prodromal PD and PD. Various tissues including skin (Kuzkina *et al.*, 2019), salivary glands (Tredici *et al.*, 2010) and gastrointestinal mucosa (Beck *et al.*, 2020) have been studied.

Abnormal alpha synuclein aggregates in cutaneous nerves of PD participants have been reported in numerous studies (Wang *et al.*, 2013; Donadio *et al.*, 2014; Doppler *et al.*, 2014). Phosphorylated alpha synuclein has also been found in cutaneous biopsies of participants with REM sleep behaviour disorder, suggesting the potential role of skin biopsies in detecting prodromal PD (Antelmi *et al.*, 2017; Doppler *et al.*, 2017). Other potential clinical applications of cutaneous alpha synuclein assessment include differentiating patients with MSA from patients with PD and orthostatic hypotension (Donadio *et al.*, 2020), PD from MSA (Zange *et al.*, 2015) and PD from tauopathies (Donadio *et al.*, 2014).

Within the gastrointestinal tract, phosphorylated alpha synuclein has been found to be concentrated in the myenteric plexus of Auerbach and the submucosal plexus of Meissner (Beach *et al.*, 2010). There are some technical challenges associated with tissue sampling which may affect the sensitivity of the investigation. Samples of small areas from the gastrointestinal tract may not contain myenteric and submucosal plexuses. Larger submucosal biopsies may enhance detection of abnormal alpha-synuclein but the morbidity related to a larger biopsy is higher than for a superficial biopsy (Sánchez-Ferro *et al.*, 2015). Further studies are required to address the technical challenges and identify the most sensitive site in the gastrointestinal tract for sampling (Tsukita *et al.*, 2019).

Submandibular gland needle biopsy has also been investigated. However in a meta-analysis of in-vivo studies involving submandibular glands, pooled sensitivity of the technique was moderate (Tsukita *et al.*, 2019). There are also technical challenges with needle biopsy. One of the difficulties, is that submandibular glandular parenchyma is not obtained in all biopsy samples (Vilas *et al.*, 2016). In addition, adverse events like bruising and swelling following biopsies are common and can occur in up to 77% of cases (Adler *et al.*, 2016).

1.2.2.3. Biomarkers to stratify disease subtypes

There is increasing evidence that there are different subtypes of idiopathic PD (Fereshtehnejad *et al.*, 2015, 2017) and biomarkers that can differentiate the clinical subtypes would aid prognostication, help clinical trial design, further our understanding of the underlying disease mechanism and eventually enable development of personalised management plans. The National Institute of Neurological Disorders and Stroke established subtype characterization as one of the top three priority areas in PD research (Sieber *et al.*, 2014).

Subtyping based on a priori hypothesis

Subtyping PD patients using variables selected a priori including age and tremor versus postural instability/gait difficulty (PIGD) predominance has been well described and used in many clinical trials (Jankovic *et al.*, 1990; Alves *et al.*, 2006). Both PIGD and tremor dominant subtypes can be classified from UPDRS III scores (Stebbins *et al.*, 2013). Tremor dominant phenotype has been associated with a more favourable prognosis compared to the PIGD phenotype (Jankovic, 2008). The PIGD subtype is associated with faster cognitive decline (Johnson *et al.*, 2016), a higher non-motor symptom burden (Ba *et al.*, 2015) and faster progression (Savica *et al.*, 2019).

Non-motor features and PD prognosis

Non-motor symptoms may also provide prognostic implications. Cardiovascular autonomic dysfunction, REM sleep behaviour disorder and gait dysfunction have been associated with a higher risk of dementia (Anang *et al.*, 2014). Peripheral neuropathy has also been reported to be an independent marker of severe PD phenotype, associated with worse axial motor, cognitive and autonomic features (Merola *et al.*, 2017).

Data-driven subtyping

Advanced statistical methods have been used to subtype PD patients as researchers recognise the complexity of PD and the limitations of single-factor subtypes (Fereshtehnejad and Postuma, 2017). A Canadian study used data from the Parkinson's Progression Markers Initiative (Parkinson Progression Marker Initiative, 2011) in a non-hypothesis driven cluster analysis. The key classifiers in the hierarchical cluster analysis were a motor score and three non-motor scores: rapid eye movement sleep behaviour disorder, cognition and dysautonomia. Three distinct subtypes of PD were defined: mild motor predominant (both composite motor score and all non-motor summary scores were below the 75th percentile), diffuse malignant (either (i) composite motor score >75th percentile and $\geq$ 1 of 3 non-motor scores >75th percentile; or (ii) all three non-motor scores >75th percentile) and intermediate (those not meeting criteria for mild motor predominant or diffuse malignant) (Fereshtehnejad *et al.*, 2017).

Biomarker-based subtyping

Advances in imaging, genomics, proteomics, metabolomics, lipodomics may aid in the differentiation of PD into different subtypes (Thenganatt and Jankovic, 2014). Horsager et al carried out a multimodal imaging study to test their hypothesis that PD comprises two different subtypes: A peripheral onset form associated with REM sleep behaviour disorder in the prodromal phase and marked autonomic neuropathy prior to involvement of the dopaminergic system and a central onset form with dopaminergic dysfunction preceding autonomic neuropathy and the absence of REM sleep behaviour disorder. PD with REM sleep behaviour disorder was compatible with a peripheral onset trajectory, characterised by loss of cardiac ^{123}I-metaiodobenzylguanidine (MIBG) signal and ^{11}C-colonic donepezil signal prior to loss of putaminal F-dopa uptake. In contrast, data from PD patients with no REM sleep behaviour

disorder was compatible with a central onset subtype, characterised by initial loss of putaminal F-dopa uptake followed by loss of cardiac MIBG signal and ^{11}C-colonic donepezil signal (Horsager *et al.*, 2020).

1.3. Corneal confocal microscopy as a biomarker in Parkinson's disease

1.3.1. Are changes in corneal innervation a potential surrogate marker of Parkinson's disease?

1.3.1.1. Peripheral nervous system involvement in Parkinson's disease

The traditional view that PD is exclusively a disease of the motor system is no longer held to be true. A wide range of associated non motor symptoms and neuropathological evidence demonstrating the presence of Lewy bodies in both central and peripheral nervous system structures outside the basal ganglia challenges the traditional view.

The presence of peripheral neuropathy in PD is multifaceted and many studies have attempted to clarify the causal mechanisms of peripheral nervous system involvement in idiopathic PD. Current studies in the literature propose the following causes of peripheral nervous system involvement in idiopathic PD: an intrinsic neuropathy driven by Parkinsonian pathology and an acquired neuropathy secondary to exposure to Levodopa therapy.

Intrinsic Neuropathy

The prevalence of peripheral neuropathy in drug naïve PD patients has recently been studied by Lee et al. 105 drug naïve patients underwent nerve conduction studies and had blood tests including vitamin B12, homocysteine and uric acid levels. In their study, 22.3% of drug naïve PD participants had features of peripheral neuropathy. PD participants with peripheral neuropathy had higher serum levels of homocysteine and uric acid compared to PD participants without peripheral neuropathy (Lee and Baik, 2020). However, the PD population with

peripheral neuropathy was significantly older than the PD population without peripheral neuropathy and carpal tunnel syndrome and ulnar neuropathy were included in the peripheral neuropathy diagnosis.

A reduction in small fibre density in skin biopsies of patients with idiopathic PD compared to patients with multiple system atrophy was described by Novak et al in 2009 (Novak *et al.*, 2009). The sample size for this study was small and was a comparison between six idiopathic PD patients and six multiple system atrophy patients. Around the same time, Nolano et al performed skin biopsies and quantitative skin testing on patients with PD and demonstrated that patients with PD had a significant increase in tactile and thermal thresholds, a significant reduction in mechanical pain perception and a significant loss of epidermal nerve fibres and Meissner's corpuscles compared to controls (Nolano *et al.*, 2008).

Several important studies have gone on to demonstrate α-synuclein deposition at the level of small nerve fibres. Wang et al found significantly higher α-synuclein deposition within cutaneous autonomic nerve fibres in skin biopsy samples of patients with PD compared to controls. No α-synuclein deposition was found within nociceptive sensory fibres in biopsy samples of all subjects (Wang *et al.*, 2013). Donadio et al demonstrated phosphorylated alpha synuclein deposits in all cervical site skin samples, 52% percent of thigh skin samples and 24% of leg skin samples in idiopathic PD patients compared to 0% in all skin sample sites of controls and participants with parkinsonisms of pathogenesis assumed not to have synuclein deposits. They also found a correlation between neuritic synuclein inclusions with small fibre neuropathy in patients with idiopathic PD and postulate the possibility of a direct role of phosphorylated alpha synuclein in peripheral nerve fibre damage (Donadio *et al.*, 2014). The pathogenesis underlying small fibre neuropathy in idiopathic PD is not definitively proven. However, the identification of alpha synuclein deposition within nerve fibres of skin biopsy samples from idiopathic PD patients strengthens the case that small fibre nerve damage is

caused by Parkinsonian pathology. Volpicelli-Daley et al showed that accumulation of pathological alpha synuclein leads to neuron death in vitro (Volpicelli-Daley *et al.*, 2011).

Acquired Neuropathy

Several studies have reported the presence of peripheral neuropathy following long term Levodopa treatment. However, the definitive role of Levodopa (L-dopa) treatment in the pathophysiology of peripheral neuropathy in PD is unclear at present and there are studies that do not support this hypothesis.

Pharmacology studies suggest that L-dopa has two metabolic fates: decarboxylation to form dopamine and O-methylation to form 3-O-methyldopa. In the O-methylation reaction, S-adenosylmethionine acts as the methyl donor and S-adenosylhomocysteine is produced as a by-product. Levodopa use increases plasma homocysteine levels as a result of hydrolysis of S-adenosylhomocysteine (Miller *et al.*, 1997). Homocysteine remethylation requires vitamin B12. Peripheral neuropathy has been associated with levodopa treatment via the different metabolic changes in the levels of homocysteine, methylmalonic acid and vitamins B6 and B12 (Toth *et al.*, 2008; Ceravolo *et al.*, 2013).

Toth et al reported the largest case series of patients with PD and peripheral neuropathy (Toth *et al.*, 2008). Out of 49 patients with PD and peripheral neuropathy, 34 patients were found to have peripheral neuropathy with no defined aetiology. The other 15 patients had peripheral neuropathy secondary to various causes including diabetes, monoclonal gammopathy of uncertain significance and probable/possible chronic inflammatory demyelinating polyneuropathy. 32 out of 34 (94%) of patients with PD and peripheral neuropathy with no defined aetiology, were found to have abnormal homocysteine or methylmalonic acid levels. The authors found that cumulative lifetime levodopa dosage and fasting methylmalonic acid levels were associated with peripheral neuropathy severity. The authors suggest that prolonged

levodopa use indirectly leads to peripheral neuropathy via elevation of cobalamin metabolite levels (homocysteine and methlymalonic acid) (Toth *et al.*, 2008).

Following the publication of Toth et al's work, Nolano et al who had previously reported significant epidermal nerve fibre and Meissner corpuscle loss in skin biopsies of patients with idiopathic PD compared to controls, performed a post hoc analysis of their population of patients and calculated the cumulative levodopa intake for each of their patients with idiopathic PD. They found that levodopa exposure did not correlate with epidermal nerve fibre density but correlated with Meissner corpuscle density. Levodopa intake also correlated with disease severity. Both Meissner corpuscle count performed in their study and nerve conduction velocity performed in Toth et al's study investigates large fibre nerves. The correlations between levodopa exposure, idiopathic PD severity and neuropathy in both sets of data, leave unclear the role of treatment and disease severity in the development of large fibre neuropathy in idiopathic PD (Nolano *et al.*, 2011). Jeziorska et al studied skin biopsies in twenty three PD patients and found that intraepidermal nerve fibre degeneration correlated with disease duration, cumulative levodopa dose, severity of motor disability and autonomic dysfunction (Jeziorska *et al.*, 2019). The role of Levodopa on small fibre degeneration is unclear from this study due to the small number of participants and the cross-sectional design of the study. Nolano et al reported loss of epidermal nerve fibres in both treated and untreated patients with idiopathic PD, whereas a loss of Meissner corpuscles was present only in treated patients (Nolano *et al.*, 2011). Therefore, the authors postulate that epidermal fibre loss is not related to drug treatment and may be an intrinsic part of Parkinsonian pathology.

In support of the levodopa induced neuropathy hypothesis, Ceravalo et al who conducted a large multicentre study reported that the duration of exposure to levodopa and age were the main risk factors for the development of neuropathy. The neuropathy that was found was predominantly a sensory axonal neuropathy. Multivariate logistic analysis indicated that the

risk of neuropathy was not influenced by disease duration, disease severity (assessed by UPDRS III total score), serum vitamin B12 level or serum homocysteine level (Ceravolo *et al.*, 2013).

Some studies did not find an association between taking oral levodopa and development of peripheral neuropathy. Shahrizaila et al compared 25 levodopa-naïve patients with 26 patients who were on ≥ 300mg/day of levodopa for at least 3 years. There was no difference in the prevalence of distal symmetric polyneuropathy between the two groups of patients (Shahrizaila *et al.*, 2013). A systematic review of randomized parallel-design trials that compared marketed antiparkinsonian drugs with placebo to quantify the frequency with which neuropathy is reported as an adverse effect in PD trials was carried out in 2011. 79 out of 795 studies satisfied inclusion criteria and were included in the analysis. These studies included 10620 patients treated with antiparkinsonian agents and 6710 patients on placebo. No reports of neuropathy as an adverse event in studies involving levodopa (and other antiparkinsonian drugs) were found in either the drug- or placebo treated subjects. However, only 7 of the included studies had follow up data beyond 52 weeks. Therefore, the review does not rule out the possibility that prolonged exposure to levodopa can cause neuropathy. The authors felt that whilst clinical trials may not be well designed to evaluate drug safety and investigators may overlook mild side effects, severe manifestations of neuropathy were most likely absent (Teodoro *et al.*, 2011).

Nolano et al carried out an interesting study in 2017 to try to clarify causation of peripheral neuropathy in PD. The group studied small and large fibre pathology in drug naïve and levodopa treated patients. 85 patients (48 participants naïve to levodopa treatment) without electrophysiological signs of neuropathy were enrolled into the study. Patients underwent clinical assessments, skin biopsies, quantitative sensory testing, dynamic sweat test and sympathetic skin response. The findings were compared to data from age- and sex-matched

healthy controls. In the drug naïve population, both small and large fibre pathology were found, leading the authors to suggest that both large and small nerve fibres are affected by the intrinsic pathological process of Parkinson's. Levodopa treated patients had a higher loss of Meissner's corpuscles, a marker of large fibre neuropathy, compared to drug naïve patients after adjusting for age, disease severity and duration. The authors suggested that there may be a selective neurotoxic effect of levodopa on large fibres (Nolano *et al.*, 2017).

Summary

Whilst levodopa may play a role in large fibre neuropathy, there is lack of evidence that it plays a role in small fibre neuropathy which studies have suggested is linked to intrinsic Parkinsonian pathology (Nolano *et al.*, 2008, 2017; Wang *et al.*, 2013; Donadio *et al.*, 2014; Doppler *et al.*, 2014). Cossu and Melis who did a literature review on studies investigating determinants of peripheral neuropathy in PD did not find any studies to support levodopa induced small fibre neuropathy (Cossu and Melis, 2016).

1.3.1.2. The potential roles of small fibre neuropathy as a biomarker in PD

Biomarker of disease progression

Doppler et al aimed to characterize alpha synuclein deposition in cutaneous nerves of patients with PD and explore whether peripheral pathology would reflect pathological features of neurodegeneration in the central nervous system (Doppler *et al.*, 2014). Wang et al previously reported alpha synuclein deposition in cutaneous autonomic nerves but used an alpha synuclein antibody that was not specific to the phosphorylated form (Wang *et al.*, 2013). Doppler et al recruited 31 patients with PD and 35 controls for their study. Skin biopsies were obtained from both cohorts of patients and phosphorylated-alpha-synuclein was quantified from the samples. Cryo-conserved sections of the substantia nigra of patients with PD containing Lewy bodies were used as controls for phosphorylated-alpha-synuclein staining. Deposits of

phosphorylated-alpha-synuclein were identified in dermal/epidermal nerve fibres of 16/31 patients with PD and 0/35 controls. Interestingly the morphological appearances of phosphorylated-alpha- synuclein deposits in the skin were identical to phosphorylated-alpha-synuclein neurites in the substantia nigra of controls. In order to evaluate the pattern of loss of different subtype of dermal fibres, double staining with antibodies against calcitonin gene-related peptide, substance P, vasoactive intestinal peptide and tyrosine hydroxylase, was performed in all phosphorylated-alpha-synuclein positive cases. Phosphorylated-alpha-synuclein was found in all four small nerve fibre subtypes. No correlation was found between cumulative levodopa dose and loss of nerve fibres. Vitamin B deficiencies were uncommon in the group of PD patients. These findings led the authors to suggest that loss of peripheral nerve fibres is driven by intrinsic Parkinsonian pathology and reflects the pathology occurring in the central nervous system (Doppler *et al.*, 2014). These findings have led to the suggestion that small fibre neuropathy may be a marker of disease progression (Siepmann *et al.*, 2017).

Biomarker of disease stratification

Small fibre degeneration in the form of autonomic dysfunction is common in PD and includes orthostatic hypotension, urinary dysfunction, sexual dysfunction, gastrointestinal dysmotility, constipation and abnormal sweating (Asahina *et al.*, 2013). De Pablo-Fernandez et al found that earlier development of autonomic dysfunction was associated with more rapid development of motor progression, cognitive impairment, global disability and shortened survival (De Pablo-Fernandez *et al.*, 2017). Peripheral neuropathy in PD participants, identified by abnormal nerve conduction studies, has been associated with cognitive impairment, worse axial motor features, dependence in activities of daily living and worse non-motor symptoms (Merola *et al.*, 2017). Histopathological studies show that changes in intraepidermal nerve fibre density correlate with disease severity, , progression and side to side asymmetry (Nolano *et al.*, 2018; Jeziorska *et al.*, 2019)

Biomarker to differentiate Parkinson's disease from atypical Parkinsonism

Multiple system atrophy (MSA) is a sporadic adult-onset neurodegenerative disease characterised clinically by Parkinsonism, autonomic failure, urogenital dysfunction, cerebellar features and corticospinal disorders. Pathologically it is defined by striatonigral and olivopontocerebellar degeneration and the presence of alpha-synuclein positive oligodendroglial cytoplasmic inclusions, neuronal loss and gliosis (Gilman *et al.*, 2008).

Progressive supranuclear palsy (PSP) is an adult onset, rapidly progressive neurodegenerative disease characterised by vertical supranuclear palsy, postural instability and falls. Pathologically it is defined by intracerebral deposition of tau protein. The predominant isoform in astrocytic tufts, neurofibrillary tangles and oligodendrocytic coils found in PSP is the four microtubule-binding repeat tau (Kovacs, 2015).

MSA and PSP can present with features of parkinsonism (Gilman *et al.*, 2008; Höglinger *et al.*, 2017) and therefore misdiagnosis of these disorders as idiopathic PD, particularly early on in the disease course, is not uncommon. This has led to an interest in developing biomarkers to differentiate the different disorders.

Cutaneous alpha synuclein assessment has shown distinct patterns of alpha synuclein deposition in patients with PD compared to patients with atypical PD. In a cross-sectional study, alpha synuclein was detected in 58% of cells in the spinous cell layer (SCL), 62% of the pilosebaceous units (PSU) and 58% in the eccrine glands (EG) of PD participants. In contrast, alpha synuclein was detected in 7% of SCL, 7% of PSU and 0% of EG in participants with atypical PD (18 with MSA and Lewy body dementia, 8 with PSP and Alzheimer's disease, 7 with secondary Parkinsonism) (Rodríguez-Leyva *et al.*, 2014). This study only investigated alpha synuclein deposition in the epidermis and PSU and did not assess intraneural deposition.

Other limitations include a relatively small sample size and heterogeneity within the atypical PD group (Siepmann *et al.*, 2017).

Central and peripheral autonomic networks are variably affected in the different alpha synucleinopathies. Autonomic dysfunction in PD is primarily caused by degeneration of peripheral autonomic structures whereas in MSA, autonomic manifestations are principally caused by degeneration of preganglionic autonomic neurons of the brainstem and spinal cord (Coon, Cutsforth-Gregory and Benarroch, 2018). In keeping with this assumption, phosphorylated alpha synuclein was found in sympathetic skin nerve fibres of PD participants but not in MSA participants (Zange *et al.*, 2015). Interestingly, Doppler et al found phosphorylated alpha synuclein in skin biopsies of 67% patients with PD and MSA and none in patients with tauopathies or controls. They found that in contrast to PD, where phosphorylated alpha synuclein clustered in autonomic nerve fibres, deposits were primarily found in unmyelinated somatosensory fibres in MSA (Doppler *et al.*, 2015). More recently, Donadio et al found mainly somatic fibre involvement with relative preservation of autonomic innervation in skin biopsies of participants with MSA-Parkinsonism subtype. In contrast, phosphorylated alpha synuclein deposits and denervation were principally found in autonomic skin fibres of participants with PD and orthostatic hypotension (Donadio *et al.*, 2020).

Studies comparing cutaneous innervation in patients with synucleinopathies and tauopathies have found denervation in patients with alpha synucleinopathies including PD and MSA and no denervation in patients with tauopathies including PSP (Doppler *et al.*, 2015; Melli *et al.*, 2018). Reddy et al studied corneal confocal microscopy changes in a small sample of participants (7 PSP, 4 PD and 5 controls) and found no differences in corneal nerve fibre between the three groups. However, this study may have been underpowered to detect any significant changes.

1.3.2. Anatomy of the cornea

The cornea has traditionally been histopathologically divided into five layers: the epithelium, Bowman's layer, stroma, Descemet's membrane and the endothelium (Figure 1-1). In 2013, a sixth layer named Dua's layer was described. Dua's layer is an acellular layer in the pre-Descemet's cornea which can be separated out along the last row of keratocytes (Dua *et al.*, 2013).

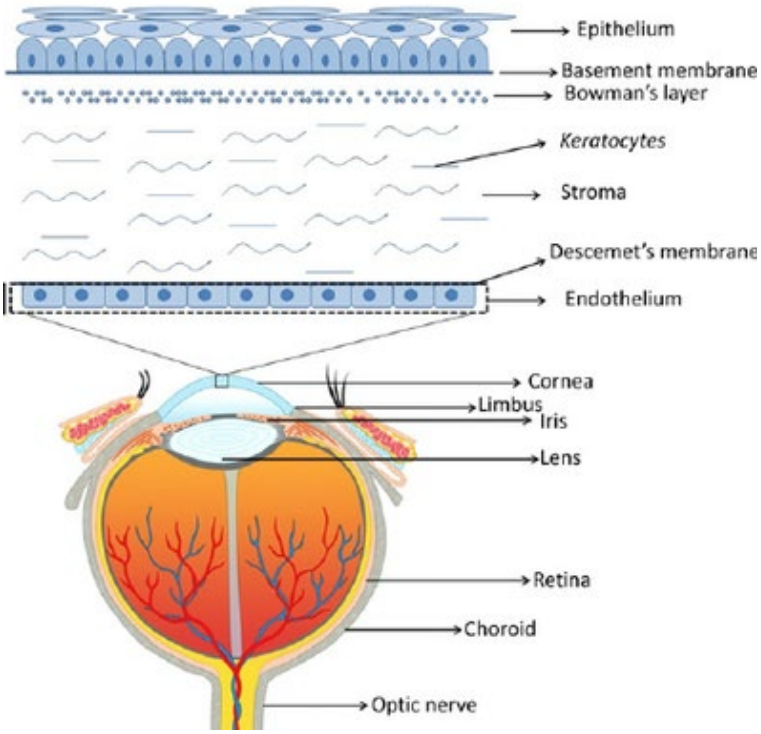

Figure 1-1 The Anatomy and Structure of the Adult Human Cornea (Parekh et al., 2016)

1.3.3. Corneal nerves: Structure and Distribution

The cornea is the most densely innervated structure in the body and is supplied by sensory and autonomic nerve fibres. Most corneal nerve fibres are sensory in origin and originate from the ophthalmic branch of the trigeminal nerve (Müller *et al.*, 2003). In mammalian corneas, sympathetic innervation is derived from the superior cervical ganglion. Sympathetic nerve fibres in humans are thought to be scarce (Ehinger, 1971). It is unclear whether human corneas receive parasympathetic innervation (Müller *et al.*, 2003).

Nerve fibres originating from trigeminal ganglion cells travel suprachoroidally and branch to form the limbal plexus which rests around the corneoscleral limbus (Al-Aqaba *et al.*, 2010). The limbal plexus divides into stromal nerve trunks which enter the cornea stroma at a depth of 293 ± 106µm (Marfurt *et al.*, 2010). Nerve bundles lose their perineum and myelin sheaths very close to the limbus and are surrounded only by Schwann cell sheaths as they continue into

the cornea in order to maintain corneal transparency (Müller *et al.*, 2003). Eventually the stromal nerves turn abruptly and proceed anteriorly towards the anterior stroma, sub-basal and epithelial layers of the cornea (Müller *et al.*, 2003; Shaheen, Bakir and Jain, 2014).

The corneal epithelium is innervated by the sub-basal plexus which originates from the peripheral stromal nerves (Oliveira-Soto and Efron, 2001). The diameter of the nerve fibres in the sub-basal plexus are mostly in the range of 0.1-0.5μm. Based on the size of the fibres, these nerve fibres are thought to be A-delta and C fibres (Müller *et al.*, 2003). Figure 1-2 summarises the different nerve fibre types.

The terminal processes of nociceptive nerve cells in the epithelial layer collectively respond to thermal, mechanical and chemical stimuli (Belmonte, Garcia-Hirschfeld and Gallar, 1997).

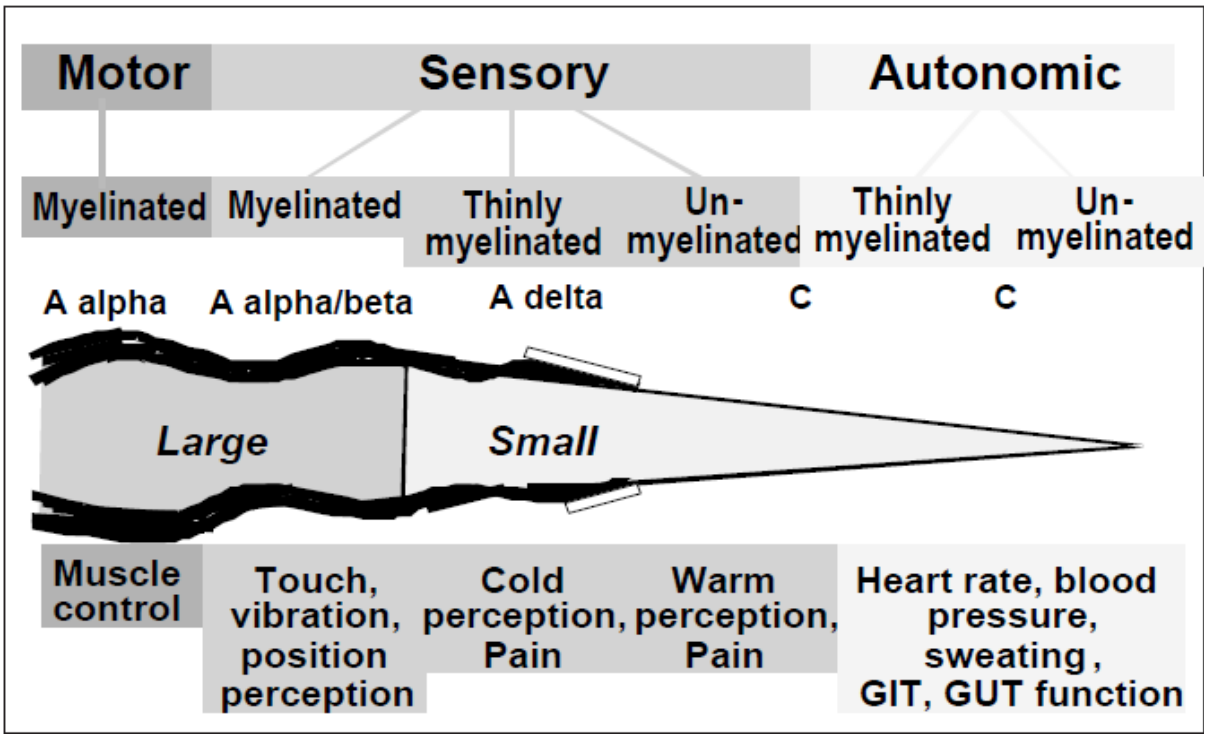

Figure 1-2 Nerve fibre types: A-alpha fibres are large, myelinated fibres predominantly involved in motor function whereas A-beta are large, myelinated fibres and function as mechanoreceptors. A-delta fibres are small, thinly myelinated fibres involved in pain and temperature perception. C fibres are predominantly unmyelinated fibres which play a role in pain and/or autonomic functions (Casellini and Vinik, 2007).

1.3.4. Methods of studying corneal innervation

Light and electron microscopy and immunohistochemical techniques have enabled us to elucidate the distribution, anatomy and neurochemical properties of corneal nerve fibres and neurotransmitters. One of the problems encountered in studying human corneal nerve architecture in vitro is the degeneration of nerves after death. Muller et al demonstrated that a great part of corneal nerves have degenerated up to 13.5 hours after death (Müller *et al.*, 1997) The more recent use of corneal confocal microscopy (CCM) allows in vivo evaluation of corneal nerves prospectively and repeatedly (Oliveira-Soto and Efron, 2001).

Epithelial nerves are unreliably visualised by CCM due to their small size (Müller *et al.*, 2003). However the sub-basal nerve fibre bundles can be visualised relatively easily and are the focus of evaluation by CCM (Linna *et al.*, 2000; Oliveira-Soto and Efron, 2001). The sub-basal nerve plexus supplies the corneal epithelium and is important in reflecting neuropathic changes in the cornea (Kass-Iliyya *et al.*, 2015).

1.3.5. Basic Principles of the corneal confocal microscope

A major limiting factor of light microscopy is that light reflected from structures surrounding the point of observation obscures the image resulting in reduced image contrast. (Jalbert *et al.*, 2003). Confocal microscopy eliminates out of focus information by using point illumination and point detection to produce high resolution images and enables study of intact tissues using optical sectioning to allow images from different depths to be obtained, thereby eliminating the need to process and section specimens (Petroll and Robertson, 2015).

The basic principle of a confocal microscope involves a point source of light created by a pinhole aperture focused by an objective lens on tissue. The light reflected by the specimen at the focal point is focused onto a separate duplicate pinhole aperture by a parallel objective lens. Light that passes the second pinhole is collected by a detector (Figure 1-3). The illumination point source and the observation aperture of the detector are focused on a single point on the specimen, hence the name ‘confocal microscopy’ (Erie, McLaren and Patel, 2009). The image produced has a very high resolution but no field of view due to a single point of illumination and detection. To resolve this problem, the confocal microscope synchronously illuminates a region of the cornea with thousands of spots of light each second. The spot images are subsequently reconstructed to create a usable field of view (Efron *et al.*, 2001).

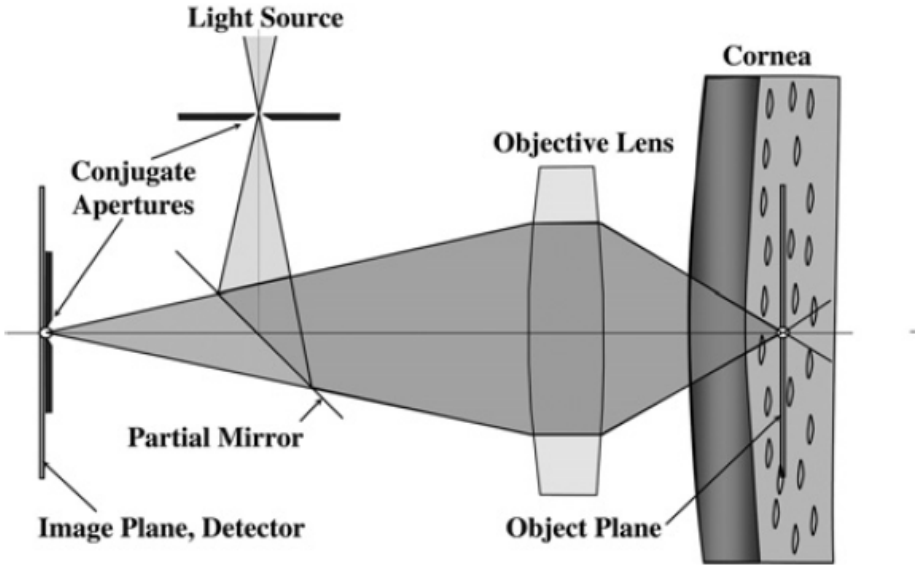

Figure 1-3 Light reflected from the light source on the specimen focal point is 'co-focused' with the detection aperture and termed 'confocal' (Erie, McLaren and Patel, 2009)

Different types of confocal microscopes have been described. The main ones are: (1) the Tandem Scanning Confocal microscope (TSCM) [Tandem Scanning, Reston, Virginia, USA], (2) the ConfoScan 4 slit-scanning confocal microscope (SSCM) [Nidek Technologies, Greensboro, North Carolina, USA] and 3) the Heidelberg Retina Tomograph Rostock III Corneal Module laser scanning confocal microscope (HRT III RCM) [Heidelberg Engineering GmbH, Heidelberg, Germany] (Erie, McLaren and Patel, 2009).

TSCM utilizes a rotating Nipkow disc that has multiple pinhole apertures arranged in an Archimedean spiral. The specimen is illuminated through the array of apertures and imaged through conjugate apertures on the opposite side. This design produces excellent lateral and axial resolution. However, the pinhole diameters are small and limit light transmission resulting in images with relatively poor contrast compared to confocal microscopes with larger pinholes.

Due to the low light throughput, a strong light source is required which causes patient discomfort and may limit examination times. TSCM is no longer commercially available (Erie, McLaren and Patel, 2009).

SSCM uses narrow vertical slit apertures for illumination and observation of the field. A rapidly oscillating two-sided mirror records images from the illuminating and displaying slit. The slit aperture allows increased light throughput compared to TSCM. Therefore, contrast is improved, and structures appear more detailed. Illumination is not as bright as TSCM which makes the procedure more comfortable for the patient (Mahelková *et al.*, 2017). The drawback of parallel illumination and detection, is reduced optical resolution as confocality is only maintained along one spatial direction, resulting in degraded depth resolution (Stachs, Guthoff and Aumann, 2019)

The HRT III RCM is a laser confocal microscope. Combining laser diodes with beam shaping optics enables spot illumination without the need for illumination pinholes. Such optical systems have superior signal to noise ratios and optimum depth sectioning can be achieved (Stachs, Guthoff and Aumann, 2019). HRT III RCM operates by scanning a 670 nm laser over the field of view in a raster pattern (Petroll and Robertson, 2015). This is a class 1 laser system and is meant to pose no danger to the eyes. (Mahelková *et al.*, 2017). The system typically uses a high numerical aperture 63x objective lens and thus produces images of excellent resolution and contrast (Petroll and Robertson, 2015).

1.3.6. CCM and the corneal layers

In vivo CCM is able to image the five main layers of the cornea: corneal epithelium, Bowman's layer, stroma, Descemet's membrane and corneal endothelium (Figure 1-4).

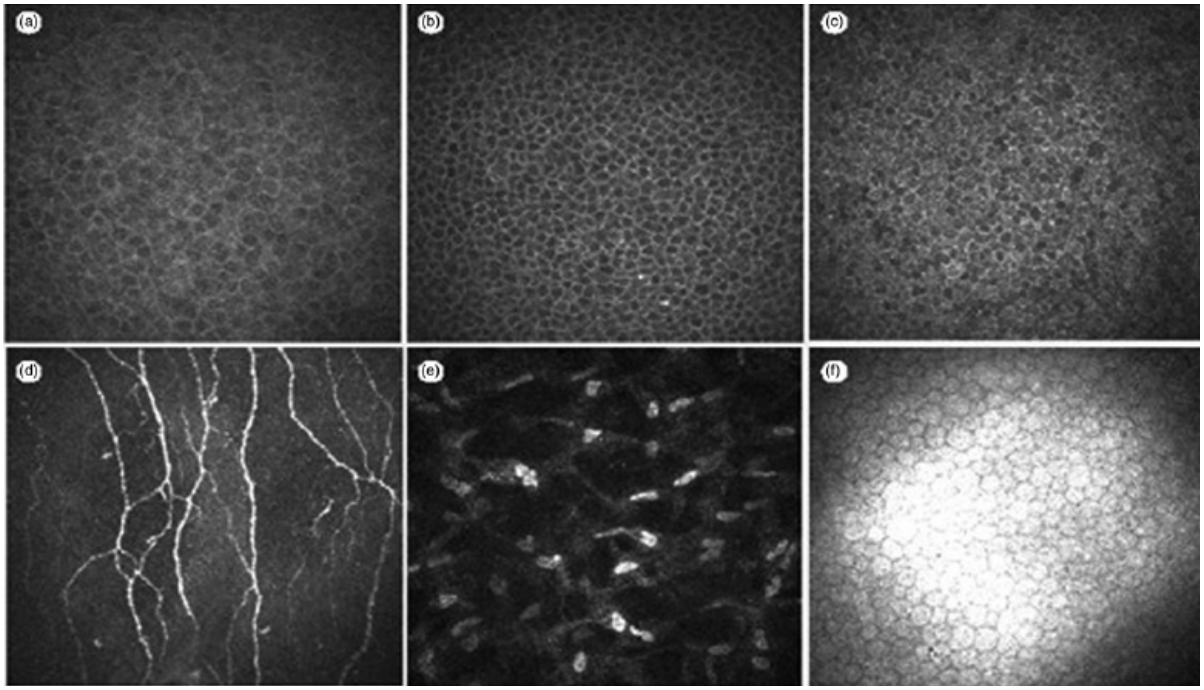

Figure 1-4 Confocal microscopy images of (a) superficial epithelial cells (b) 'wing' epithelial cells (c) basal epithelial cells (d) sub-epithelial nerve plexus (e) corneal stroma-keratocytes (f) endothelium (Kymionis *et al.*, 2015)

The corneal epithelium is made up of three layers: superficial cells, wing cells and basal cells.

Superficial cells are just below the tear film. (Masters and Thaer, 1994). The cells are polygonal and have small bright nuclei surrounded by a darker cytoplasm (Malik, 2008). Wing cells are in between superficial cells and basal cells in the epithelial layer. The cells are characterised by bright cell nuclei that are devoid of the darker band seen in superficial cells (Masters and Thaer, 1994). Basal epithelial cells do not have visible cell nuclei. The cells have a bright cell border with a dark cytoplasmic mass (Tomii and Kinoshita, 1994).

Bowman's layer is an amorphous membrane, 8-10 µm thick, located posterior to the basal epithelium, made up of collagen fibres and contains unmyelinated c-nerve fibres (Kobayashi, Yokogawa and Sugiyama, 2006). Confocal microscopic images of the Bowman's layer appears featureless and grey apart from discrete, beaded nerve fibre bundles of the sub-epithelial neural

plexus that traverse the field of view (Efron *et al.*, 2001). Keratocytes in the anterior stroma may be visible in the background (Malik, 2008).

90% of the thickness of the cornea is made up of the stroma which is composed of collagen fibres, interstitial substance, keratocytes and nerve fibres. Collagen fibres and interstitial substance cannot be visualised with the confocal microscope and appear as a grey background. Keratocytes are identified as discrete bright entities. The bright entities are the keratocyte nuclei as the cytoplasm, cell wall and processes of the keratocytes are not visible (Efron *et al.*, 2001).

Confocal images of Descemet's membrane are acquired whilst through-focussing from the posterior stroma to endothelial cells (Malik, 2008). Descemet's membrane becomes more visible with increasing age and is not visible in young subjects (Hollingsworth *et al.*, 2001).

The endothelium is made up of a single layer of hexagonal- or polygonal- shaped cells and are visualised with confocal microscopy as bright cell bodies with dark borders. The cell nuclei are rarely recognizable (Malik, 2008).

1.3.7. Corneal nerve morphology

The key parameters used to quantify corneal nerve morphology using CCM are corneal nerve fibre length (CNFL), corneal nerve fibre density (CNFD) and corneal nerve branch density (CNBD) (Figure 1-5). More recently, fully automated software has enabled the quantification of additional parameters including corneal nerve fibre area (CNFA), corneal nerve fibre width (CNFW) and corneal total branch density (CTBD) (Chen *et al.*, 2017) .

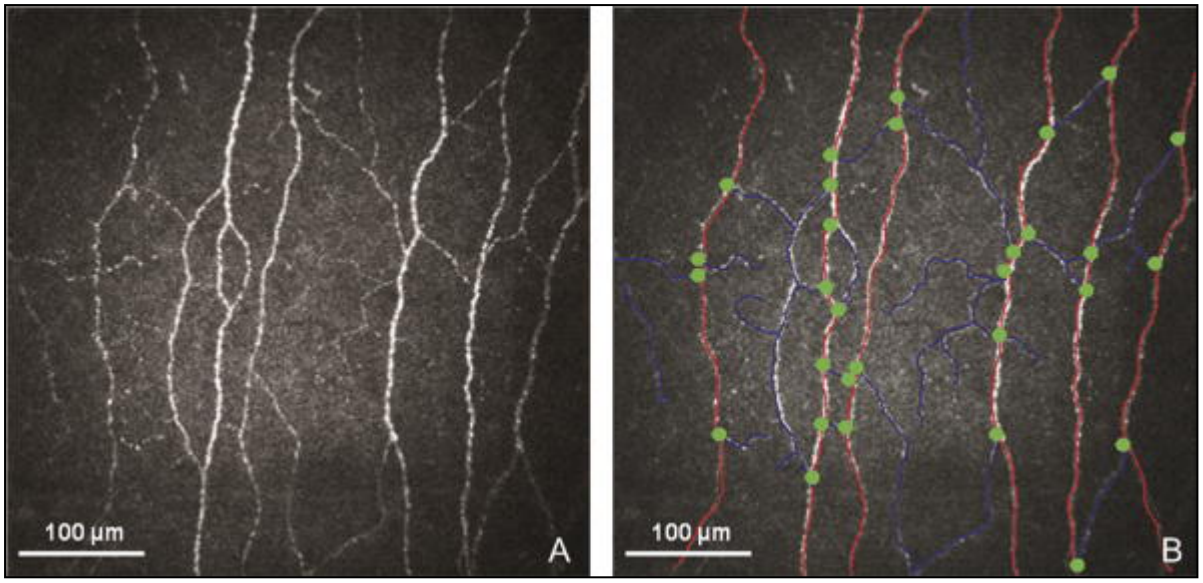

Figure 1-5 Corneal Confocal Microscopy Images. A: An original image captured with corneal confocal microscopy. B: An analysed image using CCMetrics. Red lines represent the main nerve fibres. An integrated algorithm utilises the red lines to measure corneal nerve fibre density. Blue lines represent secondary branching nerves. Corneal nerve fibre length is the summation of the length of all nerves highlighted in blue and red. Green dots represent the junction between main nerves and secondary nerves. Corneal nerve branch density is measured by the green dots (Petropoulos *et al.*, 2013).

CNFL is the length of all nerve fibres and branches measured in millimetre per square millimetre, CNFD is the number of main nerves per square millimetre and CNBD is the number of branches emanating from each main nerve per square millimetre (Petropoulos *et al.*, 2013). CNFA is the total nerve fibre area per square millimetre, CNFW is the average nerve width and CTBD is the total number of branch points per square millimetre (Chen *et al.*, 2017).

Analysis of CCM images

CCM images can be analysed using manual or fully automated software (Petropoulos *et al.*, 2014). In manual analysis, the investigator relies on luminance variation in the acquired images to identify nerve fibres from the background using a set of predefined criteria (Kim and Markoulli, 2018). The main limitation of manual analysis is that analysis of the images using the interactive software is labour intensive and requires expertise (Dabbah *et al.*, 2011). Automated analysis uses a computational modelling algorithm to detect low contrast nerve fibres from background noise. It is less precise but quicker and potentially more reliable due to its consistency and it is not subject to inter-/intra-observer variability (Dabbah *et al.*, 2011)

1.3.8. Is corneal confocal microscopy a valid and reproducible technique for quantifying corneal nerve abnormalities?

1.3.8.1. Reproducibility and validity of image acquisition, selection and analysis

CCM has been used to quantify corneal sub-basal nerve fibres in a variety of peripheral neuropathies including diabetic neuropathy idiopathic small fibre neuropathy (Tavakoli, Marshall, *et al.*, 2010), Fabry's disease (Bitirgen *et al.*, 2018) and Charcot-Marie-Tooth disease Type 1A (Tavakoli *et al.*, 2012). Previous studies shows that CCM can detect early nerve fibre regeneration after kidney and pancreas transplant in participants with diabetes (Azmi *et al.*, 2019) and after bariatric surgery in participants with obesity related neuropathy (Azmi *et al.*, 2021).

In order for CCM to be used as a reliable method to diagnose, monitor and stratify severity of peripheral neuropathies, the technique has to be reproducible. Issues such as individual anatomical variation and inter- and intra- rater consistency affect results and studies have attempted to address the reproducibility and diagnostic validity of the technique.

Image Selection

Vagenas et al carried out a study to determine the optimum number of central corneal images required to achieve an acceptable level of accuracy in quantifying CNFL and CNBD. Five to eight randomly chosen images not overlapping by more than 20% provide an acceptable level of accuracy. Sampling five images would produce a calculated mean within 13% of the true mean 80% of the times sampled and if eight images were sampled, an equivalent precision would be achieved 95% of the time for CNFL (Vagenas *et al.*, 2012).

Kalteniece et al studied the intra- and inter-rater reproducibility of using a standardized protocol for image selection. The protocol specified that the images were to be selected from the centre of the cornea, based on the orientation of corneal nerves. Images of differing number of nerves were selected for each participant and the images were required to be of high quality with paucity of pressure lines, optimal contrast and with no overlap between layers. The study employed automated image analysis to remove nerve recognition error. Four observers used a standardized protocol to select six central corneal nerve images to assess inter observer variability. The intraclass correlation coefficients (ICC) between the four observers were 0.93 for CNFD, 0.96 for CNBD and 0.95 for CNFL, thus demonstrating that implementing a standardized protocol to select images results in high intra- and inter-observer repeatability (Kalteniece *et al.*, 2017).

Image Analysis

Efron et al showed that CNFL has a high inter-observer and between-occasion repeatability in participants with Type 2 diabetes using a manual analysis software package (CCMetrics, University of Manchester, Manchester, UK). No other CCM parameter was studied in this analysis (Efron *et al.*, 2010). Hertz et al studied inter and intra-rater reproducibility of manual

image analysis using the intraclass correlation coefficient method. Only two images were selected from each eye for each participant and the images were manually analysed using CCM Image Analysis tool version 0.6. The study used a 300 µm field of view lens (producing an image area of 0.3 x 0.3 mm^2), rather than the more conventional 400 µm field of view lens (producing an image area of 0.4 x 0.4 mm^2). Images were quantified twice by one examiner and independently by a second examiner. They found that CNFL had the highest interobserver (ICC: 0.72) and intraobserver (ICC: 0.73) reproducibility. The authors hypothesized that the greater reproducibility of CNFL compared to CNFD and CNBD was because the distinction between nerve fibres and nerve branches is not consistently clear during analysis. CNFL, a measure of all nerve fibres and branches does not require discrimination between fibres and branches. The authors acknowledged that the detection of fibre or branch numbers could be limited by the smaller field lens to a greater degree than fibre length, resulting in better reproducibility with CNFL compared to the other CCM parameters (Hertz *et al.*, 2011). Petropoulos et al argued that quantifying CNFL alone limits the interpretation of corneal nerve damage and repair in disease and in their study in 2013 demonstrated that measurements of CNFD (Intraobserver ICC: 0.74, Interobserver ICC: 0.82) and CNFL (Intraobserver ICC: 0.70, Interobserver ICC: 0.66) achieved the highest values for intraobserver and interobserver agreement. Observers were least consistent in reporting CNBD (Intraobserver ICC: 0.61, Interobserver ICC: 0.54). The authors suggested that these findings highlight the importance of clear definitions for nerve fibres and nerve branches. The correct identification of nerve branches can be challenging and is affected by background contrast, image clarity and observer experience (Petropoulos *et al.*, 2013).

In order to achieve consistent results and to eliminate inter and intra-observer variability, fully automated analysis was developed. In 2014, Petropoulos carried out a study to compare fully automated and manual analysis of corneal confocal microscopy parameters in diabetic

peripheral neuropathy. Manual and automated analysis were highly correlated for CNFD (adjusted R^2=0.81, r = 0.90, p<0.0001) CNFL (adjusted R^2=0.79, r=0.89, p<0.0001) and CNBD (adjusted R^2=0.58, r=0.75, p<0.0001). CNFD manual (Area under the curve [AUC]:0.84) CNFD automated (AUC:0.8), CNFL manual (AUC: 0.82) and CNFL automated (AUC: 0.84) were associated with the highest sensitivity and specificity to diagnose diabetic sensory peripheral neuropathy (Petropoulos *et al.*, 2014). A recent study investigating the agreement of corneal nerve quantification between automated (ACCMetrics) and manual software (CCMetrics) following refractive surgery reported good agreement for CNFD (ICC: 0.811) and CNFL (ICC: 0.789). CNBD had the worst agreement (ICC: 0.642). Fully automated quantification was found to underestimate nerve measurements compared to manual quantification, although the differences were not significant. Nevertheless, the measurements obtained with different methods are not interchangeable (Chin *et al.*, 2020).

Diagnostic validity

The diagnostic performance of CCM has been compared with intraepidermal nerve fibre density (IENFD), the current standard for assessing small fibre neuropathy. Chen et al found that CCM had comparable diagnostic efficiency compared to IENFD. The area under the receiver operating characteristic curve (AUC) for identifying diabetic sensory peripheral neuropathy was 0.82 for manual CNFD, 0.80 for automated CNFD, 0.70 for manual CNFL, 0.77 for automated CNFL, 0.59 for manual CNBD, 0.70 for automated CNBD and 0.66 for IENFD. There were no significant differences between the receiver operating characteristic curves (ROC) for manual or automated CNFD and IENFD (Chen *et al.*, 2015). Alam et al found that the AUC for CNFD was 0.81 and the AUC for IENFD was 0.73 for the diagnosis of diabetic neuropathy (Alam *et al.*, 2017).

1.3.8.2. CCM studies in PD

Based on evidence that small fibre neuropathy occurs in PD, several studies have used corneal confocal microscopy to demonstrate peripheral nerve involvement in PD. In small fibre neuropathy, the unmyelinated C and the thinly myelinated A-delta fibres are affected. Corneal confocal microscopy which visualises the subbasal plexus is ideal for analysing A-delta and C fibres.

Podgorny et al's group set out to determine whether peripheral neuropathy is a feature of PD prior to treatment. The group performed assessments including a neurological examination utilising the Utah Early Neuropathy Scale, nerve conduction studies, skin biopsies and corneal confocal microscopy on 26 participants with early PD (20 participants were drug naïve, 5 participants were on low dose L-dopa and 1 participant was on Pramipexole) and 22 control subjects. No significant differences were found between participants with PD and control subjects in any nerve conduction study parameters. Epidermal nerve fibre density in skin biopsies did not differ significantly between PD and control cohorts. Automated analysis was used to analyse corneal confocal microscopy data. CNFL and CNBD were found to be significantly lower in participants with PD compared to controls. There was no difference in CNFD between subjects with PD and controls (Podgorny *et al.*, 2016).

In 2016 Misra et al's group carried out a case control study to examine the ocular surface of participants with moderately severe PD compared to a control group. 15 participants with moderately severe PD (Hoehn Yahr grade 3 or 4) and 15 control participants were recruited. Assessments including blink rate assessment, central corneal aesthesiometry and in vivo corneal confocal microscopy were carried out. The authors found that sub-basal corneal nerve density as measured by corneal confocal microscopy was significantly reduced in the PD group when compared to controls. Corneal sensitivity evaluated by directing a jet of air using a non-contact corneal aesthesiometer towards the cornea and measuring the minimum force of air (in

milibars) required to induce a sensation, did not differ significantly between the PD and control cohorts. Blink rate assessments also did not demonstrate a significant difference between the two cohorts (Misra *et al.*, 2017)

A previous study performed by our group utilised corneal confocal microscopy to determine whether it can demonstrate small nerve fibre damage in PD and to identify relationships between corneal nerve measurements, intraepidermal nerve fibre density and clinical features of PD (Kass-Iliyya *et al.*, 2015). 26 participants and 26 controls were assessed with CCM. 24 of the 26 PD participants and 10 out of the 26 controls had skin biopsies from the dorsa of both feet. PD participants also had assessments of parasympathetic function, autonomic symptoms and motor symptoms (UPDRS-III). Intraepidermal nerve fibre density (IENFD) was significantly lower in subjects with PD compared to controls (IENFD mean difference: -5.9 no. /mm^2, 95% CI [-7.9, -3.9], P<0.001). In terms of CCM parameters, CNFD was significantly lower in PD participants compared to controls (CNFD mean difference: -5.6 no./mm^2, 95% CI [-9.2, -2], p<0.001). CNBD was significantly higher in PD participants compared to controls (CNBD mean difference: 86.6 no./mm^2, 95% CI [55.9, 117.2], p<0.001). CNFL was also significantly higher in PD participants compared to controls (CNFL mean difference 3.2mm./mm^2, 95% CI [0.3, 6.1], p=0.031). The change in corneal nerve parameters was found to be independent of age, cumulative L-dopa dose, methylmalonate, homocysteine, and B12 and folate levels, suggesting that corneal nerve damage is secondary to the intrinsic pathological process of PD rather than a result of treatment.

All three studies described above demonstrate changes in corneal confocal microscopy parameters in subjects with PD compared to controls. However, it is of interest to note that whilst Podgorny et al demonstrated lower CNFL and CNBD and no difference in CNFD in subjects with PD compared to controls, Kass-Iliyya et al found the opposite: a lower CNFD and an increased CNFL and CNBD in PD participants compared to controls. Misra et al report

a reduction in sub-basal corneal nerve density which the authors report as equivalent to CNFL in the other two studies (total nerve length, measured in mm/mm^2) which is also different from the findings of Kass-Iliyya et al. The differences in their findings could be related to the different cohorts of PD participants being studied. The participants in Podgorny et al's study were predominantly untreated participants with early PD, whereas Kass-Iliyya et al recruited treated participants with early PD (Hoehn Yahr grade 1 and 2) and Misra et al recruited participants with moderately severe PD (Hoehn Yahr grade 3 and 4). Kass-Iliyya et al hypothesize that their findings reflect small fibre neuropathy characterized by reduced CNFD (the main nerves). Increased CNBD and CNFL may represent attempted nerve regeneration (Kass-Iliyya *et al.*, 2015). It is possible that nerve regeneration has not occurred in early PD and halts at later stages of the disease. The role of L-dopa also remains a subject of debate. There may also be differences in methodology in terms of acquisition of corneal images: central vs inferio-central cornea (Misra *et al.*, 2017) and calculation of nerve fibres: manual vs automated analysis. Podgorny et al used an automated analysis program to work out corneal nerve parameters compared to the other two studies who analysed corneal confocal microscopy images manually, argues that automated analysis and manual analysis were previously found to be highly correlated (Petropoulos *et al.*, 2014) (Table 1-1).

Andreasson et al studied the prevalence of small fibre neuropathy in PD participants with concurrent restless legs syndrome compared to PD participants without restless legs syndrome using CCM. The group used an image mosaicking technique to create a montage of adjacent images. A separate automated algorithm was used to measure CNFL (total nerve fibre length in a mosaic divided by the mosaic area, expressed in mm/mm^2) and CNBD (total number of nerve branching points divided by the mosaic area, expressed as the number of branching points per mm^2). No differences in CNFL, CNBD, nerve conduction studies or quantitative sensory testing were found between participants with PD and restless legs syndrome (n=21), PD

without restless legs syndrome (n=21) and healthy controls (n=13) (Andréasson *et al.*, 2021). These findings are in contrast to previous CCM studies in PD which demonstrated differences between PD participants and healthy controls (Kass-Iliyya *et al.*, 2015; Podgorny *et al.*, 2016; Misra *et al.*, 2017). The authors report that the study was not specifically designed to assess the power of CNBD and CNFL to discriminate between PD and controls. The control group was also smaller than planned due to participants fulfilling exclusion criteria and/or declining to participate (Andréasson *et al.*, 2021). The mean CNFL (17.6 mm/mm^2) obtained in the control group Andreasson et al's study was lower than the mean CNFL (24.9 mm/mm^2) in a control group from another study using mosaicking technique (Ziegler *et al.*, 2014). The differences may also lie in the methodology used. The CCM frame approach was used in the other three CCM studies in PD and the main advantages are the relatively well-established image acquisition process and the good support for morphometric analysis of images using established software. The disadvantage is the need for manual selection of non-overlapping CCM images (Allgeier *et al.*, 2018). Wide field CCM image creation using mosaic techniques was developed to increase the field of view, potentially allowing the clinician to repeatedly assess identical tissue regions. However, the current implementation of mosaicking requires a lot of expert knowledge and is prone to investigator misjudgement (Allgeier *et al.*, 2018).

	Kass-Iliyya et al. 2015	Podgorny et al. 2016	Misra et al. 2017
Participant population	27 PD (10F, 17M) 26 controls (11F, 15M)	26 PD (11F, 15M) 22 controls (14F, 8M)	18 PD (6F, 12M) 15 controls (7F, 8M)
Age	PD: 63 years, controls: 60.1 years	PD: 63 years, controls: 63 years	PD: 65.5 years, controls: 60.2 years
Duration of disease	Mean of 6.6 years	Mean of 0.92 years	-
Hoehn and Yahr Stage	I:10, II:13, III:4	-	III: 13, stage IV: 5
Dopamine therapy	All PD participants on dopamine therapy	Low dose Levodopa: 5 PD participants, Dopamine agonist: 1 PD participant, Drug naïve: 20 PD participants	Carbidopa-levodopa, Ropinirole, Levodopa-benserazide or a combination :15 patients Previous dopamine therapy:1 patient Tricyclic antidepressant:1 patient Not on dopamine therapy:1 patient
Method of analysis of CCM images	Manual	Automated	Manual
CNFD	CNFD significantly lower in PD compared to controls (CNFD mean difference: -5.6 no./mm^2, 95% CI [-9.2, -2], P = 0.003)	CNFD did not significantly differ between PD and controls	-
CNFL	CNFL significantly higher in PD participants (CNFL mean difference: 3.2 mm./mm^2, 95% CI [0.3, 6.1], P = 0.031)	CNFL significantly lower in PD (14.23 ± 0.81 mm) than in controls (16.75 ± 0.70 mm; p=0.013)	CNFL significantly lower in PD compared to controls (7.6 ± 2.4mm/mm^2 vs. 15.9 ± 2.6mm/mm^2, p < 0.0001)
CNBD	CNBD significantly higher in PD compared to controls (CNBD mean difference: 86.6 no./ mm^2, 95% CI [55.9, 117.2]), p < 0.001)	CNBD significantly lower in patients with PD (31.78 ± 2.78 mm^{-1}) than in control subjects (43.63 ± 4.54 mm^{-1}; p=0.013)	-
Correlation between CCM parameters and clinical assessments/scales	CNBD and CNFL but not CNFD correlated inversely with UPDRS-III	-	Significant correlation found between ACE-R scores and CNFL (R2 = 0.66, p = 0.02) No relationship between CNFL and SAS symptoms score

Table 1-1 . Summary of recent studies utilising corneal confocal microscopy in Parkinson's disease. CNFD: Corneal nerve fibre density, CNFL: Corneal nerve fibre length, CNBD: Corneal nerve branch density, UPDRS III: Unified Parkinson's Disease Rating Scale III, ACE-R: Addenbrooke's Cognitive Examination Revised, SAS: Survey of Autonomic Symptom

2. Research Designs and Methods

2.1. Study objectives

There is an urgent need to develop more reliable biomarkers in PD for the field of drug development in PD to progress. The research within this thesis was planned to investigate the role of corneal confocal microscopy as a biomarker in PD and atypical PD as skin biopsy studies have demonstrated potential roles for small fibre degeneration to be a marker of disease progression, disease severity and disease type. CCM is a promising tool to investigate small fibre degeneration as it is a non-invasive, rapid, and reproducible technique. Previous studies in PD cohorts of patients have been cross sectional studies with relatively small numbers of participants. Both manual and automated analysis have been utilised in previous studies. The gaps in the literature are as follows:

1. Can CCM stratify patients with PD into different subtypes (motor subtypes or fast progressors vs slow progressors)?
2. How do CCM parameters change longitudinally in subjects with PD?
3. How do CCM measures compare to clinical scales over time?
4. How do manual and automated analyses of CCM images compare in a PD cohort?
5. Which is/are the most sensitive and specific CCM parameter/s in measuring corneal nerve damage in PD?
6. How do CCM parameters in PD compare to CCM parameters in atypical Parkinsonian disorders?

The objectives of the chapters in my thesis are explored in the sections below:

Chapter 3: Automated corneal nerve analysis: A rapid and reproducible technique to quantify neurodegeneration in patients with Parkinson's disease

Previous CCM studies in PD have utilised both automated and manual analysis for quantification of corneal nerve fibres. There have been no studies comparing automated and manual analysis in PD patients. The aim of the study was to assess the validity of automated analysis in a PD cohort.

Chapter 4: Corneal confocal microscopy detects small fibre neurodegeneration in PD using automated analysis

Previous cross-sectional CCM studies in PD cohorts were done in relatively small numbers of participants, which did not enable stratification of PD participants into different subtypes. This was a cross sectional study comparing

CCM parameters between 98 PD participants and 25 healthy controls using automated analysis. The aim of the study was to demonstrate that CCM using automated analysis can identify small fibre neurodegeneration in PD patients and to investigate the association between CCM parameters, PD subtype and severity of disease.

Chapter 5: Corneal confocal microscopy identifies Parkinson's disease with more rapid motor progression**.**

Previous cross-sectional studies of CCM in PD do not enable the utility of CCM as a biomarker of PD to be explored as changes in CCM parameters and clinical scales cannot be assessed over time. This study was a longitudinal study exploring the utility of CCM as a biomarker of disease progression and disease subtype.

Chapter 6: Corneal confocal microscopy shows different degrees of nerve loss in atypical Parkinsonian disorders

Skin biopsy studies have demonstrated denervation in alpha-synucleinopathies and no denervation in tauopathies. Cutaneous biopsies have also demonstrated a differential pattern of alpha synuclein deposition and denervation in PD participants compared to MSA participants. The aim of this study was to assess CCM changes in PD, MSA and PSP to investigate the potential role of CCM as a marker of disease type.

2.2. Methods

2.2.1. Study Approval

NRES Committee/North West approved the pilot (Ref no 12/NW/0086) and larger (Ref no 17/NW/0144) study. Written informed consent was obtained from every participant. This research adhered to the tenets of the Declaration of Helsinki for clinical research involving human subjects.

2.2.2. Participant Recruitment

2.2.2.1. Participants with Parkinson’s disease

Participants with clinically established PD, defined by the UK Brain Bank diagnostic criteria and patients with probable MSA and PSP diagnosed by movement disorder neurologists according to published diagnostic criteria (Gilman *et al.*, 2008; Höglinger *et al.*, 2017) were recruited to the study. Participants were recruited from clinics across Greater Manchester and via Parkinson’s UK and Fox Trial Finder websites. Healthy controls from a pre-

existing database from Professor Rayaz Malik's University of Manchester corneal confocal microscopy group were age matched and compared to patients with PD, PSP and MSA.

Inclusion criteria for the PD participants were as follows:

1. The participant has PD

2. The participant is aged between 18 and 90 years old

3. The participant is able to understand the study and consent into the study or a consultee is able to advise on their behalf if they lack capacity

Exclusion criteria for the PD participants are as follows:

1. Diabetes

2. Active malignancy

3. Hepatic disease

4. Chronic alcoholism or any other known cause of neuropathy

5. Chronic corneal pathologies

6. History of refractive surgery

7. Systemic diseases known to affect the cornea including Fabry's disease, chronic kidney disease and autoimmune conditions such as Sjogren's syndrome.

The inclusion criteria for participants with PSP and MSA were the same as the PD group except that the participant must have PSP or MSA and not PD. The exclusion criteria were the same.

2.2.2.2. Control Participants

Control participants were selected from a pre-existing database of healthy controls by selecting participants of comparable age to participants with PD, MSA and PSP so that there was no significant difference in mean age

between the different groups of patients. A previous study found a significant decrease in corneal nerve parameters with increasing age in healthy controls (Sharma, Tobin, Prashanth R.J. Vas, *et al.*, 2018). Gender, height and weight have not been found to influence CCM results (Sharma, Tobin, Prashanth R.J. Vas, *et al.*, 2018)

2.2.3. Medical History and Demographics

Information about subjects' gender, age, height, weight, duration of diagnosis, other medical conditions, medications including dopaminergic therapies, alcohol intake and smoking history were obtained.

2.2.4. Blood Samples

Blood tests including full blood count (FBC), urea and electrolytes (UEs), glycated haemoglobin (HbA1c), immunofluorescence anti-nuclear antibodies (IFANA), B12, Folate, Immunoglobulins (IgGs), serum electrophoresis and thyroid function tests (TFTs) were performed at the first visit to exclude other known aetiologies of neuropathy.

2.2.5. Neurological Assessments

A clinical examination to detect clinical evidence of neuropathy was carried out. The Neuropathy Disability Score (NDS) (Appendix 1) was used as a means of rating the severity of neuropathy. NDS is a clinical scoring system which includes assessing ankle reflex, vibration, pin prick and temperature sensation on both great toes. The scores range from 0-10. The severity of neuropathy is divided into four groups: NDS 0-2: None, NDS 3-5: Mild, NDS 6-8: Moderate and NDS 9-10: severe.

The presence or absence of the perception of vibration was measured using a 128Hz tuning fork against the patients' great toe. The patients were asked to close their eyes and report whether the tuning fork placed on the great toe was the vibrating tuning fork or the non-vibrating tuning fork. The assessment was carried out three times and an average was obtained.

Perception of pain was assessed using a neurotip which has a sharp and blunt end. Patients were asked to close their eyes. They were presented with stimulus options A and B. Patients were asked to identify the sharp stimulus. The test was carried out three times and an average was obtained.

Two metal rods which were cold and hot respectively were used to assess perception of temperature. The patients were presented with two options, A and B, and asked to identify the hot metal rod. The test was also repeated three times to obtain an average.

Patients were scored 1 for an abnormal result and 0 for a normal result

Achilles' tendon reflexes were elicited using a tendon hammer. Patients were scored 0 if the ankle reflex was present, 1 if the reflex required reinforcement to be elicited and 2 if the reflex was absent.

Clinical progression of PD was measured using the validated MDS-UPDRS scale (Appendix 1). The MDS-UPDRS scale has four parts: I: Non motor Experiences of daily living (13 items), II: Motor Experiences of Daily Living (13 items), III: Motor Examination (33 scores for 18 items, some items requiring scores for right, left or other distribution), IV: Motor Complications (6 items). Each item scored has five possible clinical descriptors: 0=normal, 1=slight, 2=mild, 3=moderate, 4=severe. The criteria for each clinical descriptor is described for the assessor to score the item (Goetz *et al.*, 2008) .

Patients were also rated using the Hoehn and Yahr Scale (Appendix 1) which is divided into six stages: 0 - Asymptomatic; 1 - Unilateral involvement only; 2 - Bilateral involvement without impairment of balance; 3 - Mild to moderate involvement, some postural instability but physically independent, needs assistance to recover from pull test; 4 - Severe disability, still able to walk or stand unassisted; 5 - Wheelchair bound or bedridden unless aided (Goetz *et al.*, 2004).

Cognition was assessed using the Montreal Cognitive Assessment (MoCA) (Appendix 1). The MoCA assesses different cognitive domains: executive function, visuospatial skills, language, memory, attention/concentration, calculations and orientation. The total possible score is 30 points. Scores of 26 and above are considered normal (Nasreddine *et al.*, 2005).

PD participants were asked to complete a Parkinson's Disease Questionnaire-39 (PDQ-39) questionnaire (Appendix 1). PDQ-39 is a participant completed questionnaire to assess health-related quality of life in PD. There are 39 questions measuring 8 domains: mobility (10 items), activities of daily living (6 items), emotional well-being (6 items), stigma (4 items), social support (3 items), cognition (4 items), communication (3 items) and bodily discomfort (3 items). Participants were asked to rate the frequency of each item by selecting one of 5 options: never, occasionally, sometimes, often, always or cannot do at all. PDQ-39 summary index (PDQ-39 SI), the sum of dimension total scores divided by 8, was calculated for each individual participant (Jenkinson *et al.*, 1997).

2.2.6. Ophthalmic Assessments

2.2.6.1. Slit Lamp Biomicroscopy

Trained optometrists carried out the ophthalmic assessments. The eyelids, conjunctiva, sclera cornea, anterior chamber, lens and posterior chamber were assessed using a slit lamp biomicroscope (Slit Lamp BD 900®, Haag Streit International, Koeniz, Switzerland) and confirmed to be clinically normal. The examination was performed in a dark room with the participant's chin resting on the chin rest of the instrument.

2.2.6.2. Corneal Confocal Microscopy

Corneal confocal images were acquired using a laser scanning corneal confocal microscope: Heidelberg Retinal Tomograph III Rostock Cornea Module (HRT III RCM); Heidelberg Engineering GmbH, Heidelberg, Germany (Figure 3). The laser system is a class 1 laser system which uses a 670-nm helium neon diode laser. The laser beam spot was 1 µm in diameter. A x63 objective lens was used. The field of view was 400x400 µm. 2-dimensional images measuring 384 x 384 µm and 10 µm per pixel optical resolution were created. For hygienic reasons, a disposable applanating cap: TomoCap; Heidelberg Engineering GmbH, was used to cover the lens. The applanating cap has contact with the cornea. HRT III RCM uses a digital image capture system. A charged couple device camera (CCD) was attached to the confocal microscope to capture corneal images and to correctly position the applanating cap (Figure 2-1).

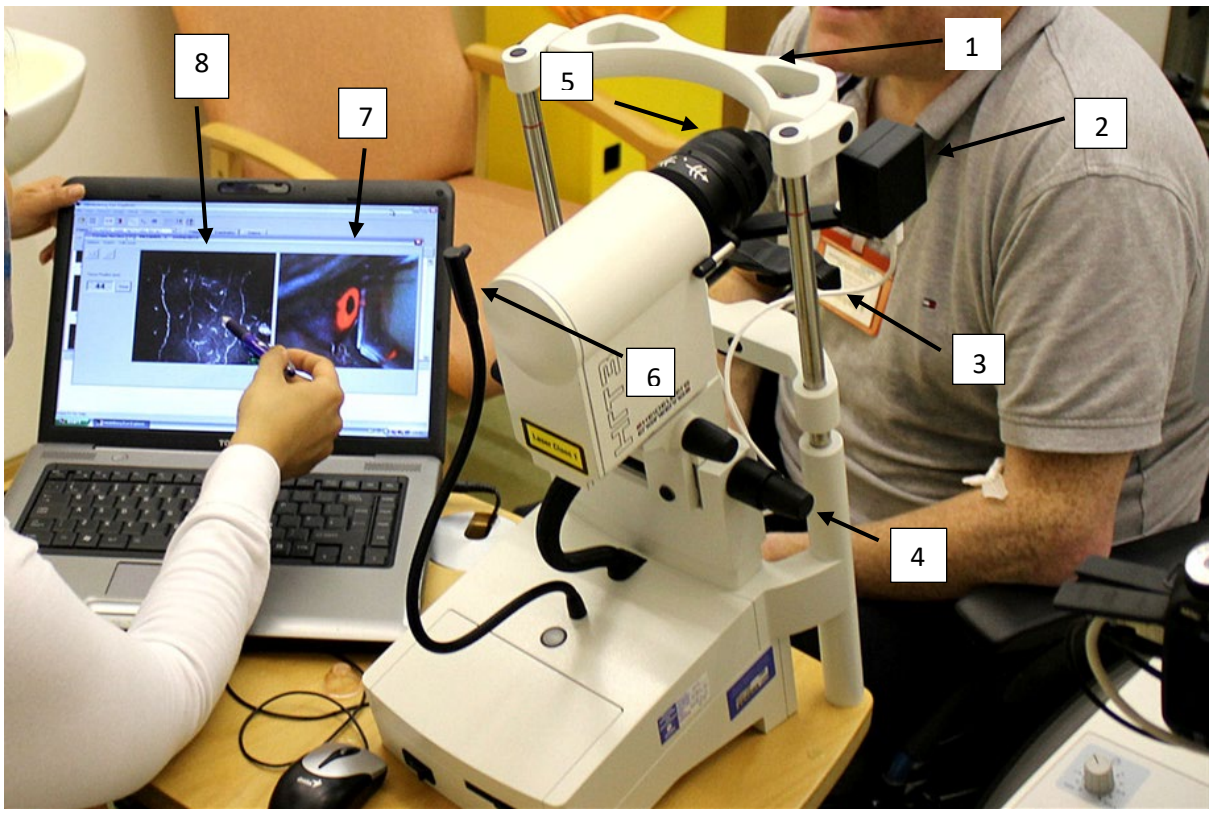

Figure 2-1 Heidelberg Retinal Tomograph III Rostock Cornea Module (HRT III RCM) Corneal Confocal Microscope (CCM). 1: forehead bar, 2: charged couple device camera, 3: chin rest, 4: Knobs to align CCM, 5: Objective lens, 6: outer fixation light, 7: charged couple device camera live image, 8: laser scanning camera live image.

2.2.6.3. Capturing Images from the Participant's Cornea using Corneal Confocal Microscopy

The procedure was explained to the participant and the examination was performed in a dark and quiet room. The participant's details including participant identifier, gender, date of birth and study identifier were entered into the software (Heidelberg Eye Explorer, Heidelberg Engineering GmBH, Heidelberg, Germany). CCM was done with PD participants in the 'ON' state to obtain high quality images and minimize interference from motor symptoms.

The objective lens of the corneal confocal microscope was set to +12 diopters and the camera was adjusted to the lowest position. The lens was then locked by rotating it anticlockwise. A drop of bubble free Viscotears (Carbomer 980, 0.2 %; Novartis, UK) was applied on the objective lens and covered by a sterile applanating cap (Figure 2-2).

The Viscotears forms a meniscus that acts as a coupling agent between the lens and the applanating cap. The applanating cap was pushed as far back as possible over the holder and care was taken not to touch the front surface during mounting. The laser scanning camera was then moved as far back as possible on the camera mount and the focal plane of the adjustment wheel was adjusted until a bright reflection was observed, indicating that the lens was focused within the front of the applanating cap. The depth setting was reset to zero.

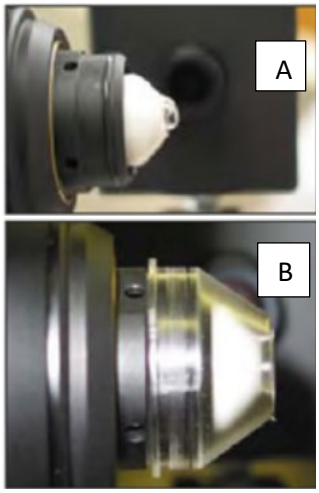

Figure 2-2 Preparation of the objective lens with (A) a drop of viscotears and (B) applanating cap (Tavakoli and Malik, 2010)

The participant was then prepared for the procedure. A drop of 0.4% benoxinate hydrochloride was used to anaesthetize each eye. Viscotears (Carbomer 980, 0.2 %; Novartis, UK) was also applied to the participants' eyes to reduce any discomfort and to act as a coupling agent between the applanating cap of the device and the cornea. The position of the participant's head was stabilised by getting the participant to place his/her chin on a chin rest and press their forehead against the forehead bar. Participants were asked to fixate on an outer fixation light with the eye contralateral to the one being examined.

The CCD camera was positioned so that its optical axis was perpendicular to the optical axis of the laser scanning camera. The CCD camera provided a lateral view of the position of the objective lens relative to the surface of the cornea, via a live image, which enabled the operator to monitor and adjust the position of the lens. The laser scanning camera was moved forward until it was approximately 5-10 mm from the applanating cap. At that distance, the laser camera was moved up/down and left/right until the applanating cap was positioned at the centre

of the cornea. The reflection of the laser beam from the cornea was checked so that it was at the anterior pole of the cornea (Figure 2-3). The laser scanning camera was then moved further forwards until there was good contact between the applanating cap and the cornea. Care was taken not to apply too much pressure on the cornea to prevent pressure lines from appearing on the images

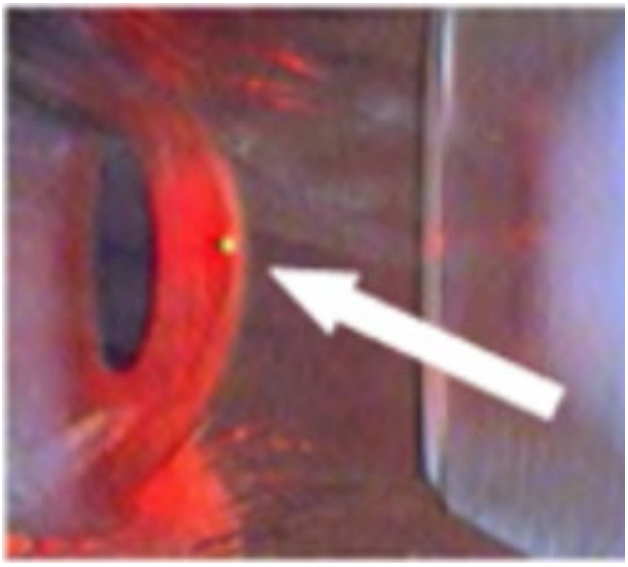

Figure 2-3 Alignment of the laser beam reflection at the anterior pole of the cornea to ensure central images of the cornea are captured (Tavakoli and Malik, 2010)

The laser camera was turned on and the image acquisition window was opened. Images from all corneal layers (epithelium, Bowman's layer, sub-basal layer, stroma and endothelium) were captured using the 'section' mode. The section mode is used to capture and store a single image with each press of the foot switch, enabling images from the whole corneal layer to be acquired. The distance between each image was approximately 1µm; therefore, if the sub-basal layer were approximately 10 µm, 10 images could be captured. Other modes of image acquisition include sequence scan mode and volume scan mode. The sequence scan mode enables a sequence of up to 100 images to be obtained with an adjustable frame rate which can be set between 1 frame to 30 frames per second. In volume scan mode, a series of 40 images at consecutive focal planes is captured.

Once the examination was complete, the camera was turned off and the participant was advised not to rub his/her eyes until the local anaesthetic had worn off. The applanating cap was removed and the instrument was cleaned.

2.2.6.4. Selection of images

Approximately 6 images (3 per eye) of the sub-basal layer from the central cornea were selected, based on the number of vertical nerves, depth, contrast and quality of the images. Corneal nerves are usually vertically orientated at the centre of the cornea and obliquely orientated at the peripheries. Therefore, images with vertically orientated nerves were selected. Images selected had to be of high quality with paucity of pressure lines and of optimal contrast (Figure 2-4).

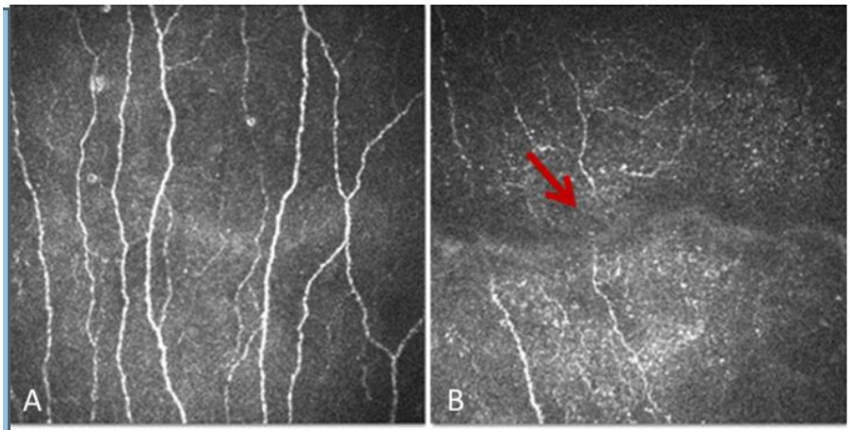

Figure 2-4 Corneal confocal microscopy images demonstrating (A) a good-quality image from the central cornea with vertically orientated nerves and (B) a poor-quality image from the central cornea with a pressure line denoted by the red arrow.

Analysis of the images

Manual and automated analysis of the images have been utilised in the studies described in this thesis (Figure 2-5)

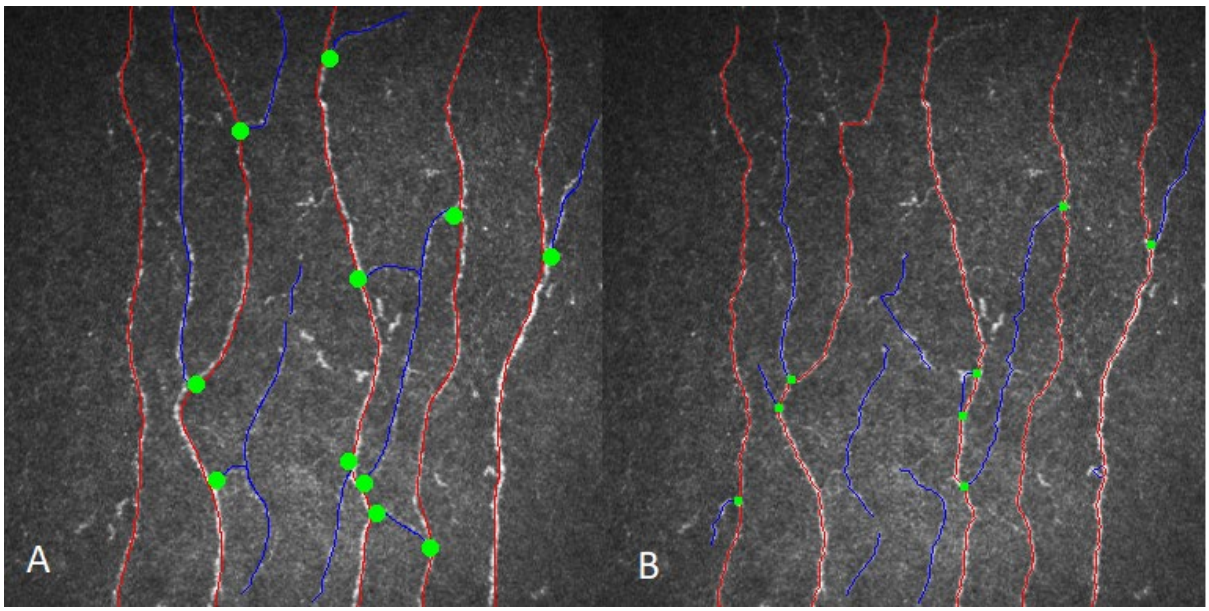

Figure 2-5 CCM images analysed using (A) manual analysis (CCMetrics Image Analysis Tools version 1.1) and (B) automated analysis (ACCmetrics version 2).

2.2.6.5. Manual image analysis

Manual analysis was carried out using purpose designed software (CCMetrics Image Analysis Tools v 1.1.)

The following corneal nerve parameters were measured using manual analysis:

1. Corneal nerve fibre density (CNFD): number of main nerve fibres per frame (no/mm^2)

2. Corneal nerve branch density (CNBD): number of intersections between main nerves and secondary nerves per frame (no/mm^2)

3. Corneal nerve fibre length (CNFL): the total length of all nerve fibres per frame (mm/mm^2)

Images were opened in the software and each corneal parameter was measured by selecting the relevant option in the metrics box. A digital pen was used to trace nerves and identify intersections between main nerves and branches. Each CCM parameter was identified by a different colour in the software: main nerves in red, nerve branches in blue and intersections between main nerves and nerve branches in green (Figure 2-6). Once tracing of the nerves was completed for each image, the measurements for each parameter was automatically generated by

the software and the result would appear in the measurement box. The measurements for all 6 images for each participant was saved in a text file and copied into an excel spreadsheet, and the average value for each parameter was calculated.

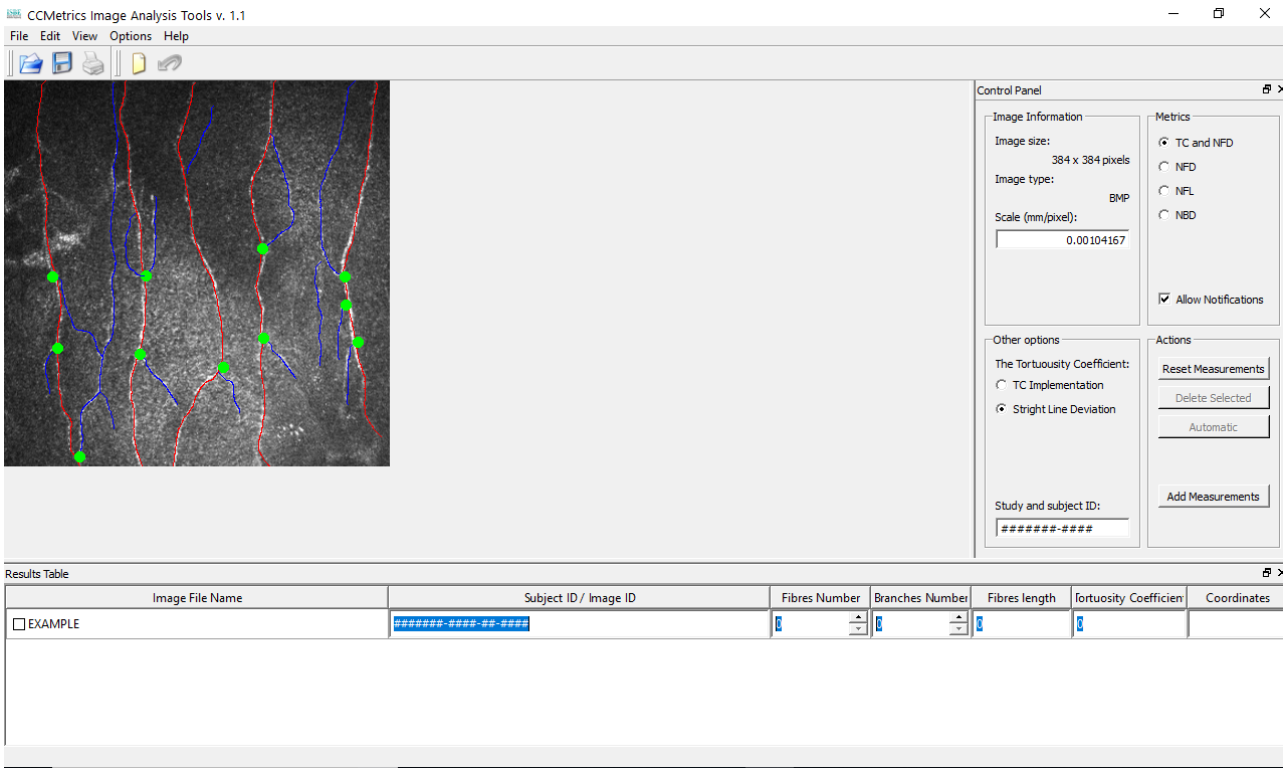

Figure 2-6 CCMetrics image analysis tools window. Nerves and branch points are manually traced (red: main nerves, green: branch points, blue: nerve branches) and the results are generated by the software in the results table.

2.2.6.6. Automated image analysis

Automated analysis was carried out using ACCmetrics V2 (M.A. Dabbah, Imaging Science, The University of Manchester, 2010). The software uses a multi scale dual model detection algorithm to detect nerves and can be used for either single image analysis or multiple image analysis (Figure 2-7). Using the multiple image analysis mode, a dataset of participants' images was imported into the software. A results folder was created in the investigator's laptop. The software would automatically analyse the images and save the annotated images and corresponding analysis results as a text file in the designated folder. In addition to the three parameters measured by manual analysis, the automated software generates 3 other CCM parameters as outlined below:

1. Corneal total branch density (CTBD): the total number of branch points per frame (no/mm^2)

2. Corneal nerve fibre area (CNFA): The total fibre area per frame (mm^2/mm^2)

3. Corneal nerve fibre width (CNFW): The average nerve fibre width per frame (mm/mm^2)

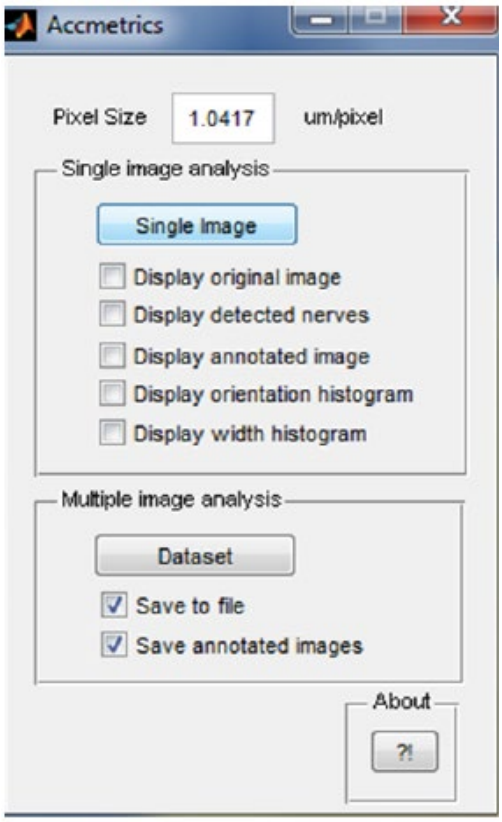

Figure 2-7 Accmetrics Version 2 window

2.2.6.7. Addressing potential bias in image selection and quantification of corneal nerves

Potential bias may arise during selection of images and quantification of corneal nerves. Therefore images were selected using an established protocol (Kalteniece *et al.*, 2017) to minimize selection bias. Corneal nerve quantification performed using automated analysis ensured blinded quantification of corneal nerves. Manual quantification of corneal nerves was performed using a strict protocol to minimize bias and inter-/intra-rater variability.

3. Automated corneal nerve analysis: A rapid and reproducible technique to quantify neurodegeneration in participants with Parkinson's disease

Authors' contributions

Sze Hway Lim recruited PD participants, performed neurological assessments, analysed images using automated analysis, did statistical analysis, interpreted data, drafted manuscript and integrated author comments to produce final draft.

Maryam Ferdousi and Alise Kalteniece performed corneal confocal microscopy assessments. Maryam Ferdousi analysed images manually.

Ziyad Mahfoud provided statistical input.

Ioannis Petropoulos, Professor Rayaz Malik, Dr Kobylecki and Professor Silverdale reviewed and critiqued manuscript.

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3.2. Abstract

Objective: Corneal nerve loss has been proposed as an objective surrogate marker of neurodegeneration in participants with Parkinson's disease (PD). We have compared manual and fully automated analysis of corneal confocal microscopy (CCM) images in a cohort of participants with Parkinson's disease and control subjects.

Methods: Sixty-four participants with PD and twenty-five healthy controls underwent CCM. Corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD) and corneal nerve fibre length (CNFL) were quantified manually by an expert assessor and using fully automated software. Intraclass correlation (ICC) and Bland Altman plots were used to assess agreement and ROC curves were used to assess the ability of manual and automated analysis to differentiate participants with PD from controls. Furthermore, the ability to identify significant corneal nerve loss (<2SD of the control group) in PD participants was compared between manual and automated analysis.

Results: CNFD, CNBD and CNFL were lower in participants with PD compared to controls. The values were lower in automated compared to manual analysis, but there was good agreement between manual and automated analysis for CNFD (ICC: 0.812, 95% confidence interval (CI): 0.709-0.882) and CNFL (ICC: 0.821, 95% CI: 0.721- 0.887) and moderate agreement for CNBD (ICC: 0.640, 95% CI: 0.469-0.765). The area under the curve (AUC) for identifying PD participants from controls for CNFD manual (0.756 (95%CI 0.637, 0.835)) and automated (0.685 (95% CI 0.568, 0.802) and CNBD manual (0.781 (95% CI 0.678, 0.884)) and automated (0.707 (95% CI 0.595, 0.819)) were comparable, but was significantly better for CNFL manual (0.828 (95%CI 0.734, 0.922)) compared to automated (0.701 (95% CI 0.583, 0.818), diff =0.127, p<0.001).

Conclusion: Both manual and automated CCM analysis identifies a loss of corneal nerve fibres in participants with PD with overall good agreement between automated and manual corneal nerve quantification. Automated corneal nerve analysis identifies more participants with an abnormal CNFD compared to manual analysis.

3.3. Introduction

Parkinson's disease (PD) is a heterogenous clinical syndrome which presents primarily as a movement disorder, but non-motor features such as autonomic dysfunction and peripheral neuropathy are increasingly recognised for their prognostic and subtyping value in PD (Nolano *et al.*, 2018; Borghammer and Berge, 2019). Intraepidermal nerve fibre loss and phosphorylated alpha synuclein has been detected within dermal nerve fibres of participants with PD (Donadio *et al.*, 2014; Doppler *et al.*, 2014) and higher alpha synuclein ratios have been associated with more advanced PD (Wang *et al.*, 2013). Differences in the patterns of cutaneous denervation and alpha synuclein deposition may also help differentiate participants with PD from other atypical forms of Parkinsonism (Melli *et al.*, 2018; Donadio *et al.*, 2020). Intraepidermal nerve fibre (IENF) degeneration and impaired regeneration has been shown to correlate with somatic and autonomic symptoms and deficits in participants with PD (Jeziorska *et al.*, 2019). These findings have led to an interest in exploring small fibre morphology as a biomarker for neurodegeneration in PD. However, skin biopsy is an invasive procedure that requires expert laboratory assessment and is not widely available.

Corneal confocal microscopy is a rapid non-invasive ophthalmic technique that enables in vivo visualisation of small nerve fibres (Kalteniece *et al.*, 2017) and has been used to detect neurodegeneration in a wide range of peripheral neuropathies (Petropoulos *et al.*, 2020) including diabetic neuropathy (Tavakoli, Quattrini, *et al.*, 2010), idiopathic small fibre neuropathy (Tavakoli, Marshall, *et al.*, 2010), Charcot Marie Tooth disease (Tavakoli *et al.*, 2012), chronic inflammatory demyelinating peripheral neuropathy (Stettner *et al.*, 2016) and HIV neuropathy (Kemp *et al.*, 2017). Corneal confocal microscopy and IENFD also have comparable diagnostic utility for diabetic neuropathy (Chen *et al.*, 2015; Alam *et al.*, 2017).

The quantification of corneal nerve morphology has been shown to have good reproducibility for evaluating corneal nerve fibre density (CNFD) and corneal nerve fibre length (CNFL) and to a lesser extent corneal nerve branch density (CNBD), using manual analysis (Kalteniece *et al.*, 2017). Manual analysis is however, labour intensive and subject to interobserver variability due to the subjective criteria applied to identify each nerve structure (Petropoulos *et al.*, 2013). Automated nerve analysis represents a less precise but faster and more

consistent and unbiased approach for quantifying corneal nerve fibres and has the advantage of scalability and improved reproducibility for use in large cohort studies and clinical trials. Studies have shown that manual and automated evaluation of corneal nerve fibre length have comparable utility in the diagnosis of diabetic neuropathy (Petropoulos *et al.*, 2014; Dehghani *et al.*, 2016).

Several recent CCM studies have demonstrated significant corneal nerve loss in participants with PD using manual (Kass-Iliyya *et al.*, 2015; Misra *et al.*, 2017) and automated analysis (Podgorny *et al.*, 2016; Lim *et al.*, 2020). Previous studies assessing the validity of fully automated CCM analysis have primarily focused on cohorts of participants with diabetes (Petropoulos *et al.*, 2014; Pacaud *et al.*, 2015). There are no studies to date, comparing the utility of manual and automated quantification of corneal nerve fibre parameters in a PD cohort. This study aims to compare manual and automated corneal nerve image analysis in a cohort of participants with PD.

3.4. Methods

3.4.1. Ethics

NRES Committee/North West approved the study (Ref no 17/NW/0144).

3.4.2. Study subjects

Participants with PD aged 18-90 years fulfilling Queen Square Brain Bank criteria were invited to participate. Participants were recruited from clinics across Greater Manchester and via Fox Trial Finder and Parkinson's UK websites between September 2017 and September 2018. The exclusion criteria were concurrent diagnosis of diabetes, active malignancy, hepatic disease, any other known cause of neuropathy, chronic corneal pathology, history of refractive surgery and any systemic disease known to affect the cornea. Healthy aged match volunteers were used as controls. Written informed consent was obtained from all participants.

3.4.3. Corneal Confocal Microscopy

CCM examination using laser scanning corneal confocal microscopy HRT III (Heidelberg Retinal Tomograph III Rostock Cornea Module, Heidelberg Engineering, Heidelberg, Germany) was performed for both eyes (Kalteniece *et al.*, 2017). Six images (3 per eye) from the central sub-basal nerve plexus were selected by a single expert in a

masked fashion taking into account the quality, depth and variability according to our established protocol (Kalteniece *et al.*, 2017).

3.4.4. Image Analysis

Images were analysed manually by a single experienced examiner (MF) using a custom designed nerve analysis software package (CCMetrics, MA Dabbah; Imaging Science and Biomedical Engineering, University of Manchester, UK). Fully automated analyses of the same images were performed using ACCMetrics (M.A. Dabbah, Imaging Science, The University of Manchester, 2010) software (Dabbah *et al.*, 2010, 2011). The parameters measured were corneal nerve fibre density (CNFD): number of nerve fibres per square millimetre (no/mm^2), corneal nerve branch density (CNBD): number of intersections between main nerves and branches per square millimetre (no/mm^2) and corneal nerve fibre length (CNFL): total length of all nerve fibres and branches per square millimetre (mm/mm^2).

3.4.5. Statistical Analysis

All analyses were performed using IBM SPSS version 25. Shapiro-Wilk test was used to assess normality of distribution. Independent samples t-test was used to compare means of normally distributed data and Mann Whitney U test was used for non-parametric data. Intraclass correlation coefficient (ICC) was used as a measure of repeatability between manual and automated analysis and Bland and Altman plots were computed to demonstrate the agreement between manual and automated analysis methods. ICC was considered excellent if the value was greater than 0.9, good for values between 0.75-0.9, moderate for values between 0.5-0.75 and poor for values less than 0.5 (Koo and Li, 2016). Receiver operating characteristic curves (ROC) analysis was performed to compare the discriminatory ability of automated and manual quantification of corneal nerve parameters to differentiate PD participants from healthy controls. The optimum cut-off points for the identification of PD participants from controls using CCM parameters were determined by selecting values where the sensitivity and specificity were at a ratio of 1:1. Automated and manual AUCs were compared based on empirical ROC curve estimation described by Zhou et al (Zhou, Obuchowski and McClish, 2011). Graphs were created using Graphpad Prism (Version 9.0 for windows, Graphpad Software, La Jolla California, USA)

A corneal nerve parameter was considered abnormal if it was <2 standard deviations (SD) of the mean value in controls. Cohen's Kappa was used to determine if there was agreement between manual and automated analysis in the identification of abnormal CCM parameters. Kappa values between 0.81-1.00 were considered very good, 0.61-0.80 were considered good, 0.41-0.60 were considered moderate, 0.21-0.40 were considered fair and less than 0.20 were considered poor (Altman, 1991).

3.5. Results

3.5.1. Demographics

The demographic and clinical parameters in participants with PD (n=64) and controls (n=25) are given in Table 3-1.

	PD participants	Controls	P
Age (years)	64.1 ± 7.8	63.1 ± 6.8	0.564
Gender	49M 15F	14M 11F	0.055
Disease duration (months)	56.9 ± 42.6	N/A	N/A
MDS UPDRS III	27.5 ± 10.3	N/A	N/A
Hoehn and Yahr Stage	I:9; II:45; III: 10	N/A	N/A
MoCA	26.5 ± 0.4	N/A	N/A

Table 3-1 Demographics and clinical characteristics of participants. Data shown as mean ± SD. PD: Parkinson's disease, MDS UPDRS: Movement disorder society unified Parkinson's disease score, MoCA: Montreal Cognitive Assessment

3.5.2 Corneal nerve morphology in PD participants compared to controls using manual and automated analysis

There was a significant reduction in manual and automated CNFD ($p<0.001$, $p=0.006$), CNBD ($p<0.001$, $p=0.007$) and CNFL ($p<0.001$, $p=0.002$) in participants with PD compared to controls (Figure 3-1).

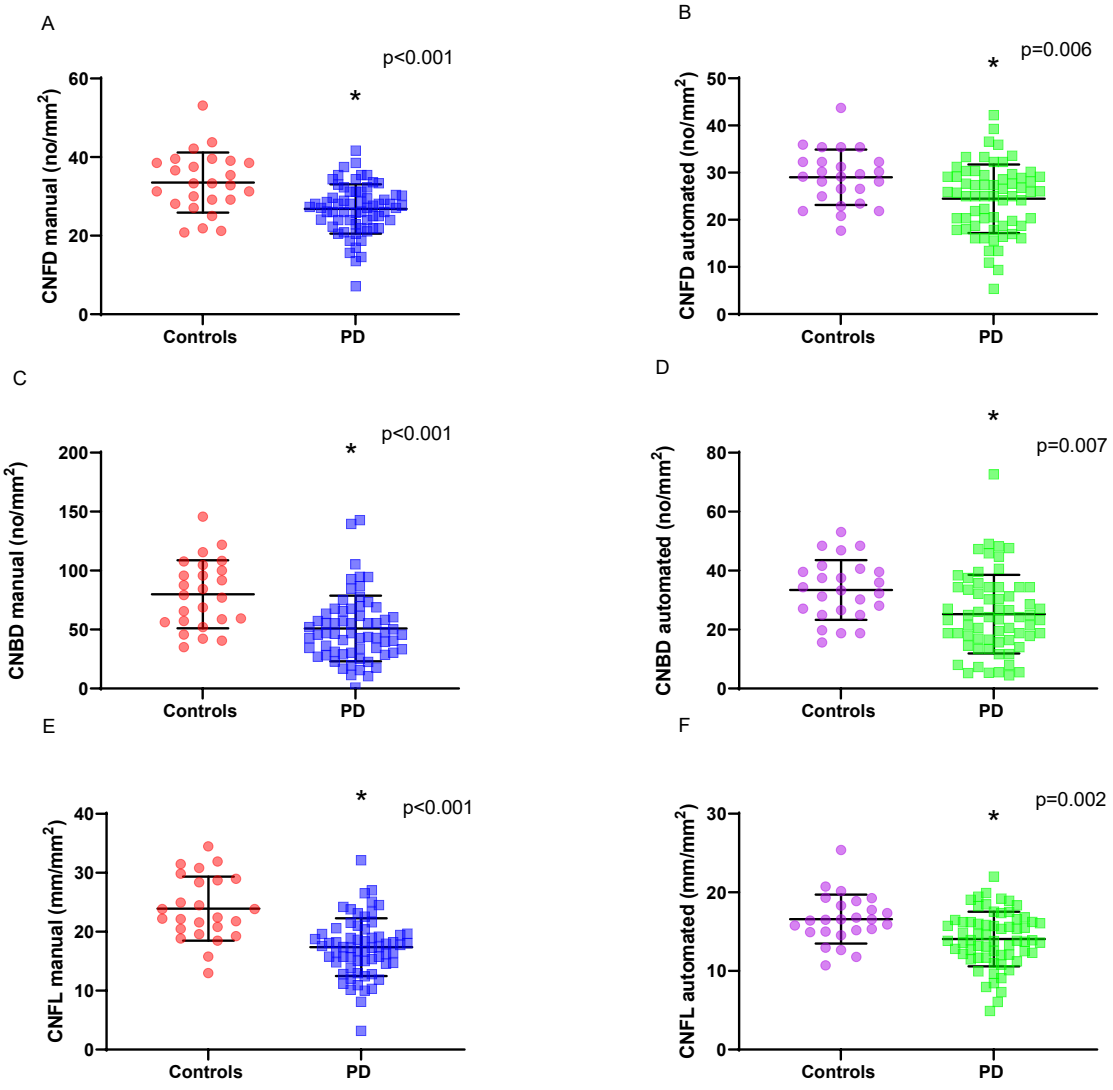

Figure 3-1 Corneal nerve parameters in controls compared to participants with Parkinson's disease using manual and automated analysis. Mean ± SD of corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD) and corneal nerve fibre length (CNFL) using manual (A, C, E) and automated (B, D, F) analysis in participants with Parkinson's disease (PD) compared to controls with significance levels.

3.5.3. Automated vs manual analysis in PD participants

Manual measurements were higher than automated measures for all CCM parameters (Table 3-2). The mean differences (±SD) were 2.34 no/mm^2 ± 4.17 for CNFD, 25.65 no/mm^2 ± 18.53 for CNBD and 3.31 ± 2.55mm/mm^2 for CNFL.

	Manual	Automated	ICC (95% CI)
CNFD (no/mm^2)	26.81 ± 6.29	24.47 ± 7.28	0.812 (0.709, 0.882)
CNBD (no/mm^2)	50.89 ± 27.85	25.24 ± 13.32	0.640 (0.469, 0.765)
CNFL (mm/mm^2)	17.38 ± 4.91	14.07 ± 3.48	0.821 (0.721, 0.887)

Table 3-2 CCM measurements and ICC in participants with Parkinson's disease analysed using manual and automated analysis. Data shown as mean ± SD and intraclass correlation coefficient (95% confidence interval). CNFD: Corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, ICC: intraclass correlation coefficient, CI: Confidence interval.

3.5.4. Intraclass correlation coefficient for manual vs automated CCM

The ICC for CNFD and CNFL were good, whereas the ICC for CNBD was moderate (Table 3-2). Figure 3-2 show Bland-Altman plots to demonstrate the agreement between manual and automated analysis for all CCM parameters.

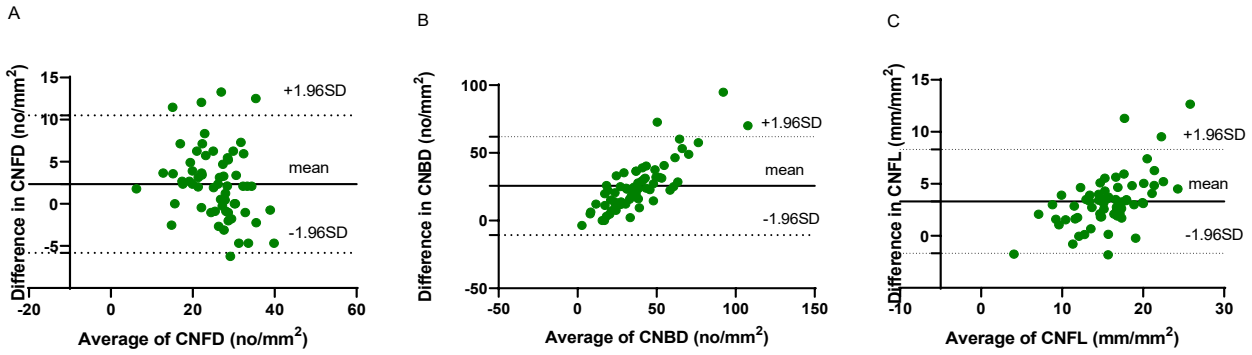

Figure 3-2 Bland-Altman plots for CNFD (A) CNBD (B) and CNFL (C) indicating the level of agreement between fully automated and manual measurements. The continuous line demonstrates the mean difference between manual and automated measurements (manual - automated). The dashed lines demonstrate the 95% limits of agreement.

3.5.5. Diagnostic ability for manual vs automated CCM

ROC curves to demonstrate the ability of automated and manual analysis of CNFD, CNBD and CNFL in identifying participants with PD compared to controls are shown in Figure 3-3. The area under the curve (AUC) for CNFD manual was 0.756 (95%CI 0.637, 0.835) with an optimum cut off point of 29.17 no/mm^2, sensitivity of 69% and specificity of 72%, and for automated CNFD it was 0.685 (95% CI 0.568, 0.802) with an optimum cut off point of 27.9 no/mm^2, sensitivity of 67% and specificity of 64%, with no significant difference between manual and automated CNFD (difference= 0.071, p=0.154). The AUC for CNBD manual was 0.781 (95% CI 0.678, 0.884) with an optimum cut off point of 58.54 no/mm^2, sensitivity of 70% and specificity of 72%, and for automated it

was 0.707 (95% CI 0.595, 0.819) with an optimum cut off point of 27.60 no/mm^2, sensitivity of 64% and specificity of 68% and did not differ significantly between manual and automated CNBD (difference=0.074, p=0.088). The AUC for CNFL manual was 0.828 (95%CI 0.734, 0.922) with an optimum cut off point of 20.43mm/mm^2, sensitivity of 78% and specificity of 76% and for CNFL automated it was 0.701 (95% CI 0.583, 0.818) with an optimum cut off point of 15.76mm/mm^2, sensitivity of 67% and specificity of 64%, which was significantly better for manual compared to automated analysis (difference =0.127, p<0.001).

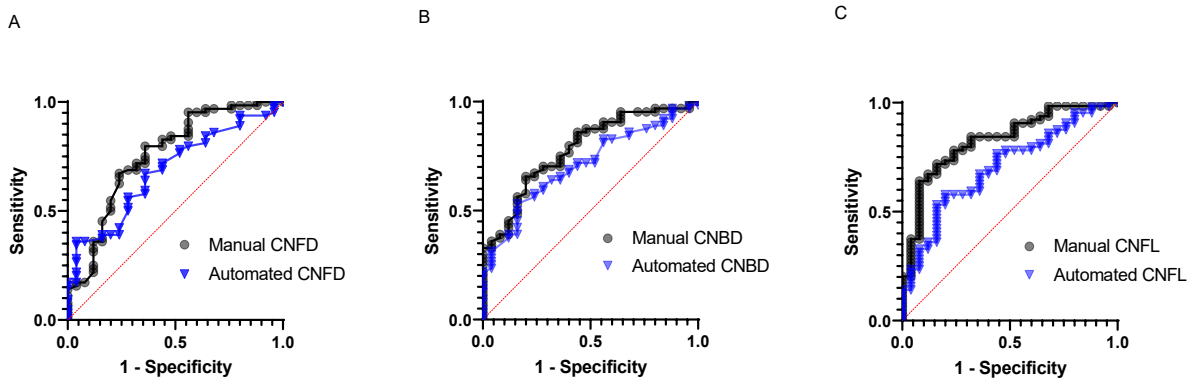

Figure 3-3 Receiver Operating Curves for manual and automated analysis for identifying participants with PD compared to controls. Receiver operating characteristic curves for manual (black) and automated (blue) CNFD (A), CNBD (B) and CNFL (C).

3.5.6. Detection of abnormal CCM parameters between automated and manual analysis

A CCM value <2 standard deviations (SD) of the mean value in controls was considered abnormal in participants with PD (Table 3-3). Cohen's Kappa was used to determine if there was agreement between identification of abnormal CCM parameters in the PD cohort using manual and automated analysis. For CNFD, manual and automated analysis agreed on 5 abnormal results. Automated analysis classified an additional 6 abnormal CNFD results, classified as normal by manual analysis. There was moderate agreement between manual and automated analysis, K=0.580. For CNBD, manual and automated analysis agreed on 4 abnormal results. Automated analysis classified 6 results as abnormal when manual analysis classified the results as normal. Manual analysis classified

3 results as abnormal classified as normal by automated analysis. There was fair agreement between manual and automated analysis, K=0.392. For CNFL, manual and automated analysis agreed on 8 abnormal results. Automated analysis classified 1 result as abnormal when manual analysis classified the result as normal. Manual analysis classified 5 results as abnormal when automated analysis classified the results as normal. There was good agreement between manual and automated analysis, K=0.673.

	Manual (mean ± SD)	**<2SD cut-off**	**Automated (mean ± SD)**	**<2SD cut-off**
CNFD (no/mm^2)	33.53 ± 7.63	18.27	29.02 ± 5.86	17.31
CNBD (no/mm^2)	79.89 ± 28.80	22.30	33.41 ± 10.13	13.16
CNFL (mm/mm^2)	23.92 ± 5.43	13.07	16.59 ± 3.11	10.37

Table 3-3 Manual and automated results of CCM parameters in control group with abnormal cut off values CNFD: Corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, SD: standard deviation.

3.6. Discussion

In the present study we show evidence of varying degrees of corneal nerve loss using both manual and automated analysis as demonstrated by the overlap in CCM parameters between healthy controls and PD participants. There is good agreement between automated and manual corneal nerve analysis for corneal nerve fibre density and length and moderate agreement for corneal nerve branch density. This confirms previous findings in participants with diabetic neuropathy (Petropoulos *et al.*, 2014) reflecting the limitations of automated analysis to accurately detect branches. This may have particular relevance in PD as previous studies have shown variability with some studies showing a decrease (Podgorny *et al.*, 2016) whilst our previous pilot study showed an increase (Kass-Iliyya *et al.*, 2015) and our recent study using automated analysis showed a decrease (Lim *et al.*, 2020) in corneal nerve branch density. The ability to identify participants with PD from healthy controls was higher for manual analysis and this difference was significant for CNFL.

There is a substantial body of work demonstrating cutaneous phosphorylated alpha synuclein deposition and small fibre degeneration in PD populations compared to healthy controls (Wang *et al.*, 2013; Donadio *et al.*, 2014; Doppler *et al.*, 2014; Kuzkina *et al.*, 2019). Intriguingly, studies have also demonstrated structural similarities between Lewy body pathology in the brain and cutaneous nerve deposits of alpha synuclein (Doppler *et al.*, 2014; Kuzkina *et al.*, 2019). These findings suggest the possibility of measuring small fibre degeneration to monitor disease progression or identify a severe disease subtype. Some studies have shown a difference in small fibre loss between participants with PD and participants with atypical Parkinsonism (Melli *et al.*, 2018; Donadio *et al.*, 2020), suggesting the possible utility of small fibre degeneration in differentiating the conditions.

However, the quantification of small fibre dysfunction or degeneration can be challenging. Nerve conduction studies cannot be utilised as they assess large fibre dysfunction. Whilst quantitative sensory testing (QST) can be used to evaluate for small fibre neuropathy (Fruhstorfer, Lindblom and Schmidt, 1976) it has several limitations as the findings can be influenced by psychogenic conditions (Verdugo and Ochoa, 1993) and abnormal results do not localise to peripheral or central nervous system aetiologies (Maier *et al.*, 2010). Skin biopsies allow a reliable

diagnosis of small fibre neuropathy (McCarthy *et al.*, 1995), however, they are invasive, labour intensive and costly.

Corneal confocal microscopy enables non-invasive visualisation of small nerve fibres in the cornea. Manual analysis by a trained observer entails the identification of nerve structures based on a subjective set of criteria which is time consuming and can result in intra- and inter-observer variability. The automated detection of corneal nerves relies on identifying low contrast nerve structures from a noisy background, using a combination of detection methods and predefined criteria such as nerve fibre orientation and axon reflectivity (Dabbah *et al.*, 2010). Multiple computational methods using a variety of quantification algorithms and multiple scale image analysis have shown a very low error rate (15.44%) for automated detection of corneal nerve morphology(Ruggeri, Scarpa and Grisan, 2006; Dabbah *et al.*, 2010) and is available as a standard software application, ACCMetrics (University of Manchester, UK). In participants with diabetic neuropathy, automated and manual corneal nerve analysis has shown excellent agreement, especially for CNFD and CNFL and to a lesser extent CNBD and automated analysis with consistently lower values for all three parameters, especially CNBD (Dehghani *et al.*, 2014; Petropoulos *et al.*, 2014; Pacaud *et al.*, 2015). In participants with PD, we also find good agreement between manual and automated analysis for CNFD and CNFL and moderate agreement for CNBD. The Bland Altman plots demonstrate that manual analysis is likely to produce higher values for more complex features such as CNBD and CNFL, but not for CNFD. The lower absolute values for CNBD and CNFL also reflects the adoption by the automated algorithm of a higher threshold for detecting branches to limit incorrect detection of features such as Langerhans cells adjacent to nerves that can be misinterpreted as branches. Automated analysis also identified a higher percentage of participants considered to have a significant loss of corneal nerve fibres based on CNFD, but not CNFL or CNBD. The derived cut off for automated and manual analysis was comparable for CNFD, whilst it was lower for CNBD and CNFL.

In conclusion, both manual and automated corneal nerve analysis show a significant loss of corneal nerve fibres in participants with PD compared to control subjects. Automated corneal nerve analysis is rapid and reproducible,

eliminates inconsistencies, and enables CCM to be scalable for widespread use in clinical practice and clinical trials.

3.7. Acknowledgements

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3.8. References

Please refer to Chapter 8: References

4. Corneal confocal microscopy detects small fibre neurodegeneration in Parkinson's disease using automated analysis

This manuscript has been published in Scientific Reports

Authors' contributions

Sze Hway Lim recruited PD participants, performed neurological assessments, analysed images using automated analysis, did statistical analysis, interpreted data, drafted manuscript and integrated author and reviewer comments to produce final draft.

Maryam Ferdousi and Alise Kalteniece performed corneal confocal microscopy assessments and selection of images.

Lewis Kass-Iliyya recruited participants and performed neurological assessments for the pilot study.

Ioannis Petropoulos, Professor Rayaz Malik, Dr Kobylecki and Professor Silverdale reviewed and critiqued manuscript.

Full citation

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4.2. Abstract

OBJECTIVE: We studied the utility of corneal confocal microscopy (CCM) in detecting a reduction in corneal nerve parameters in a large cohort of participantss with Parkinson's disease (PD) compared to controls using a fully automated potentially scalable method of analysis. We also assessed if CCM parameters are related to the severity and sub-type of PD.

METHODS: 98 participants with PD and 26 healthy controls underwent CCM with automated corneal nerve quantification**,** MDS-UPDRS III, Hoehn and Yahr scale, Montreal Cognitive Assessment (MoCA), Parkinson's Disease Questionnaire -39 (PDQ-39) and PD subtype assessment.

RESULTS: Corneal nerve fibre density (mean difference: -5.00 no/mm^2, 95% confidence interval (CI) [-7.89, -2.12], p=0.001), corneal nerve branch density (mean difference: -10.71 no/mm^2, 95% CI [-16.93, -4.48], p=0.003), corneal total branch density (mean difference: -14.75 no/mm^2, 95% CI [-23.58, -5.92], p=0.002), and corneal nerve fibre length (mean difference: -2.57 mm/mm^2, 95% CI [-4.02, -1.12], p=0.001) were significantly lower in PD participants compared to controls. There was no correlation between corneal nerve parameters and duration, severity or subtype of PD, cognitive function or quality of life.

CONCLUSIONS: CCM with automated corneal nerve analysis identifies nerve fibre damage and may act as a biomarker for neurodegeneration in PD.

4.3. Introduction

Traditionally, Parkinson's disease (PD) was thought to be a motor disorder caused primarily by degeneration of the dopaminergic nigrostriatal pathway, but it is now increasingly viewed as a multisystem disease secondary to widespread deposition of alpha-synuclein (Schulz-Schaeffer, 2010). Indeed, there is substantial neuropathological evidence of Lewy bodies in both central extra-nigral and peripheral nervous system structures (Fujishiro *et al.*, 2008; Lebouvier *et al.*, 2010; Wang *et al.*, 2013; Donadio *et al.*, 2014). Phosphorylated alpha-synuclein has been demonstrated in autonomic nerves of the colon (Lebouvier *et al.*, 2010), cardiac plexus (Fujishiro *et al.*, 2008) and cutaneous c-fibres (Donadio *et al.*, 2014). This departure from the basal ganglia-centric model of PD, allows us to explore the utility of peripheral nerve damage as a biomarker in PD.

The heterogenous clinical features and different rates of progression in PD suggests that there may be distinct subtypes under the umbrella of a PD diagnosis. Research on peripheral nerve involvement in PD is improving our understanding of the pathological mechanisms in PD and enabling better stratification of disease subtypes for prognostication of disease progression. Peripheral neuropathy (Merola *et al.*, 2017) and autonomic involvement (De Pablo-Fernandez *et al.*, 2017) in PD have been associated with faster disease progression, as well as certain clinical subtypes such as postural instability/gait disturbance (Allcock, Kenny and Burn, 2006). Small fibre neuropathy has traditionally been studied using skin biopsies and skin biopsies in people with PD demonstrate alpha-synuclein deposition and small nerve fibre degeneration (Wang *et al.*, 2013; Donadio *et al.*, 2014; Kass-Iliyya *et al.*, 2015). However, skin biopsy is an invasive procedure, requiring manual tissue processing and expertise for quantification, which is time consuming. The development of less invasive methods for assessing small fibre neuropathy is crucial for widespread clinical and research use. Corneal confocal microscopy (CCM) is a non-invasive ophthalmic imaging technique that can visualise corneal sub-basal nerve fibres in vivo (Auran *et al.*, 1995) and has been used to identify small fibre damage in a range of peripheral neuropathies including diabetic neuropathy (Petropoulos *et al.*, 2014), idiopathic small fibre neuropathy (Tavakoli, Marshall, *et al.*, 2010), Fabry's disease (Tavakoli *et al.*, 2009) and Charcot Marie Tooth disease (Tavakoli *et al.*, 2012).

To date three studies utilising CCM in small cohorts of PD participants have shown small fibre damage in PD participants compared to controls (Kass-Iliyya *et al.*, 2015; Podgorny *et al.*, 2016; Misra *et al.*, 2017). We previously showed a reduction in corneal nerve fibre density which correlated with the severity of autonomic dysfunction and motor severity of PD (Kass-Iliyya *et al.*, 2015). Podgorny et al demonstrated a reduction in corneal nerve fibre length and branch density in PD participants compared to controls (Podgorny *et al.*, 2016). Misra et al demonstrated a reduction in corneal nerve fibre length in PD participants with more advanced PD (Hoehn Yahr stage III and IV) (Misra *et al.*, 2017).

In this study we have utilised automated analysis to objectively compare corneal nerve parameters in PD participants to healthy controls and the association between CCM measures and clinical parameters in the PD cohort.

4.4. Methods

4.4.1. Ethics

NRES Committee/North West approved the pilot (Ref no 12/NW/0086) and larger (Ref no 17/NW/0144) study. Written informed consent was obtained from every participant. This research adhered to the tenets of the Declaration of Helsinki for clinical research involving human subjects.

4.4.2. Subjects

Participants with PD aged between 18 and 90 years, fulfilling Queen Square Brain Bank Criteria (Hughes *et al.*, 1992) were recruited from neurology clinics across Greater Manchester and via Fox Trial Finder and Parkinson's UK websites between September 2017 and September 2018. Key exclusion criteria included concurrent diagnoses of diabetes, active malignancy, hepatic disease, any other known cause of neuropathy, chronic corneal pathologies, history of refractive surgery and any systemic disease known to affect the cornea such as Fabry's disease, chronic kidney disease, and Sjogren's disease. Eighty-four PD participants were screened. Five participants were excluded due to abnormal blood tests suggestive of other causes of neuropathy, two were excluded as they had normal DaT scans, one was unable to undergo CCM and one participant had been concurrently enrolled in a disease modifying

drug trial. Seventy-five participants were enrolled into the study in addition to twenty-three PD participants from our pilot study using the same key inclusion and exclusion criteria. Twenty- six healthy age matched volunteers were used as controls. Written informed consent was obtained from all participants.

4.4.3. Medical History and Demographics

Subjects' gender, age, other medical conditions, medications including dopaminergic therapies, alcohol intake and smoking history was recorded. Duration of disease was calculated from the date of diagnosis to date of assessment. Full blood count, urea and electrolytes, glycated haemoglobin, immunofluorescence anti-nuclear antibodies, B12, Folate, immunoglobulins, serum electrophoresis and thyroid function tests were performed to exclude other known aetiologies of neuropathy.

4.4.4. Neurological Assessment

A clinical examination to detect evidence of peripheral neuropathy was carried out. Movement Disorder Society Unified Parkinson's Rating Scale part III (MDS-UPDRS III) was used to assess motor severity in the 'ON' state. PD participants in the pilot study were assessed in the 'ON' state using the Unified Parkinson's Disease Rating Scale part III (UPDRS III) and the data was converted to MDS-UPDRS III using the conversion formula described by Goetz et al (Goetz, Stebbins and Tilley, 2012). PD stage was determined using the Hoehn and Yahr scale. Participants recruited between September 2017-September 2018 also had cognition assessed using the Montreal Cognitive Assessment (MoCA) scale and were asked to complete a PDQ-39 questionnaire to assess health related quality of life in PD. PDQ39 summary index (PDQ39 SI), a validated summary score that is derived from the eight-dimension scores gained from the PDQ-39 questionnaire, was calculated for each PD participant.

4.4.5. Subtypes

Participants recruited between September 2017-September 2018 were subtyped into tremor dominant and postural instability with gait disturbance, based on the ratio of MDS-UPDRS scores as described by Stebbins et al (Stebbins *et al.*, 2013).

4.4.6. Ophthalmic Assessment

Participants underwent a comprehensive ophthalmic assessment by trained optometrists. Both eyes were assessed initially using a slit lamp biomicroscope (Slit Lamp BD 900, Haag Streit) to exclude pathology in the anterior segment of the eye. Corneal confocal images were acquired using a laser scanning corneal confocal microscope: Heidelberg Retinal Tomograph III Rostock Cornea Module (HRT III RCM); Heidelberg Engineering GmbH, Heidelberg, Germany. A x63 objective lens was used. The field of view was 400x400 μm. 2-dimensional images measuring 384 x 384 μm and 10 μm per pixel optical resolution were created.

During the examination, head/chin frames were used to help stabilize the position of the participant's head. The alignment of the participant's eyes was maintained by asking the participant to fixate on a white light with the eye contralateral to the one being examined. In addition, a charged couple device (CCD) camera was used to monitor the exact location of the camera on the corneal surface during the examination. Several images were taken from the central cornea of each eye and six images (three per eye) were selected based on standardised criteria (Kalteniece *et al.*, 2017).

4.4.7. Corneal Nerve Quantification

CCM images were analysed using fully automated software ACCMetrics (M.A. Dabbah, Imaging Science, The University of Manchester, 2010) (Dabbah *et al.*, 2010; Chen *et al.*, 2017). Corneal nerve fibre density (CNFD): number of main nerve fibres per frame (no/mm^2), corneal nerve branch density (CNBD): number of intersections between main nerves and secondary nerves per frame (no/mm^2), corneal total branch density (CTBD): the total number of branch points per frame (no/mm^2) and corneal nerve fibre length (CNFL): the total length of all nerve fibres per frame (mm/mm^2) were quantified and a mean was derived for each parameter.

4.4.8. Statistical analysis

Based on our published pilot study (Kass-Iliyya *et al.*, 2015) we calculated that at least 80 participants with PD were required (4:1 split, 64 PD, 16 control) with an alpha error of 0.05 and a beta error of 0.8 to demonstrate a difference in corneal nerve metrics between participants with PD and controls. A larger PD group was recruited

to enable stratification of the PD participants into different Hoehn Yahr stages, disease subtypes and cognitive status.

IBM SPSS version 25 was used to analyse the results. Chi square test was used to assess for a statistical difference between categorical data. Normality of distribution was assessed by the Shapiro-Wilk test. Independent samples t-test was used to compare means of normally distributed data and the Mann-Whitney U test was used for non-parametric data. Cohen d was calculated to measure effect size: d=0.2 (small), d=0.5 (medium), d=0.8 (large). Two tailed Spearman's correlation was used to ascertain relationships between continuous variables. One-way ANOVA was used to compare means between groups.

4.5. Results

4.5.1. Study population

Ninety-eight PD participants were compared with twenty-six controls. The demographics and clinical characteristics of PD participants and controls are shown in Table 4-1

	PD participants (n=98)	Controls (n=26)	P value
Gender	28F 70M	10F 16M	0.33
Age (years)	64.0 ± 0.82 (42-81)	62.0 ±1.40 (49-76)	0.24
MDS-UPDRS III	29.0 ± 1.18 (7-65)		
Disease Duration (months)	58.0 ± 4.8 (2-249)		
Hoehn and Yahr Stage	I:21 II:63 III:14		
MoCA (n=77)	26.0 ± 0.3 (17-30)		

Table 4-1 Demographics and clinical characteristics of Parkinson's disease participants and controls. Data shown as mean ± SEM (range). PD: Parkinson's disease, MDS UPDRS III: Movement Disorder Society Unified Parkinson's Disease Rating Scale part III, MoCA: Montreal Cognitive Assessment

4.5.2. Corneal nerve morphology in PD participants compared to controls

The quality of the images acquired for the PD participants was comparable to the controls as PD participants were assessed in the 'ON' state to minimize fatigue and interference from motor symptoms. CNFD, CNBD, CTBD and CNFL were significantly lower in participants with PD compared to controls (CNFD mean difference: -5.00 no/mm^2, 95% confidence interval (CI) [-7.89, -2.12], d=0.79; CNBD mean difference: -10.71 no/mm^2, 95% CI [-

16.93, -4.48], d=0.68; CTBD mean difference: -14.75 no/mm^2, 95% CI [-23.58, -5.92], d=0.68 and CNFL mean difference: -2.57 mm/mm^2, 95% CI [-4.02, -1.12], d=0.76) (Figure 4-1, Figure 4-2).

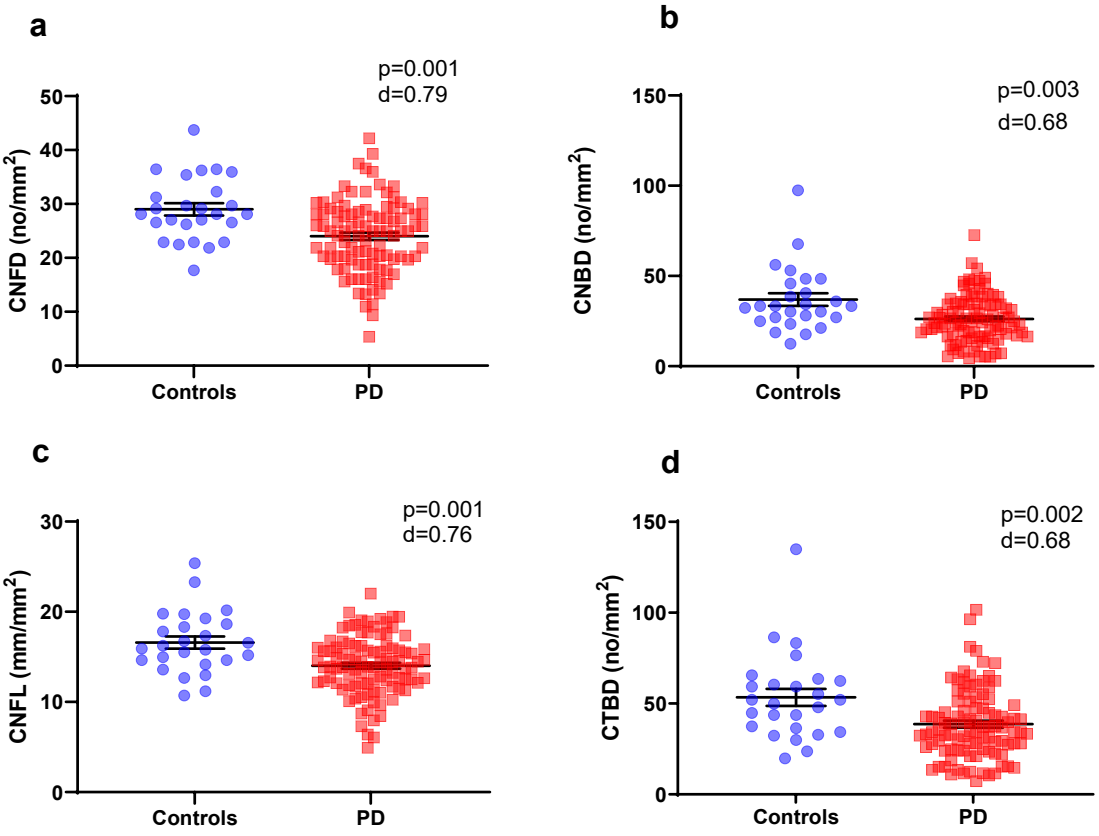

Figure 4-1 Mean ± SEM of corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD), corneal nerve fibre length (CNFL) and corneal nerve total branch density (CTBD) in participants with Parkinson's disease compared to controls with significant levels and Cohen d effect size.

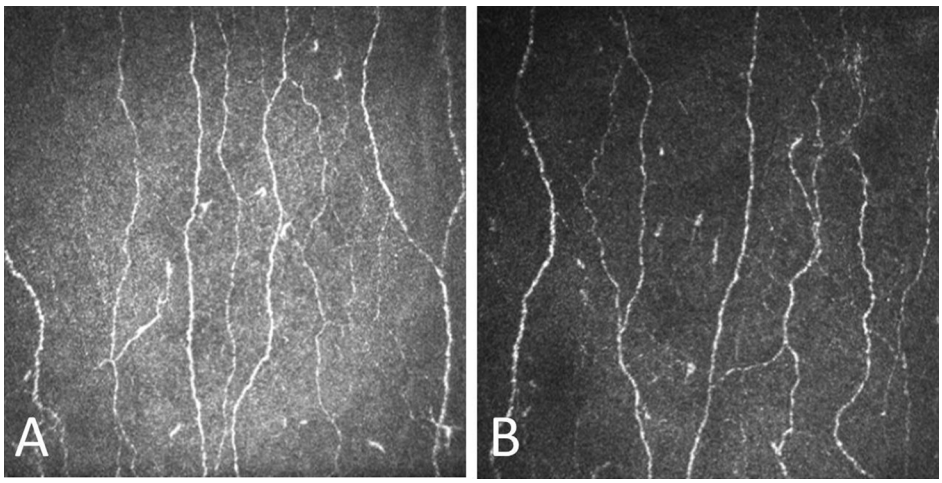

Figure 4-2 Corneal confocal image of a healthy control (A) compared to a participant with Parkinson's disease (B) showing an overall reduction in corneal nerve fibre density, corneal nerve branch density, corneal nerve total branch density and corneal nerve fibre length.

4.5.3. Corneal nerve morphology in PD subtypes

There was no significant difference in CCM parameters between tremor dominant (TD) (n=42), postural instability, gait difficulty (PIGD)(n=25) and indeterminate (n=8) subtypes of PD (Table 4-2)

CCM parameter	PIGD (n=25)	TD (n=42)	P value
CNFD (no/mm^2)	25.33 ± 1.49	22.57 ±1.02	0.12
CNBD (no/mm^2)	27.57 ± 3.27	23.33 ± 1.64	0.20
CNFL (mm/mm^2)	14.54 ± 0.75	13.39 ± 0.48	0.18
CTBD (no/mm^2)	38.51 ± 4.65	34.51 ± 2.16	0.93

Table 4-2 Corneal Confocal Microscopy parameters in postural instability/gait disturbance vs tremor dominant participants with Parkinson's disease. Data shown as mean ± SEM. CCM: Corneal confocal microscopy, PIGD: Postural instability/gait disturbance, TD: Tremor dominant, CNFD: Corneal nerve fibre density, CNBD: Corneal nerve branch density, CNFL: Corneal nerve fibre length, CTBD: Corneal total branch density.

4.5.4. Corneal nerve morphology between different Hoehn Yahr stages

Although participants in Hoehn Yahr stage II and III had lower values of CCM parameters compared to stage I (Table 4-3), one-way ANOVA did not demonstrate a significant difference in CNFD ($F=0.218$, $p=0.804$), CNBD ($F=0.792$, $p=0.456$), CNFL ($F=0.448$, $p=0.641$) and CTBD ($F=0.790$, $p=0.457$) between the different Hoehn Yahr stages.

	Hoehn Yahr Stage I (n=21)	Hoehn Yahr Stage 2 (n=63)	Hoehn Yahr Stage 3 (n=14)
CNFD (no/mm^2)	24.88 ± 1.35	23.77 ± 0.88	23.75 ± 1.88
CNBD (no/mm^2)	29.21 ± 2.82	25.08 ± 1.67	26.94 ± 3.65
CNFL (mm/mm^2)	14.61 ± 0.69	13.88 ± 0.43	13.72 ± 0.87
CTBD (no/mm^2)	43.09 ± 3.92	37.04 ± 2.46	39.29 ± 5.23

Table 4-3 CCM parameters in participants Hoehn Yahr stage I, II and III. Data reported as mean ± SEM. CNFD: Corneal nerve fibre density, CNBD: Corneal nerve branch density, CNFL: Corneal nerve fibre length, CTBD: Corneal total branch density.

4.5.5. Correlation between corneal nerve morphology and clinical data

Ninety-eight participants with PD were included in the analysis for correlation between corneal nerve measures, duration of disease and MDS UPDRS III. Seventy-five participants were analysed for correlation between corneal nerve measures with MoCA and PDQ39 SI. MDS-UPDRS, duration of disease, MoCA and PDQ39 SI were not normally distributed. There were no statistically significant correlations between corneal nerve measures and clinical measures of PD (Table 4-4).

CCM parameter	Duration of disease	MDS-UPDRS III 'ON'	MoCA	PDQ39 SI
CNFD	0.024	-0.109	0.202	0.010
CNBD	-0.041	-0.087	0.195	-0.054
CNFL	0.006	-0.098	0.184	-0.045
CTBD	-0.059	-0.041	0.179	-0.127

Table 4-4 Correlation between corneal nerve measures, duration of disease, MDS UPDRS III, MoCA and PDQ39-SI. Data reported as Spearman's correlation coefficient. No correlations were significant (p>0.05). CCM: Corneal confocal microscopy, MDS UPDRS III: Movement Disorder Society Unified Parkinson's Disease Rating Scale part III, MoCA: Montreal Cognitive Assessment, PDQ39 SI: Parkinson's Disease Questionnaire-39 summary index.

4.6. Discussion

This study shows a significant reduction in corneal nerve parameters in participants with PD compared to controls. Three previous studies using the Heidelberg HRTIII CCM demonstrated a loss of corneal nerves in participants with PD (Kass-Iliyya *et al.*, 2015; Podgorny *et al.*, 2016; Misra *et al.*, 2017). However, the cohorts studied were relatively small and different methods of corneal nerve quantification were used. Although image selection has been shown to be reproducible between investigators (Kalteniece *et al.*, 2017), quantification of corneal nerves can vary according to the protocol used for image selection (Schaldemose, E L. Fontain FI, Karlsson P, 2017) and whether the analysis is manual, semi-automated or fully automated (Li *et al.*, 2019). Manual analysis is labour intensive, subject to inter/intra-rater variability and requires training to limit variability. Fully-automated CCM analysis using ACCMetrics (M.A. Dabbah, Imaging Science, The University of Manchester, 2010) (Dabbah *et al.*, 2010; Chen *et al.*, 2017) has been successfully used by our group and others in several previous studies to demonstrate small fibre degeneration (Dabbah *et al.*, 2011; Petropoulos *et al.*, 2014; Podgorny *et al.*, 2016). It has the advantage of being reproducible and the images can be rapidly analysed (Chen *et al.*, 2017), enabling

scalability of the technique. Here, we use the same fully automated analysis to quantify corneal sub basal nerve parameters in a large cohort of PD participants compared to controls.

Previously we undertook manual corneal nerve quantification and showed a reduction in CNFD but increased CNBD and CNFL in PD participants with relatively mild PD, suggestive of proximal corneal nerve degeneration with more distal regeneration (Kass-Iliyya *et al.*, 2015). Misra et al showed reduced CNFL in participants with more advanced PD (Misra *et al.*, 2017), whilst Podgorny et al showed a significant reduction in CNFL and CNBD, indicative of distal degeneration in participants with recently diagnosed PD (Podgorny *et al.*, 2016). Arrigo et al demonstrated increased corneal nerve tortuosity and beading and altered trigeminal nerve diffusion on MRI in participants with PD (Arrigo *et al.*, 2018).

In the present cohort of PD participants, we show a global reduction in all corneal nerve parameters using automated analysis. Disease duration and severity as well as the method of analysis can affect the outcome when evaluating corneal nerve degeneration in PD. Nerve degeneration and regeneration is a dynamic process. A previous study by Nolano et al showed that cutaneous nerve regeneration may accompany degeneration early on in the disease process, but may become less efficient as PD progresses (Nolano *et al.*, 2018). We have also recently shown evidence of intraepidermal nerve fibre degeneration with impaired regeneration, which correlated to disease severity in PD (Jeziorska *et al.*, 2019). Regeneration may cause the number of branches and the total length of nerves to vary depending on stage of disease which may explain the variations in CNFL and CNBD seen between the different studies. Despite the differences in method of analysis between our current study and our previous study (Kass-Iliyya *et al.*, 2015), CNFD is consistently reduced in PD participants compared to controls.

PD is a widely heterogenous disorder. Identifying subtypes and features that determine faster rates of progression is a high priority clinical and research area. Subtyping participants based on motor symptoms was one of the initial methods used to describe different phenotypes in PD. In this analysis, we explored whether there were any differences in CCM parameters between motor subtypes, as PD participants presenting with tremor dominant symptoms are thought to have a more benign course of disease and slower rate of progression compared to the PIGD subtype (Aleksovski *et al.*, 2018). There were no significant differences in CCM parameters between

different subtypes classified by motor symptoms. The difference in rate of progression between the motor subtypes may not be caused by the overall extent of neurodegeneration as there is evidence that there is differential involvement of neurotransmitter systems and brain structures between the subtypes (Rossi *et al.*, 2010) Pathological studies demonstrate less cell loss in the substantia nigra pars compacta and the locus coeruleus, with more cell loss in the retrorubral area of the midbrain in participants with tremor dominant compared to non-tremor dominant PD (Jellinger, 1999). Motor subtyping has also been criticised for confounding by disease stage and inconsistent reliability as participants with initial tremor dominant symptoms can switch subtypes in later stages and vice versa (Nutt, 2016).

As our understanding of PD has progressed, several studies have shown that non-motor symptoms and involvement of the peripheral nervous system (Anang *et al.*, 2014; Merola *et al.*, 2017) provide additional prognostic value beyond the traditional tremor vs PIGD subtyping. This study demonstrates that some PD participants have fairly marked corneal nerve degeneration whereas others have CCM parameters within the normative range for healthy subjects. It has recently been proposed that there are two separate forms of PD: A peripheral onset form associated with marked autonomic neuropathy prior to involvement of the dopaminergic system and a central onset form with dopaminergic dysfunction preceding autonomic neuropathy (Borghammer and Berge, 2019). Peripheral neuropathy is associated with a more severe Parkinson's phenotype (Merola *et al.*, 2017). Small fibre damage in the form of autonomic dysfunction such as orthostatic hypotension, constipation, sweating abnormalities and erectile dysfunction in males has also been associated with more rapid disease progression and shorter survival (De Pablo-Fernandez *et al.*, 2017). Thus, earlier identification of a peripheral onset form of PD may enable the identification of a 'fast progressors' cohort.

Whilst there was an overall trend for a reduction in CCM parameters with higher Hoehn Yahr stages, this was not significant. This may be because the Hoehn Yahr scale is a categorical scale that describes clinical status which is weighted heavily towards postural instability. Each increment on the scale does not necessarily represent a higher degree of overall disability and non-motor features are not captured by the scale (Goetz *et al.*, 2004). There can also be a large variation of impairment severities within each Hoehn Yahr category (Goetz *et al.*, 2004). The

Hoehn Yahr scale may not be a sufficiently nuanced scale to tease out the association between corneal nerve changes and features of severe disease phenotype.

We have found no correlation between CCM parameters and disease duration, severity of motor or cognitive impairment, or quality of life. However, motor score or quality of life at a single time point are not in themselves markers of severe PD phenotypes as the scores can be confounded by stage of disease, age and intra-rater variability. The lack of association between CCM parameters and MoCA scores is likely because the PD participants in this cohort had minimal cognitive impairment as demonstrated by the high MoCA scores. Small fibre degeneration may be more closely related to different disease phenotypes i.e. those with and without small fibre degeneration as opposed to disease severity (Kass-Iliyya *et al.*, 2015). These participants are being followed up longitudinally to determine how CCM parameters change and correlate with clinical changes over time.

This study was not designed to assess the utility of CCM as a biomarker in PD as a cross sectional study does not enable CCM changes to be monitored and compared to clinical progression. MDS-UPDRS III was also assessed in the 'ON' state which may have affected the interpretation of motor severity scores. The assessments were done in the 'ON' state to enable the acquisition of high quality CCM images. Autonomic function was not assessed in this study. However, we have previously demonstrated a correlation between autonomic dysfunction and CCM parameters (Kass-Iliyya *et al.*, 2015). We were able to achieve our primary objective which was to show differences in CCM parameters using automated analysis in PD participants compared to healthy controls, with significant effect size.

Due to the overlap in CCM parameters between healthy controls and PD participants, CCM cannot be considered to be a diagnostic tool for PD. Nevertheless, the study demonstrates that automated CCM can detect small nerve fibre degeneration in PD. Longitudinal assessment of this cohort may help to define whether CCM allows objective identification of a 'fast progressor' cohort and / or an objective measure of disease progression in PD. Further longitudinal studies are required to study the relationship between corneal nerve changes and other markers of severe disease phenotype.

4.7. Acknowledgements

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4.8. References

Please refer to Chapter 8: References

5. Corneal confocal microscopy identifies Parkinson's disease with more rapid motor progression

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Authors' contributions

Sze Hway Lim recruited PD participants, performed neurological assessments, analysed images using automated analysis, did statistical analysis, interpreted data, drafted manuscript and integrated author and reviewer comments to produce final draft.

Maryam Ferdousi and Alise Kalteniece performed corneal confocal microscopy assessments.

Ziyad Mahfoud provided statistical input.

Ioannis Petropoulos, Professor Rayaz Malik, Dr Kobylecki and Professor Silverdale reviewed and critiqued manuscript.

Full citation

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5.2. Abstract

Background: Corneal confocal microscopy (CCM) is a non-invasive, reproducible ophthalmic technique to quantify corneal small nerve fibre degeneration. CCM demonstrates small nerve fibre damage in Parkinson's disease (PD), but its role as a longitudinal biomarker of PD progression has not been explored.

Objective: To assess corneal nerve morphology using CCM in relation to disease progression in PD.

Methods: Sixty-four PD participants were assessed at baseline and at 12-month follow up. Participants underwent CCM with automated corneal nerve quantification and assessment of Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS UPDRS), Hoehn and Yahr stage and Montreal Cognitive Assessment (MoCA).

Results: Corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD), corneal nerve fibre length (CNFL), corneal total branch density (CTBD) and corneal nerve fibre area (CNFA) were significantly lower in PD participants compared to healthy controls. Worsening of MDS UPDRS III score over 12 months was significantly greater in participants with a CNFD in the lowest compared to the highest quartile at baseline (mean difference: 6.0, 95% CI [1.0, 10.9], p=0.019). There were no significant changes in CNFD, CNBD, CNFL, CTBD, CNFA or CNFW between baseline and 12-month follow up.

Conclusion: CCM identifies neurodegeneration in participants with PD, especially those who show the greatest progression in neurological disability. CCM may be a useful tool to help enrich clinical trials with those likely to exhibit more rapid progression and reduce required sample size and cost of studies.

5.3. Introduction

Parkinson's disease (PD) is a heterogenous clinical syndrome in relation to both movement disorder and associated non-motor manifestations. Indeed, non-motor features such as autonomic dysfunction (De Pablo-Fernandez *et al.*, 2017), sleep disorders (Rolinski *et al.*, 2014) and peripheral neuropathy (Merola *et al.*, 2017) have prognostic value and may indicate distinct subtypes of PD (Horsager *et al.*, 2020). Peripheral nerves are a target for alpha synuclein deposition (Fujishiro *et al.*, 2008; Lebouvier *et al.*, 2010; Donadio *et al.*, 2014) and peripheral neuropathy (Merola *et al.*, 2017) and autonomic dysfunction (De Pablo-Fernandez *et al.*, 2017) have been associated with more severe disease phenotypes. Subtyping PD may enable a better understanding of disease mechanisms and prediction of disease progression.

Skin biopsies in people with PD demonstrate alpha synuclein deposition and small fibre neurodegeneration (Wang *et al.*, 2013; Donadio *et al.*, 2014; Nolano *et al.*, 2018). Higher alpha synuclein ratios have been correlated with more advanced disease in PD (Wang *et al.*, 2013) and cutaneous small fibre degeneration has been correlated with motor severity (Nolano *et al.*, 2018).

Corneal confocal microscopy (CCM) is a novel non-invasive ophthalmic method that enables in vivo quantification of small nerve fibre damage. It was initially developed to overcome the limitations of light microscopy which can only study corneal nerve architecture in vitro and produces poor resolution images (Jalbert *et al.*, 2003). The cornea has the densest small fibre innervation in the body and has a central corneal nerve density of approximately 7000 nociceptors per square millimetre, resulting in the cornea being 300 to 600 times more sensitive than skin (Yang, Chow and Liu, 2018). CCM has been used to detect small fibre degeneration in a range of peripheral neuropathies including diabetic neuropathy (Tavakoli, Quattrini, *et al.*, 2010), idiopathic small fibre neuropathy (Tavakoli, Marshall, *et al.*, 2010), chronic inflammatory demyelinating polyneuropathy (Stettner *et al.*, 2016) and Charcot Marie Tooth disease (Tavakoli *et al.*, 2012). The key parameters to quantify corneal nerve morphology are corneal nerve fibre density (CNFD); a measure of the number of main nerves, corneal nerve branch density (CNBD); a measure of the number of branch points and corneal nerve fibre length (CNFL); a measure of the total length of main nerves and branches (Figure 2). More recently, fully automated analysis has

enabled the quantification of corneal nerve fibre area (CNFA), corneal nerve fibre width (CNFW) and corneal total branch density (CTBD) (Chen *et al.*, 2017). CNFD has been shown to have a better sensitivity/specificity compared to intraepidermal nerve fibre density from skin biopsies in the diagnosis of diabetic polyneuropathy (Chen *et al.*, 2015; Alam *et al.*, 2017). CCM can also identify early nerve regeneration evidenced by an increase in CNFD and CNFL after simultaneous kidney and pancreas transplantation in participants with type 1 diabetes (Azmi *et al.*, 2019) and CNFD, CNBD and CNFL after bariatric surgery in participants with obesity (Azmi *et al.*, 2021). CCM has undergone multiple validation studies and has been shown to be a reliable and highly reproducible corneal nerve imaging technique (Kalteniece *et al.*, 2017; Petropoulos *et al.*, 2020).

Several cross-sectional studies using corneal confocal microscopy have demonstrated corneal nerve fibre degeneration in PD participants compared to controls (Kass-Iliyya *et al.*, 2015; Podgorny *et al.*, 2016; Misra *et al.*, 2017). Our initial pilot study of CCM in 26 PD participants demonstrated a decrease in CNFD and an increase in CNBD and CNFL compared to controls, indicative of proximal nerve degeneration with more distal nerve regeneration (Kass-Iliyya *et al.*, 2015). Several skin biopsy studies have reported cutaneous denervation in PD compensated by nerve regeneration (suggested by the presence of increased nerve branching) which declines over time (Nolano *et al.*, 2008; Jeziorska *et al.*, 2019). Our more recent study of 98 PD participants demonstrated a reduction in all CCM parameters compared to controls (Lim *et al.*, 2020). Nerve regeneration may result in an increase in branches and total length of nerves, thus CNBD and CNFL may vary according to the stage of disease.

All PD studies utilising CCM to date have been cross sectional which does not allow an assessment of the utility of quantifying CCM parameters to predict disease progression. In this study we have assessed corneal nerve morphology at baseline and over 12 months in relation to change in disease severity in participants with PD.

5.4. Methods

NRES Committee/North West approved the study (Ref no 17/NW/0144). Written informed consent was obtained from each participant. This research adhered to the tenets of the Declaration of Helsinki for clinical research involving human subjects.

5.4.1. Subjects

Participants with PD, fulfilling Queen Square Brain bank criteria (Hughes *et al.*, 1992) were recruited from clinics across Greater Manchester and via Fox Trial Finder and Parkinson's UK websites between September 2017 and September 2018. Eighty-four participants were screened based on their clinical history and blood tests (full blood count, urea and electrolytes, glycated haemoglobin, immunofluorescence anti-nuclear antibodies, B12, folate, immunoglobulins, serum electrophoresis and thyroid function tests). Exclusion criteria were concurrent diagnosis of diabetes, active malignancy, hepatic disease, any known cause of neuropathy, chronic corneal pathologies, history of refractive surgery and any systemic disease known to affect the cornea such as Fabry's disease, chronic kidney disease, and Sjogren's disease. Seventy-five PD participants were enrolled into the study and sixty-four were followed up after 12 months (Figure 5-1).

Twenty-five healthy volunteers were recruited as controls and compared to the baseline CCM parameters of the sixty-four PD participants.

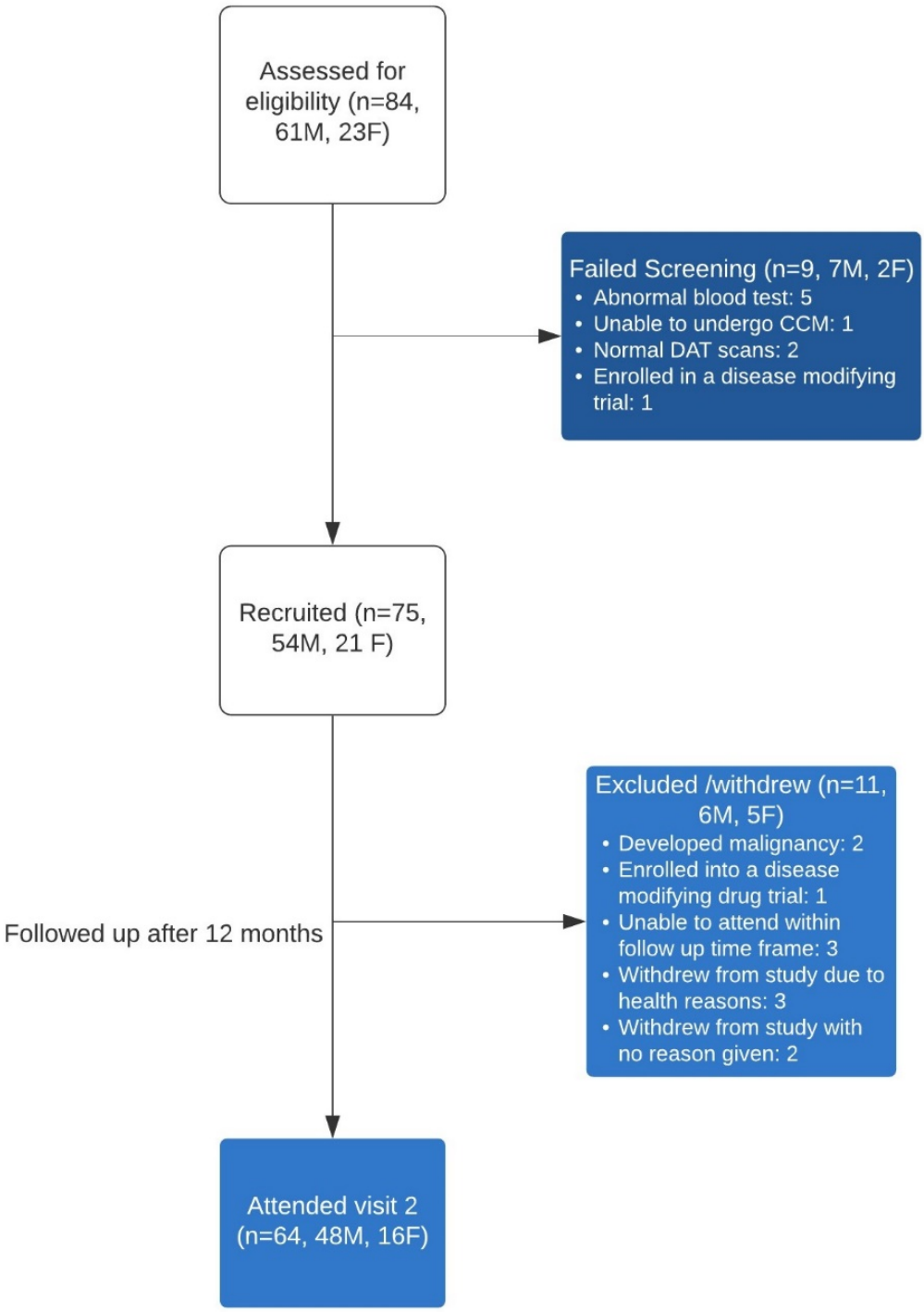

Figure 5-1 Flow diagram of recruitment of PD participants

5.4.2. Medical history and neurological assessment

Participants' age, gender, medical history, and medications including dopaminergic therapy were documented. Levodopa equivalent daily dose was calculated according to validated conversion tables (Tomlinson *et al.*, 2010; Fabbri *et al.*, 2018; Schade, Mollenhauer and Trenkwalder, 2020). Disease duration was calculated from the date of diagnosis to the date of assessment. All participants underwent a neurological examination to exclude participants with clinically manifest peripheral neuropathy. All parts of the Movement Disorder Society Unified Parkinson's Rating Scale (Goetz, C. G., Tilley, B. C., Shaftman, 2008) were performed on participants in the 'ON' state, the Hoehn Yahr scale was used to assess disease stage and cognitive function was assessed using the Montreal Cognitive Assessment (MoCA) (Nasreddine *et al.*, 2005) scale.

5.4.3. Ophthalmic Assessment

All ophthalmic assessments were performed by trained optometrists. Both eyes were first assessed with a slit lamp biomicroscope (Slit Lamp BD 900, Haag Streit) to exclude anterior eye pathology. Laser scanning corneal confocal microscopy (Rostock Cornea Module/Heidelberg Retina Tomograph lll; Heidelberg Engineering GmbH) was performed at baseline and after 12 months of follow-up. Corneal confocal microscopy was performed with participants in the 'ON' state to minimize interference from motor symptoms. A drop of 0.4% benoxinate hydrochloride (Oxybuprocaine Hydro 0.4%, Bausch & Lomb, UK) was used to anaesthetize each eye. Viscotears (Carbomer 980, 0.2 %; Novartis, UK) was also applied to the participants' eyes to reduce any discomfort. Head/chin frames were used to stabilise the position of the participant's head. The participants were asked to fixate on an outer fixation target with the contralateral eye and a charge couple device was used to identify the exact location of the camera on the corneal surface during the examination.

The full thickness of the central cornea was scanned using the section mode and 2D images measuring 384 x 384 µm with optical resolution of 10 µm per pixel were obtained. Multiple images of the sub-basal plexus were taken and stored in a database. The total time taken to acquire CCM images for each participant was ~10 minutes.

Six high-quality (three per eye) images of the subbasal nerve plexus were selected for eachparticipant, following an established protocol to eliminate any variability in image selection (Kalteniece *et al.*, 2017). Automated

CCMetrics software, version 2.0 (University of Manchester, Manchester, UK) was used to quantify the nerve fibers. This fully automated analysis ensures blinded quantification of six corneal nerve parameters: corneal nerve fiber density (CNFD), the number of main nerves per frame (no/mm^2); corneal nerve branch density (CNBD), the number of branches arising from major nerves (no/mm^2); corneal nerve fiber length (CNFL), the total length of all nerve fibers and branches (mm/mm^2); corneal total branch density (CTBD), the total number of branches per frame (no/mm^2); corneal nerve fiber area (CNFA), the total area of nerve fibers per frame ($\mu m^2/mm^2$) and corneal nerve fiber width (CNFW), the average axial diameter of nerve fibers per frame (μm). A mean was derived for each parameter.

5.4.4. Statistical Analysis

IBM SPSS version 25 was used to analyse the results. Normality of distribution was assessed by the Shapiro-Wilk test. Means of continuous data for PD participants and controls at baseline were compared using independent t-test for normally distributed data and Mann-Whitney U test for non-parametric data. Cohen d was calculated to measure effect size: d=0.2 (small), d=0.5 (medium), d=0.8 (large) (Sullivan GM, 2012). Chi square was used to compare categorical data. Paired samples t-test was used to compare means of normally distributed data at baseline and 12-month follow up. McNemar-Bowker test was used to compare proportions of paired categorical outcomes

In order to compare rate of disease progression in participants with the most and least corneal nerve damage at baseline, participants were divided into four quartiles based on their baseline CCM parameter values. An independent t-test was used to compare the means of change in MDS UPDRS III scores after 12 months between participants in quartile 1 and quartile 4.

Linear regression was used to measure the variation in change in MDS UPDRS III scores after 12 months in participants with the lowest number of nerves (CNFD quartile 1) compared to the highest number of nerves (CNFD quartile 4) after adjusting for the effects of age, gender and disease duration. The first linear regression model consisted of ‘CNFD quartile 1 vs CNFD quartile 4’ as the independent variable. The second linear regression model studied the effects of ‘CNFD quartile 1 vs CNFD quartile 4’ on change in MDS UPDRS III after 12 months, after adjusting for the effects of age, disease duration and gender by entering all four factors as independent

variables. Tests for linearity, homoscedasticity, multicollinearity, influential data points and normality showed that the assumptions of the regression analysis were met.

Spearman's correlation was used to assess for correlation between change in levodopa equivalent daily dose, change in CCM parameters, change in MDS UPDRS scores, and change in MoCA over 12 months.

Data was reported as mean ± SD and $p<0.05$ was considered statistically significant.

5.5 Results

5.5.1 Demographics and clinical characteristics of participants

Sixty-four PD participants (sixteen female, forty-eight male), with a mean age of 64.1 ± 7.8 years and twenty-five control participants (eleven female, fourteen male), with a mean age of 63.1 ± 6.8 years were recruited to the study. There was no significant difference in age ($p=0.56$) or gender ($p=0.08$) between the PD cohort and the control cohort. The PD participants were followed up after a mean duration of 12.0 ± 1.0 months. The mean duration of PD was 56.9 ± 42.6 months at visit 1 (baseline). Clinical characteristics at visit 1 and visit 2 (12-month follow up) are summarised in Table 5-1.

Clinical Scores/LEDD			
	Baseline	12-month follow up	p
MDS UPDRS II	10.5 ± 6.7	11.1 ± 6.8	0.203
MDS UPDRS III	27.4 ± 10.3	31.5 ± 12.3	<0.001*
Full MDS UPDRS	52.0 ± 19.4	58.1 ± 20.0	<0.001*
Hoehn Yahr stage	I:9 II:45 III: 10 2 (2, 2)	I:7 II:49 III:7 IV:1 2 (2, 2)	0.593
MoCA	26.5 ± 2.8	26.1 ± 3.4	0.297
LEDD	483.5 ± 260.7	578.5 ± 312.8	<0.001*
CCM parameters			
	Baseline	12-month follow up	p
CNFD (no./mm^2)	24.47 ± 7.28	24.75 ± 7.80	0.707
CNBD (no./mm^2)	25.24 ± 13.32	27.12 ± 15.86	0.191
CNFL (mm/mm^2)	14.06 ± 3.48	14.17 ± 3.63	0.703
CTBD (no./mm^2)	36.22 ± 18.53	38.95 ± 20.16	0.170
CNFA ($\mu m^2/mm^2$)	5234 ± 1419	5200 ± 1611	0.812
CNFW (µm)	20.67 ± 0.98	20.5 ± 1.00	0.273

Table 5-1 . Clinical characteristics and corneal confocal microscopy parameters of PD participants at baseline and 12-month follow up. Data shown as mean ± SD apart from Hoehn Yahr (median with interquartile range). MDS UPDRS: Movement Disorder Society Unified Parkinson's Disease Rating Scale, MoCA: Montreal Cognitive Assessment, LEDD: Levodopa Equivalent Daily Dose, CNFD: corneal nerve fibre density; CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, CTBD: corneal total branch density, CNFA: corneal nerve fibre area, CNFW: corneal nerve fibre width, *p<0.05 considered statistically significant.

5.5.2. Corneal nerve morphology in PD participants at baseline and controls

CNFD, CNBD, CNFL, CTBD and CNFA were significantly lower in PD participants at baseline compared to controls (CNFD mean difference: 4.55 no/mm^2, 95% confidence interval (CI) [1.31, 7.79], d= 0.7, p=0.006); CNBD mean difference: 8.18 no/mm^2, 95% CI [2.31, 14.05], d= 0.7, p=0.003); CNFL mean difference: 2.53 mm/mm^2, 95% CI [0.94, 4.11], d= 0.8, p=0.002); CTBD mean difference: 11.19 no/mm^2, 95% CI [2.92, 19.45], d= 0.7, p=0.003); CNFA mean difference: 773.9µm^2/mm^2, 95% CI [97.0, 1450.8], d= 0.5, p=0.026). CNFW did not differ significantly between PD participants and controls (CNFW mean difference: -0.257µm, 95% CI [0.23, 0.20], d=0.3, p=0.158) (Figure 5-2, Figure 5-3).

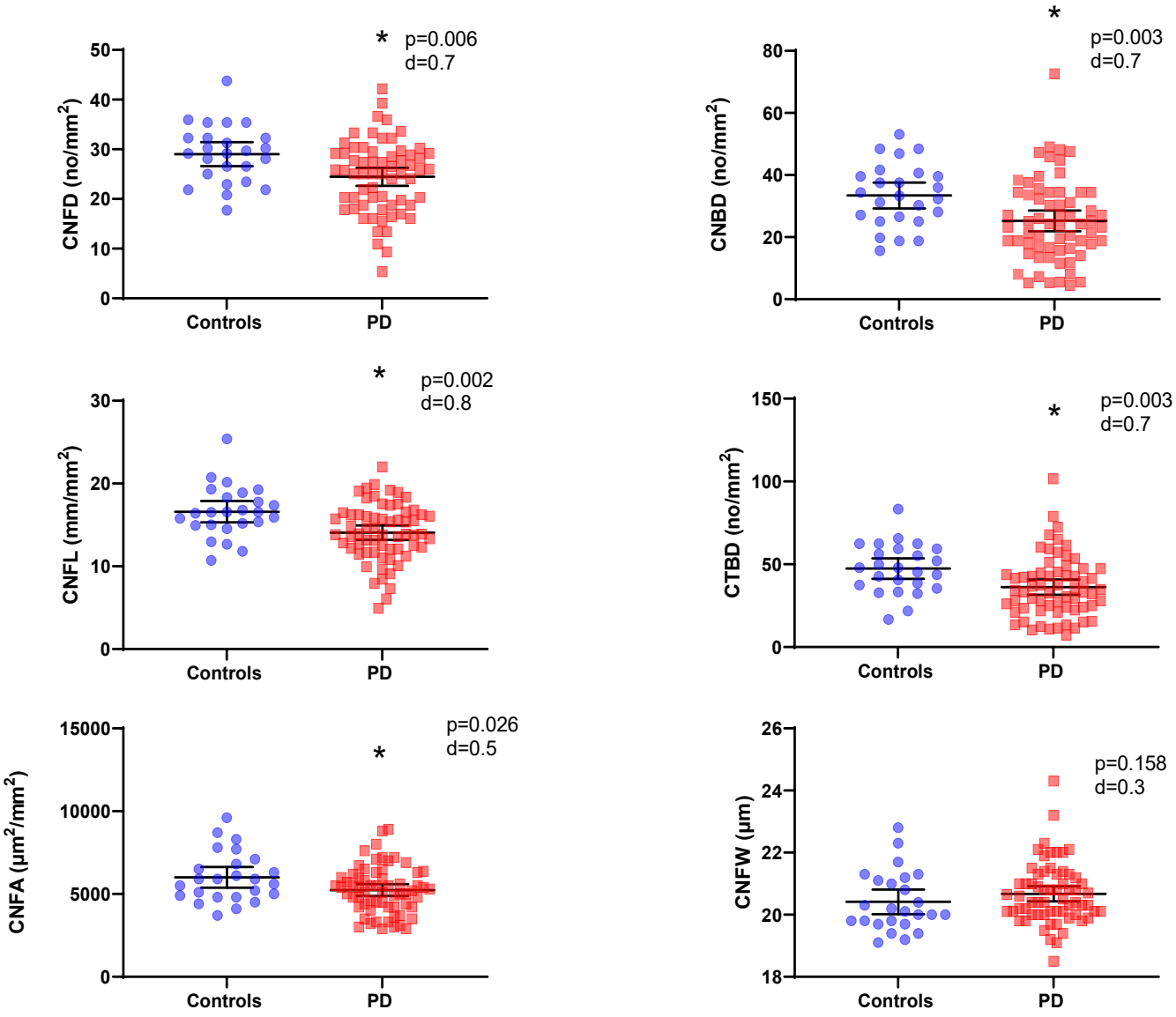

Figure 5-2 Corneal confocal microscopy parameters in Parkinson's disease participants compared to controls. Mean ± 95% confidence interval of corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD), corneal nerve fibre length (CNFL), corneal nerve total branch density (CTBD), corneal nerve fibre area (CNFA) and corneal nerve fibre width (CNFW) in participants with Parkinson's disease compared to controls with significance levels and Cohen d effect size.

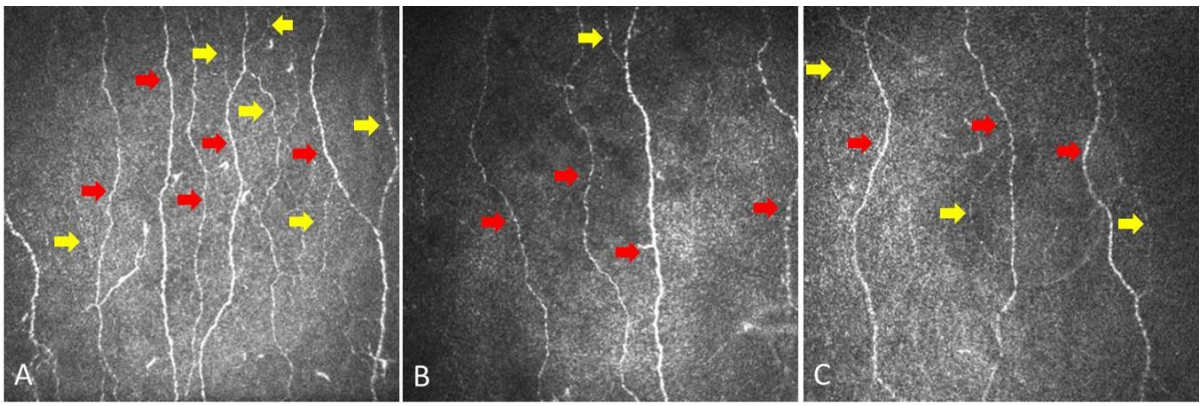

Figure 5-3 Corneal confocal microscopy images. Corneal confocal microscopy image of a healthy control (A), an age-matched participant with Parkinson's disease (B) and the participant with Parkinson's disease after 12 months (C). Corneal nerve fibre density is the total number of main nerves (indicated by red arrows) per square millimetre (no./mm^2), corneal nerve branch density is the total number of junction between branches (indicated by yellow arrows) and main nerves (red arrows) per square millimetre (no./mm^2), corneal nerve fibre length is the total length of main nerves and nerve branches per square millimetre (mm/mm^2).

5.5.3. Corneal nerve morphology in PD participants at baseline and 12 months follow up

Across the whole PD cohort, there were no significant changes in CCM parameters between baseline and follow-up at 12 months (Table 5-1).

5.5.4. Disease progression between PD participants based on severity of baseline impairment of corneal nerve parameters

The change in MDS UPDRS III over 12 months was significantly different between PD participants in quartile 1 (most severe corneal nerve degeneration) compared to quartile 4 (least severe corneal nerve degeneration) for CNFD and did not differ for CNFL, CNBD, CTBD, CNFW and CNFA (Table 5-2).

	Number of participants	Change in MDS UPDRS III after 12 months	p
CNFD Quartile 1	17	6.9 ± 8.0	0.019*
CNFD Quartile 4	18	0.9 ± 6.3	
CNBD Quartile 1	16	5.4 ± 7.8	0.406
CNBD Quartile 4	17	3.1 ± 7.5	
CNFL Quartile 1	16	5.6 ± 8.1	0.367
CNFL Quartile 4	16	3.0 ± 7.7	
CTBD Quartile 1	16	3.5 ± 9.7	0.658
CTBD Quartile 4	16	2.3 ± 5.7	
CNFA Quartile 1	17	4.9 ± 9.3	0.796
CNFA Quartile 4	16	4.1 ± 8.6	
CNFW Quartile 1	16	5.9 ± 7.7	0.228
CNFW Quartile 4	16	1.9 ± 10.5	

Table 5-2 Change in MDS UPDRS III over 12 months between participants corneal nerve parameters in quartile 1 (most severe corneal nerve degeneration) compared to quartile 4 (least severe corneal nerve degeneration) at baseline. Data reported as mean ± SD. CNFD: corneal nerve fiber density; CNBD: corneal nerve branch density, CNFL: corneal nerve fiber length, CTBD: corneal total branch density, CNFA: corneal nerve fiber area, CNFW: corneal nerve fiber width, MDS UPDRS III: Movement Disorder Society Unified Parkinson's Disease Rating Scale part III, *p<0.05 considered statistically significant.

5.5.5. Regression Analysis

Compared to PD participants in quartile 4, those in quartile 1 of CNFD had a significantly greater increase in MDS UPDRS III after 12 months (mean difference: 5.99, p=0.019). This difference remained significant even after adjusting for age, gender, and duration of the disease of the participants (adjusted mean difference: 5.55, p=0.036) (Table 5-3).

CNFD (Quartile 1 vs. Quartile 4)	Mean difference in change in MDS UPDRS III over 12 months (B)	Standard error	p-value	R^2
Unadjusted	5.99	2.4	0.019*	15.6%
Adjusted for age, gender, and disease duration	5.55	2.5	0.036*	22.4%

Table 5-3 Regression Analysis. CNFD: corneal nerve fibre density; MDS UPDRS III: Movement disease society unified Parkinson's disease rating scale part III; *p<0.05, considered statistically significant.

5.5.6. Correlations between change in levodopa equivalent daily dosage, MDS UPDRS scores and change in CCM parameters

There were no correlations between change in levodopa equivalent daily dose and change in CNFD (Rho=-0.143, p=0.260), MDS UPDRS II (Rho=-0.155, p=0.221), MDS UPDRS III (Rho=-0.047, p=0.715), full MDS UPDRS (Rho=-0.168, p=0.185) and MoCA (Rho=-0.047, p=0.715) over 12 months.

5.6. Discussion

This study confirms previous findings by our group (Kass-Iliyya *et al.*, 2015; Lim *et al.*, 2020) and others (Podgorny *et al.*, 2016; Misra *et al.*, 2017) of corneal nerve damage in participants with PD compared to healthy controls. Whilst there was no significant decline in corneal nerve parameters over 12 months, intriguingly, participants with a baseline CNFD in the lowest quartile (most severe corneal nerve degeneration) compared to the highest quartile (least severe corneal nerve degeneration) showed the most rapid clinical deterioration based on an increase in MDS UPDRS III. Studies in participants with diabetic neuropathy have shown that it may take 2-4 years for a significant reduction in corneal nerve parameters (Dehghani *et al.*, 2016; Edwards *et al.*, 2017). A recent study in 590 participants with diabetes followed over ~5 years demonstrated more rapid corneal nerve loss in a sub-group of participants who showed more rapid worsening of neuropathy and were referred to as progressors (Lewis *et al.*, 2020).

In this study, there was overlap in CCM parameters between the control and PD cohort, suggesting that there are subgroups of PD participants with different degrees of corneal nerve denervation. Indeed, we show that the severity of small nerve fibre degeneration at baseline may confer a poorer prognostic outcome in relation to greater worsening of motor disability over 12 months after adjusting for age, gender, and disease duration. The 6 point increase in MDS UPDRS III after 12 months, between participants with the least and most corneal nerves at baseline exceeds the margin of 4.6, which is considered to be a clinically important worsening of the MDS UPDRS III score (Horváth *et al.*, 2015).

Extra-nigral involvement and non-motor features have been increasingly used to subtype PD and assess rates of disease progression. A recent cluster analysis study identified three non-motor features (rapid eye movement sleep behaviour disorder, mild cognitive impairment and orthostatic hypotension) at baseline predict the most rapidly progressive subtype termed the ‘diffuse malignant subtype’ (Fereshtehnejad *et al.*, 2015). The authors have suggested that the ‘diffuse malignant subtype’ may represent diffuse neurodegenerative pathology as the features involve the simultaneous dysfunction of different anatomical regions (Fereshtehnejad and Postuma, 2017). Other studies have also demonstrated that autonomic dysfunction is associated with a more severe PD phenotype with a

higher risk of falls, wheelchair dependence and cognitive impairment(Oliveira *et al.*, 2019). Neuropathy is associated with worse motor and cognitive scores and non-motor disability (Merola *et al.*, 2017). Interestingly, a recent study has shown that the reduction in the ganglion cell-inner plexiform layer and peripapillary retinal nerve fibre layer thickness over 3 years was related to cognitive decline but not motor deterioration in participants with PD (Murueta-Goyena *et al.*, 2021). In the present study CNFD had prognostic value for motor deterioration as it is a more stable measure of proximal nerve degeneration, whilst CNFL, CNBD and CTBD are more variable due to ongoing distal nerve regeneration (Nolano *et al.*, 2018). Indeed, our previous study showed a decrease in CNFD but an increase in CNBD and CNFL in PD[15].

PD related peripheral neuropathy may be due to the iatrogenic effects of dopaminergic therapies and intrinsic neurodegeneration. Studies have demonstrated an association between therapy with levodopa and large fibre neuropathy (Toth *et al.*, 2008) but not small fibre neuropathy (Nolano *et al.*, 2017). Many studies have suggested that small fibre neuropathy is an intrinsic part of the disease process in PD (Nolano *et al.*, 2011, 2017; Doppler *et al.*, 2014; Kass-Iliyya *et al.*, 2015). Nolano et al found large and small fibre neuropathy in drug naïve participants and showed that large but not small fibre pathology worsened with levodopa use (Nolano *et al.*, 2017). Doppler et al found no correlation between intraepidermal nerve fibre density and the cumulative levodopa intake (Doppler *et al.*, 2014). Our previous study demonstrated no correlation between corneal nerve parameters and cumulative levodopa dose (Kass-Iliyya *et al.*, 2015). This study also demonstrates no correlation between change in levodopa daily dose and change in corneal nerve parameters after 12 months.

Some limitations should be noted. It was not possible to establish a robust link between peripheral and central neurodegeneration as we have not directly compared CCM parameters with imaging markers of central dopaminergic neuronal integrity. The number of participants in the highest and lowest quartiles of CNFD was relatively small and the findings require validation in other PD cohorts. A longer follow up period will also be required to fully assess progression of corneal nerve degeneration in PD.

This study confirms corneal nerve loss in participants with PD and further suggests that CCM may be a useful marker of neurodegeneration to identify PD participants with a more progressive and severe disease phenotype,

termed ‘fast progressors’. Identification of slow and fast progressors may allow the identification and recruitment of PD participants who are more or less responsive to disease modifying therapies (Espay *et al.*, 2017) to enable the design of shorter, more cost-effective clinical trials and to eliminate heterogeneity in the PD cohort

5.7. Acknowledgements

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5.8. References

Please refer to chapter 8: References

6. Corneal confocal microscopy shows different degrees of nerve loss in atypical Parkinsonian disorders

Authors' contributions

Sze Hway Lim recruited PD participants, performed neurological assessments, did statistical analysis, interpreted data, drafted manuscript and integrated authors' comments to produce final draft.

Maryam Ferdousi and Alise Kalteniece performed corneal confocal microscopy assessments.

Sze Hway Lim and Maryam Ferdousi analysed images manually

Ziyad Mahfoud provided statistical input.

Ioannis Petropoulos, Professor Rayaz Malik, Dr Kobylecki and Professor Silverdale reviewed and critiqued manuscript.

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6.2. Abstract

Background: Cutaneous studies show different patterns of small fibre degeneration between participants with Parkinson's disease (PD), multiple system atrophy (MSA), and progressive supranuclear palsy (PSP).

Objective: We studied corneal nerve loss using corneal confocal microscopy (CCM) as a potential marker of neurodegeneration in PD, MSA and PSP compared to healthy controls.

Methods: Participants underwent CCM with corneal nerve quantification and clinical assessments.

Results: Corneal nerve fibre density was significantly reduced in participants with PD (p=0.005), PSP (p=0.005) and MSA (p=0.0003) compared to controls. Corneal nerve branch density was significantly reduced in participants with PD (p=0.01) and MSA (p=0.019) but not in participants with PSP (p=0.662) compared to controls. CNFL was significantly reduced in participants with PD (p=0.002) and MSA (p=0.001) but not in participants with PSP (p=0.191) compared to controls.

Conclusion: Corneal nerve loss is primarily detected in participants with PD and MSA and is less marked in PSP.

6.3. Introduction

Intracellular deposition of histopathologically misfolded proteins typifies many neurodegenerative disorders. Parkinson's Disease (PD) and multiple system atrophy (MSA) are characterized by the presence of oligomers or multimers of alpha synuclein (Gilman *et al.*, 2008; Schulz-Schaeffer, 2010), whilst progressive supranuclear palsy (PSP) is associated with intracellular deposition of tau protein (Kovacs, 2015).

Participants with PSP and MSA can present with parkinsonism (Gilman *et al.*, 2008; Höglinger *et al.*, 2017), particularly in multiple system atrophy-parkinsonism (MSA-P) and progressive supranuclear palsy-parkinsonism (PSP-P) subtypes. Participants with idiopathic PD can also have features such as autonomic dysfunction which may overlap with the clinical presentation of MSA (Coon, Cutsforth-Gregory and Benarroch, 2018). The similar clinical presentations particularly in early-stage disease can result in delays in diagnosing participants with PSP and MSA.

Skin biopsy studies have shown small fibre denervation in participants with alpha synuclein pathology and no denervation in participants with tauopathies including PSP (Donadio *et al.*, 2014). Alpha synuclein deposition occurs in autonomic nerve fibres in PD and somatic nerve fibres in MSA (Zange *et al.*, 2015; Donadio *et al.*, 2018). These findings have led to an interest in small nerve fibre degeneration as a marker of disease type.

Corneal confocal microscopy (CCM) has been used to identify small nerve fibre degeneration in a range of neuropathies including diabetic neuropathy (Ferdousi *et al.*, 2021), HIV neuropathy (Kemp *et al.*, 2017), chemotherapy induced peripheral neuropathy (Ferdousi *et al.*, 2015), fibromyalgia (Evdokimov *et al.*, 2019) and Friedreich's ataxia (Pagovich *et al.*, 2018).

More recently, CCM has also demonstrated corneal nerve loss in conditions with central neurodegeneration, such as multiple sclerosis (Petropoulos *et al.*, 2017) and dementia (Ponirakis *et al.*, 2019). Corneal nerve loss has also been observed in participants with PD (Kass-Iliyya *et al.*, 2015; Podgorny *et al.*, 2016; Lim *et al.*, 2020). A small study of 7 participants with PSP and 4 participants with PD showed decreased corneal sensitivity but no reduction in corneal nerve density(Reddy *et al.*, 2013) suggesting that small nerve fibre pathology may differ between

subtypes of PD. The aim of this study was to assess whether corneal nerve morphology could be used to differentiate participants with PD from those with PSP and MSA.

6.4. Methods

6.4.1. Ethics

NRES Committee/North West approved the study (Ref no 17/NW/0144).

6.4.2. Subjects

Participants with PSP, MSA and PD were recruited from clinics across Greater Manchester and via Fox Trial Finder and Parkinson's UK websites between September 2017 and March 2020. Participants had a clinical diagnosis of PD fulfilling UK Brain Bank diagnostic criteria (Hughes *et al.*, 1992). Participants with probable MSA and PSP were diagnosed by movement disorder neurologists according to published diagnostic criteria (Gilman *et al.*, 2008; Höglinger *et al.*, 2017). Participants with a known history of cancer, diabetes, alcoholism, hepatic disease, previous refractive surgery, vitamin deficiencies, autoimmune conditions, chronic corneal pathologies, and other known causes for peripheral neuropathy were excluded. Healthy age matched volunteers were used as controls. All subjects provided written consent.

Subjects' gender, age, medical history, medications, alcohol intake and smoking history were documented. Disease duration was calculated from date of diagnosis of parkinsonism to date of assessment. Subjects were screened with blood tests including full blood count, urea and electrolytes, glycated haemoglobin, immunofluorescence anti-nuclear antibodies, B_{12}, Folate, immunoglobulins, serum electrophoresis and thyroid function tests to exclude other known causes of neuropathy.

6.4.3. Neurological Assessment

PD, PSP and MSA participants were assessed with all four parts of the Movement Disorder Society Unified Parkinson's Rating Scale (MDS UPDRS) in the 'ON' state in a clinical research facility. The Hoehn and Yahr scale was used to determine stage of disease and the Montreal cognitive (MoCA) assessment was performed in participants with PD, PSP and MSA.

6.4.4. Corneal Confocal Microscopy

Corneal confocal microscopy was performed using a Heidelberg Retinal Tomograph III with Rostock Cornea Module (HRT III RCM) (Heidelberg Engineering GmbH, Heidelberg, Germany) according to our previously established protocol (Kalteniece *et al.*, 2017). Six high resolution images of the sub-basal corneal nerve plexus from the central cornea were selected. Corneal nerve parameters were quantified manually using purpose-designed software (CCMetrics, M.A. Dabbah, Imaging Science, The University of Manchester, Manchester, UK). Corneal nerve fibre density (CNFD): number of main nerve fibres per frame (no/mm^2), corneal nerve branch density (CNBD): number of intersections between main nerves and secondary nerves per frame (no/mm^2) and corneal nerve fibre length (CNFL): the total length of all nerve fibres per frame (mm/mm^2) were quantified.

6.4.5. Statistical Analysis

Statistical analysis was performed using IBM SPSS for windows (Version 25, IBM SPSS statistics, Armonk, NY: IBM Corp). Shapiro Wilk test was used to assess whether the data were normally distributed. One-way analysis of variance (ANOVA) with post hoc tests were used to compare means of continuous variables. Homogeneity of variance was calculated using Levene's test. If there was homogeneity of variance, one-way ANOVA was reported, and Tukey post hoc test was used for multiple comparisons between groups. If homogeneity of variance was violated, Welch ANOVA was reported, and Games Howell post hoc test was used for multiple comparisons between groups. Fisher exact test was used to compare categorical variables.

Analysis of covariance (ANCOVA) with post hoc LSD was used to compare variables between groups whilst statistically controlling for the effects of age and gender.

6.5. Results

6.5.1. Demographics

Participants with PD (n=19), PSP (n=11), MSA (n=8) and controls (n=18) were recruited. The demographic data and clinical assessments in each group are summarized in Table 6-1. Age was comparable between controls and participants with PD (p=0.99), PSP (p=0.352) and MSA (p=0.357) and between participants with PD compared

to PSP (p=0.382) and MSA (p=0.316). Participants with MSA were significantly younger than participants with PSP (p=0.028). Disease duration did not differ significantly between groups (p=0.092). Motor disability as measured by the MDS UPDRS III was significantly greater in the MSA (p=0.001) and PSP (p=0.003) groups compared to the PD group; but did not differ between the PSP and MSA groups (p=0.732). The Hoehn and Yahr scale was significantly greater in the PSP and MSA groups compared to the PD group (p=0.014). The MoCA score was significantly lower in the PSP group compared to the PD group (p=0.018) but did not differ between PD and MSA (p=0.669) or PSP and MSA (p=0.158) groups.

	Controls	PD	PSP	MSA	p value
Age (years)	64.9 ± 1.1	65.1 ± 1.3	68.6 ± 1.6	60.8 ± 3.1$^{\$}$	0.04*
Gender	10M 8F	14M 5F	9M 2F	4M 4F	0.330
Disease duration (months)	N/A	51.2 ± 5.3	30.6 ± 7.5	46.4 ± 9.1	0.092
MDS-UPDRS III	N/A	26.0 ± 2.4	44.3 ± 5.1 ^	49.0 ± 5.3 ^	<0.001*
Hoehn Yahr scale	N/A	I:1 II:16 III:2 IV: 0	I:0 II: 5^ III: 4 IV:1 V:1	I: 1 II: 2^ III: 2 IV: 2^ V:1	0.014*
MoCA	N/A	26.9 ± 0.5	21.6 ± 1.5^	25.6 ± 1.4	0.022*

Table 6-1 Demographic data of participants undergoing corneal confocal microscopy. Data shown as mean ± SEM apart from Hoehn Yahr (frequency). PD: Parkinson's disease; MSA: Multiple system atrophy; PSP: progressive supranuclear palsy; MDS UPDRS III: Movement Disorder Society Unified Parkinson's Disease Rating Scale part III, MoCA: Montreal Cognitive Assessment. *p<0.05 considered statistically significant. $ Represents statistically significant compared to PSP, ^ Represents statistically significant compared to PD.

6.5.2. Corneal nerve parameters

ANCOVA showed significant differences in CNFD (F=6.70, p=0.001), CNBD (F=3.52, p=0.021) and CNFL (F=5.49, p=0.002) between the different groups after controlling for age and gender. CNFD (no/mm^2) was significantly lower in participants with PD (27.19 ± 1.70, p=0.005), PSP (25.65 ± 2.4, p=0.005) and MSA (21.86 ± 2.74, p=0.0003) compared to controls (34.44 ± 1.76) but did not differ significantly between participants with PD and PSP (p=0.595) or MSA (p=0.107) or between participants with MSA and PSP (p=0.319). CNBD (no/mm^2) was significantly lower in participants with PD (53.38 ± 7.01, p=0.01) and MSA (32.27 ± 13.28, p=0.019), but not PSP (75.05 ± 9.64, p=0.662) compared to controls (80.43 ± 7.24); and showed a non-significant trend to be higher in participants with PSP compared to participants with PD (p=0.073) and MSA (p=0.089). CNFL (mm/mm^2) was significantly lower in participants with PD (17.63 ± 1.25, p=0.002) and MSA (15.49 ± 2.02, p=0.001) compared to controls (23.55 ± 5.38). It did not differ between participants with PSP (20.67 ± 1.72, p=0.191) and controls and showed a non-significant trend to be higher compared to participants with MSA (p=0.067) (Figure 6-1, Figure 6-2).

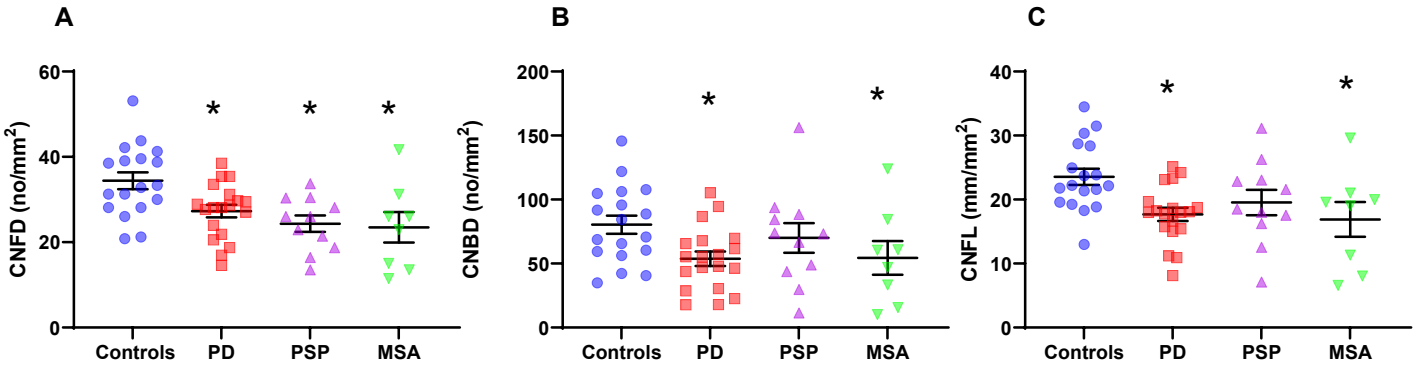

Figure 6-1 Mean ± SEM of (A) corneal nerve fibre density (CNFD), (B) corneal nerve branch density (CNBD) and (C) corneal nerve fibre length (CNFL) in participants with Parkinson's disease (PD), progressive supranuclear palsy (PSP) and multiple system atrophy (MSA) compared to healthy controls with significance level. *p<0.05 represents statistically significant compared to controls.

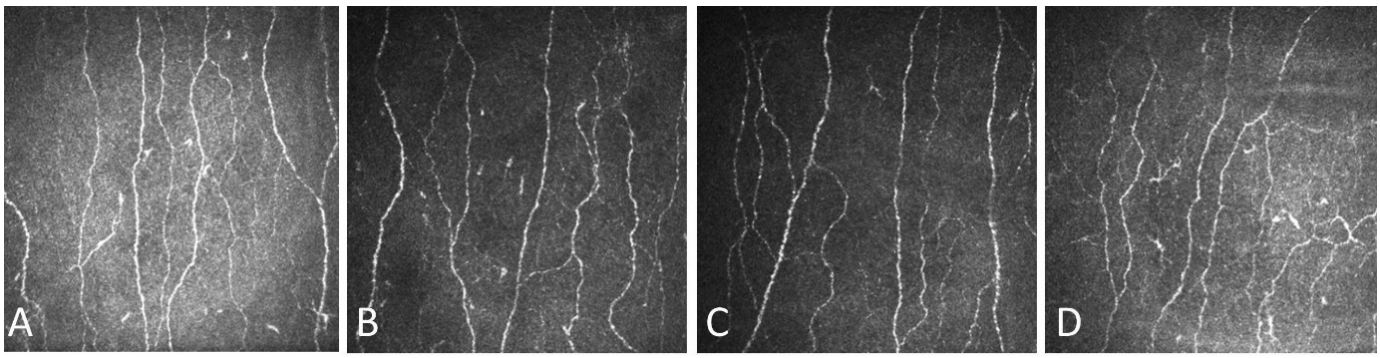

Figure 6-2 Corneal Confocal Microscopy image of a healthy control (A) compared to participants with Parkinson's disease (B), multiple system atrophy (C) and progressive supranuclear palsy (D) showing a global reduction in corneal nerve fibre density in (B), (C) and (D).

6.6. Discussion

We show that corneal confocal microscopy can rapidly and objectively demonstrate corneal nerve loss in participants with PD, MSA and PSP. This confirms previous studies showing corneal nerve loss in PD (Podgorny *et al.*, 2016; Lim *et al.*, 2020). However, we show differences between these groups with relative preservation of corneal nerves in participants with PSP, whilst participants with MSA show the most severe small nerve fibre loss. Previously, Melli et al also found marked skin denervation in participants with clinical syndromes consistent with alpha-synuclein pathology but not in tauopathies, including PSP (Melli *et al.*, 2018).

Corneal nerve loss was most evident in MSA followed by PD and PSP and may be related to the deposition of peripheral alpha synuclein. Both Donadio et al (Donadio *et al.*, 2018) and Zange et al (Zange *et al.*, 2015) have reported alpha synuclein in cutaneous autonomic nerves of participants with PD and mainly in sensory nerves of participants with MSA. However, Donadio et al found no nerve fibre loss in participants with tauopathies (Donadio *et al.*, 2014). Somatic nerve fibre involvement with relative preservation of autonomic innervation has been shown in participants with MSA-P. In contrast, phosphorylated alpha synuclein deposition and denervation predominantly involved autonomic nerve fibres in participants with PD and orthostatic hypotension (Donadio *et al.*, 2020). This study showed that participants with MSA have the most extensive corneal nerve loss. This may

be because most corneal nerves are somatic nerves (Müller *et al.*, 2003) and somatic nerve fibres are predominantly affected in MSA whereas autonomic nerves are predominantly affected in PD (Donadio *et al.*, 2020).

The relative sparing of corneal nerves in PSP may reflect a lack of peripheral involvement whereas there is evidence for combined central and peripheral degeneration in MSA and PD. In PD, Lewy body pathology has been found in the cardiac plexus (Fujishiro *et al.*, 2008), myenteric plexus (Lebouvier *et al.*, 2010), autonomic nerves innervating the submandibular glands (Tredici *et al.*, 2010) and in cutaneous biopsies (Doppler *et al.*, 2014). Autonomic dysfunction in MSA has been attributed to both central and peripheral involvement, although peripheral involvement is less marked compared to PD (Coon, Cutsforth-Gregory and Benarroch, 2018) and skin biopsy studies have demonstrated reduced intraepidermal nerve fibre density in participants with MSA (Donadio *et al.*, 2018).

Corneal nerve loss may be driven by direct damage or as a consequence of systemic metabolic processes as evidenced by the loss of corneal nerves in a range of neurological (Petropoulos *et al.*, 2020) and metabolic disorders such as diabetes (Ferdousi *et al.*, 2021), Fabry's disease (Bitirgen *et al.*, 2018) and hypothyroidism (Sharma, Tobin, Prashant R J Vas, *et al.*, 2018). The reduction in CNFD in participants with PSP may also be related to local ocular damage, related to reduced blink rate and/or dry eyes (Williams and Lees, 2010). Of note participants with PSP have relative preservation of more distal corneal nerves as evidenced by higher corneal nerve branch density and length compared to the alpha-synucleinopathies of PD and MSA, which is consistent with greater small nerve fibre loss in these conditions.

A limitation of this study is the small sample size of participants with atypical parkinsonism, especially participants with MSA. Despite this we show corneal nerve loss in participants with PD, especially those with MSA, with relative preservation in PSP. A bigger sample size may have demonstrated a significant difference between participants with MSA and PSP. It would also be useful to have long-term follow up data on these cohorts including pathology, if possible, to verify diagnosis. CCM may provide further insight into the differential involvement of small fibre damage between tauopathies and alpha-synucleinopathies.

6.7. Acknowledgement

The authors thank the Manchester Wellcome Trust Clinical Research Facility for providing the facilities to conduct this research.

6.8.. References

Please refer to Chapter 8: References

7. Discussion and Conclusions

Parkinson's disease is now known to be a multisystem disorder and alpha synuclein pathology is not limited to the dopaminergic nigrostriatal system. Phosphorylated alpha synuclein has been found in multiple areas of the central, autonomic and peripheral nervous system including the retina (Beach *et al.*, 2014), enteric nervous system (Lebouvier *et al.*, 2010), cutaneous c-fibres (Donadio *et al.*, 2014) and cardiac plexus (Fujishiro *et al.*, 2008).

Cutaneous deposition of alpha synuclein and denervation has generated interest in the role of small fibre degeneration as a biomarker in PD (Donadio *et al.*, 2014; Doppler *et al.*, 2014; Kuzkina *et al.*, 2019). The cornea receives the densest small fibre innervation in the body (Yang, Chow and Liu, 2018) and CCM enables in vivo, non-invasive qualitative and quantitative characterisation of corneal nerves. An MRI imaging study of drug naïve PD participants compared to controls demonstrated alterations in diffusion based parameters of the trigeminal nerve from which corneal innervation is derived, reinforcing the hypothesis of trigeminal and corneal denervation affecting participants with PD (Arrigo *et al.*, 2018).

CCM was initially developed for studying corneal disease (Cruzat, Qazi and Hamrah, 2017). It was later shown to have utility in demonstrating a reduction in corneal nerve parameters in participants with diabetic neuropathy (Alam *et al.*, 2017) and identifying subclinical or early stages of diabetic neuropathy (Tavakoli, Marshall, *et al.*, 2010). CCM has been shown to have comparable diagnostic utility compared to intraepidermal nerve fibre density in the diagnosis of diabetic neuropathy (Chen *et al.*, 2015). Subsequently, alterations in corneal sub-basal nerves have been detected in a range of other hereditary, inflammatory and metabolic neuropathies (Tavakoli *et al.*, 2012; Stettner *et al.*, 2016; Bitirgen *et al.*, 2018).

The studies in this thesis investigate the role of CCM as a biomarker in PD and atypical parkinsonism. Cross sectional studies have demonstrated significant differences in CCM parameters of PD participants compared to healthy controls (Kass-Iliyya *et al.*, 2015; Podgorny *et al.*, 2016; Misra *et al.*, 2017). Differences in methodology and small sample sizes limit any definitive conclusions to be drawn.

The first paper in this thesis compares fully automated analysis and manual analysis of CCM images in a PD cohort. Whilst the agreement between automated and manual analysis has been studied in adults and children with diabetes (Petropoulos *et al.*, 2014; Pacaud *et al.*, 2015) previously, no study has investigated the reliability and

reproducibility of automated analysis in PD participants. Unique challenges arise when a new diagnostic test is used in different populations of participants and should be specifically validated for different participant cohorts. In a PD cohort, it was important to ensure that motor symptoms such as tremor and stiffness do not interfere with the reproducibility of the technique. The study found good agreement between automated and manual analysis for CNFD and CNFL and moderate agreement for CNBD which is in keeping with the results of similar methodology studies in diabetic cohorts (Petropoulos *et al.*, 2014; Pacaud *et al.*, 2015). CNBD, a measure of the number of nerve branches connected to main nerve fibres has previously been reported to be variable and has modest validity in diagnosing neuropathy (Petropoulos *et al.*, 2013, 2014). Both automated and manual analysis identified corneal nerve loss in PD participants. Automated analysis has the advantage of being a rapid technique and is not subject to inter-/intra-rater variability. Validation of the reliability and reproducibility of automated analysis is important to enable the use of CCM to be extended to widespread clinical and research use involving multiple sites and longitudinal studies.

The next paper in this thesis (Lim *et al.*, 2020) investigates a large cohort of PD participants using fully automated image analysis. The main advantage of fully automated image analysis is the reproducibility of the technique which enables scalability. The study established corneal morphology changes in PD using automated image analysis. The large number of participants enabled stratification of PD participants into different subtypes (tremor dominant vs postural instability and gait difficulty) which has not been investigated previously, although no significant differences in CCM parameters between the two subtypes were found. The overlap between CCM parameters of PD participants and healthy controls suggests that the severity of corneal nerve loss varies in a PD population. Therefore, CCM may not be the ideal technique for diagnosing PD. However, the presence of small fibre damage may confer prognostic value as autonomic abnormalities such as orthostatic hypotension, constipation, sweating abnormalities, urinary symptoms and erectile dysfunction are associated with more rapid disease progression and shorter survival (Oliveira *et al.*, 2019).

Interparticipant variability in cross sectional studies does not allow us to study the association between corneal nerve changes and clinical scales accurately. The next study in this thesis is the first study to investigate

longitudinal CCM changes in a PD cohort. No significant decline in CCM parameters was found over a 12-month interval, although there was a significant change in MDS-UPDRS scores. This suggests that CCM parameters may not be an ideal marker of overall disease progression. Interestingly, PD participants with the most corneal nerve degeneration as measured by CNFD were found to have a faster rate of motor progression after 12 months, indicating that more extensive corneal nerve loss at baseline may be a marker of poor prognosis. Identifying PD subtypes by defining groups of PD participants with shared biological, clinical or genetic features is an area of considerable research interest. PD subtyping aids clinical prognostication and helps identify patients with evidence of vulnerability to the molecular mechanisms of potential disease modifying interventions (Fearon, Lang and Espay, 2021). Difficulties demonstrating meaningful changes in study end points of trialled putative disease modifying agents may be due to heterogeneity of disease progression and/or response to treatment in PD cohorts studied (Mollenhauer, Zimmermann, *et al.*, 2019). Studies on the annual rate of change of motor and non-motor symptoms demonstrate large interparticipant variability indicating heterogenous trajectories of progression (Biundo *et al.*, 2016; Simuni *et al.*, 2018). There are multiple studies which report predictors for worse motor progression (Fereshtehnejad *et al.*, 2017) and early cognitive decline (Ray *et al.*, 2018). As the scope of available data expands, composite scores taking into account multiple predictors of 'fast progressor' phenotype such as CCM data, neuroimaging, clinical and demographic features and CSF studies may help produce a clearer picture of disease subtypes.

This thesis also explores CCM changes in MSA and PSP participants compared to PD and healthy controls. No other CCM study has investigated changes in atypical parkinsonism apart from a small study by Reddy et al which investigated 7 PSP, 4 PD and 5 healthy control participants. Reddy et al did not find a significant difference in CNFD between PSP, PD and control participants (Reddy *et al.*, 2013). However, the study may have been underpowered to identify significant differences. The study in this thesis involved 19 PD, 11 PSP, 8 MSA and 18 control participants. Corneal nerve loss was identified primarily in MSA and PD participants and to a lesser degree in PSP participants. The differential nerve loss is in keeping with our current understanding of peripheral nerve damage whereby tauopathies such as PSP lack peripheral nerve involvement (Gawel *et al.*, 2013).

CCM enables in vivo assessment of corneal nerves and has been shown to be a reproducible technique, but there are some limitations requiring further development of the technology. Currently, CCM has a relatively small field of view resulting in only a proportion of the total sub basal nerve plexus to be scanned at a given time. A standardized protocol to select and analyse multiple images has been developed to overcome this limitation (Kalteniece *et al.*, 2017). Mosaicking techniques have been developed to increase the field of view, but currently requires a lot of expert knowledge and are subject to investigator misjudgement (Allgeier *et al.*, 2018). There is also currently an absence of clinically validated reference values for CCM parameters. Despite the limitations, CCM has been shown to reliably demonstrate nerve loss in many peripheral neuropathies (Stem *et al.*, 2014; Chen *et al.*, 2015) and more recently in neurodegenerative conditions (Kass-Iliyya *et al.*, 2015; Bitirgen G, Akpinar Z, Malik RA, 2017; Ponirakis *et al.*, 2019). CCM has also been shown to detect nerve regeneration following intervention (Azmi *et al.*, 2019).

The use of CCM in PD is intriguing and the studies in this thesis explored its role as a biomarker in PD. However, there is further work to be done. The association between the presence of more extensive corneal nerve loss and more rapid motor progression requires validation in other PD cohorts. Future work should also study the association between autonomic dysfunction, CCM changes, disease severity and progression over time. Larger cohorts of participants with atypical parkinsonism are required to confirm if there is a differential loss of corneal nerves between disease types and to enable CCM changes to be compared between the different subtypes of MSA and PSP. In addition, other structures within the corneal layers such as Langerhans cells should be studied. The density and morphological features of Langerhans cells such as shape, size and presence or absence of dendritic cells may provide important information about chronicity of disease and disease severity. Assessing different areas of the cornea in addition to the central cornea such as the inferior whorl, the most distal area of the sub basal plexus, may provide information about the pattern of corneal nerve loss in PD and atypical Parkinsonism. Further work is also required to establish reference values for CCM parameters in PD participants and healthy controls to enable clinical application of the tool.

8. References

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9. Appendices

9.1. Revised Neuropathy Disability Score

		Right	Left
Vibration Perception Threshold 128-Hz tuning fork; apex of big toe: normal= can distinguish vibrating/not vibrating	Normal 0 Abnormal 1		
Temperature perception on dorsum of the foot Use tuning fork with beaker of ice/warm water	Normal 0 Abnormal 1		
Pin Prick Apply pin proximal to big toe nail just enough to deform the skin; trial pair=sharp/blunt; normal=can distinguish sharp/not sharp	Normal 0 Abnormal 1		
Achilles Reflex	Present=0 Present with reinforcement=1 Absent=2		
NDS Total out of 10 _____/10		_____/5	_____/5

9.2. MDS UPDRS scores and Hoehn Yahr scale

MDS UPDRS
Part I: Non-Motor Aspects of Experiences of Daily Living (nM-EDL)

Part 1A: Complex behaviors: [completed by rater]

Primary source of information:

☐ Patient ☐ Caregiver ☐ Patient and Caregiver in Equal Proportion

To be read to the patient: I am going to ask you six questions about behaviors that you may or may not experience. Some questions concern common problems and some concern uncommon ones. If you have a problem in one of the areas, please choose the best response that describes how you have felt MOST OF THE TIME during the PAST WEEK. If you are not bothered by a problem, you can simply respond NO. I am trying to be thorough, so I may ask questions that have nothing to do with you.

	SCORE
1.1 COGNITIVE IMPAIRMENT Instructions to examiner: Consider all types of altered level of cognitive function including cognitive slowing, impaired reasoning, memory loss, deficits in attention and orientation. Rate their impact on activities of daily living as perceived by the patient and/or caregiver. *Instructions to patients [and caregiver]: Over the past week have you had problems remembering things, following conversations, paying attention, thinking clearly, or finding your way around the house or in town?* [If yes, examiner asks patient or caregiver to elaborate and probes for information.] 0: Normal: No cognitive impairment. 1: Slight: Impairment appreciated by patient or caregiver with no concrete interference with the patient's ability to carry out normal activities and social interactions. 2: Mild: Clinically evident cognitive dysfunction, but only minimal interference with the patient's ability to carry out normal activities and social interactions. 3: Moderate: Cognitive deficits interfere with but do not preclude the patient's ability to carry out normal activities and social interactions. 4: Severe: Cognitive dysfunction precludes the patient's ability to carry out normal activities and social interactions.	

	SCORE
1.2 HALLUCINATIONS AND PSYCHOSIS Instructions to examiner: Consider both illusions (misinterpretations of real stimuli) and hallucinations (spontaneous false sensations). Consider all major sensory domains (visual, auditory, tactile, olfactory and gustatory). Determine presence of unformed (for example sense of presence or fleeting false impressions) as well as formed (fully developed and detailed) sensations. Rate the patient's insight into hallucinations and identify delusions and psychotic thinking. *Instructions to patients [and caregiver]: Over the past week have you seen, heard, smelled or felt things that were not really there?* [If yes, examiner asks patient or caregiver to elaborate and probes for information.] 0: Normal: No hallucinations or psychotic behavior. 1: Slight: Illusions or non-formed hallucinations, but patient recognizes them without loss of insight. 2: Mild: Formed hallucinations independent of environmental stimuli. No loss of insight. 3: Moderate: Formed hallucinations with loss of insight. 4: Severe: Patient has delusions or paranoia.	☐
1.3 DEPRESSED MOOD Instructions to examiner: Consider low mood, sadness, hopelessness, feelings of emptiness or loss of enjoyment. Determine their presence and duration over the past week and rate their interference with the patient's ability to carry out daily routines and engage in social interactions. *Instruction to the patient (and caregiver): Over the past week have you felt low, sad, hopeless or unable to enjoy things? If yes, was this feeling for longer than one day at a time? Did it make it difficult for you carry out your usual activities or to be with people?* [If yes, examiner asks patient or caregiver to elaborate and probes for information.] 0: Normal: No depressed mood. 1: Slight: Episodes of depressed mood that are not sustained for more than one day at a time. No interference with patient's ability to carry out normal activities and social interactions. 2: Mild: Depressed mood that is sustained over days, but without interference with normal activities and social interactions. 3: Moderate: Depressed mood that interferes with, but does not preclude, the patient's ability to carry out normal activities and social interactions. 4: Severe: Depressed mood precludes patient's ability to carry out normal activities and social interactions.	☐

	SCORE
1.4 ANXIOUS MOOD Instructions to examiner: Determine nervous, tense, worried or anxious feelings (including panic attacks) over the past week and rate their duration and interference with the patient's ability to carry out daily routines and engage in social interactions. *Instructions to patients [and caregiver]: Over the past week have you felt nervous, worried or tense? If yes, was this feeling for longer than one day at a time? Did it make it difficult for you to follow your usual activities or to be with other people*? [If yes, examiner asks patient or caregiver to elaborate and probes for information.] 0: Normal: No anxious feelings. 1: Slight: Anxious feelings present but not sustained for more than one day at a time. No interference with patient's ability to carry out normal activities and social interactions. 2: Mild: Anxious feelings are sustained over more than one day at a time, but without interference with patient's ability to carry out normal activities and social interactions. 3: Moderate: Anxious feelings interfere with, but do not preclude, the patient's ability to carry out normal activities and social interactions. 4: Severe: Anxious feelings preclude patient's ability to carry out normal activities and social interactions.	☐
1.5 APATHY Instructions to examiner: Consider level of spontaneous activity, assertiveness, motivation and initiative and rate the impact of reduced levels on performance of daily routines and social interactions. Here the examiner should attempt to distinguish between apathy and similar symptoms that are best explained by depression. *Instructions to patients (and caregiver): Over the past week, have you felt indifferent to doing activities or being with people?* [If yes, examiner asks patient or caregiver to elaborate and probes for information.] 0: Normal: No apathy. 1: Slight: Apathy appreciated by patient and/or caregiver, but no interference with daily activities and social interactions. 2: Mild: Apathy interferes with isolated activities and social interactions. 3: Moderate: Apathy interferes with most activities and social interactions. 4: Severe: Passive and withdrawn, complete loss of initiative.	☐

1.6 FEATURES OF DOPAMINE DYSREGULATION SYNDROME

SCORE

Instructions to examiner: Consider involvement in a variety of activities including atypical or excessive gambling (e.g. casinos or lottery tickets), atypical or excessive sexual drive or interests (e.g., unusual interest in pornography, masturbation, sexual demands on partner), other repetitive activities (e.g. hobbies, dismantling objects, sorting or organizing), or taking extra non-prescribed medication for non-physical reasons (i.e., addictive behavior). Rate the impact of such abnormal activities/behaviors on the patient's personal life and on his family and social relations (including need to borrow money or other financial difficulties like withdrawal of credit cards, major family conflicts, lost time from work, or missed meals or sleep because of the activity).

Instructions to patients [and caregiver]: Over the past week, have you had unusually strong urges that are hard to control? Do you feel driven to do or think about something and find it hard to stop? [Give patient examples such as gambling, cleaning, using the computer, taking extra medicine, obsessing about food or sex, all depending on the patients.]

0: Normal: No problems present.

1: Slight: Problems are present but usually do not cause any difficulties for the patient or family/caregiver.

2: Mild: Problems are present and usually cause a few difficulties in the patient's personal and family life.

3: Moderate: Problems are present and usually cause a lot of difficulties in the patient's personal and family life.

4: Severe: Problems are present and preclude the patient's ability to carry out normal activities or social interactions or to maintain previous standards in personal and family life.

The remaining questions in Part I (Non-motor Experiences of Daily Living) [Sleep, Daytime Sleepiness, Pain and Other Sensation, Urinary Problems, Constipation Problems, Lightheadedness on Standing, and Fatigue] are in the **Patient Questionnaire** along with all questions in Part II [Motor Experiences of Daily Living].

Patient Questionnaire:

Instructions:

This questionnaire will ask you about your experiences of daily living.

There are 20 questions. We are trying to be thorough, and some of these questions may therefore not apply to you now or ever. If you do not have the problem, simply mark 0 for NO.

Please read each one carefully and read all answers before selecting the one that best applies to you.

We are interested in your average or usual function over the past week including today. Some patients can do things better at one time of the day than at others. However, only one answer is allowed for each question, so please mark the answer that best describes what you can do most of the time.

You may have other medical conditions besides Parkinson's disease. Do not worry about separating Parkinson's disease from other conditions. Just answer the question with your best response.

Use only 0, 1, 2, 3, 4 for answers, nothing else. Do not leave any blanks.

Your doctor or nurse can review the questions with you, but this questionnaire is for patients to complete, either alone or with their caregivers.

Who is filling out this questionnaire (check the best answer):

☐ Patient ☐ Caregiver ☐ Patient and Caregiver in Equal Proportion

Part I: Non-Motor Aspects of Experiences of Daily Living (nM-EDL)	
1.7 SLEEP PROBLEMS Over the past week, have you had trouble going to sleep at night or staying asleep through the night? Consider how rested you felt after waking up in the morning. 0: Normal: No problems. 1: Slight: Sleep problems are present but usually do not cause trouble getting a full night of sleep. 2: Mild: Sleep problems usually cause some difficulties getting a full night of sleep. 3: Moderate: Sleep problems cause a lot of difficulties getting a full night of sleep, but I still usually sleep for more than half the night. 4: Severe: I usually do not sleep for most of the night.	**SCORE** ☐
1.8 DAYTIME SLEEPINESS Over the past week, have you had trouble staying awake during the daytime? 0: Normal: No daytime sleepiness. 1: Slight: Daytime sleepiness occurs but I can resist and I stay awake. 2: Mild: Sometimes I fall asleep when alone and relaxing. For example, while reading or watching TV. 3: Moderate: I sometimes fall asleep when I should not. For example, while eating or talking with other people. 4: Severe: I often fall asleep when I should not. For example, while eating or talking with other people.	☐

	SCORE
1.9 PAIN AND OTHER SENSATIONS Over the past week, have you had uncomfortable feelings in your body like pain, aches tingling or cramps? 0: Normal: No uncomfortable feelings. 1: Slight: I have these feelings. However, I can do things and be with other people without difficulty. 2: Mild: These feelings cause some problems when I do things or am with other people. 3: Moderate: These feelings cause a lot of problems, but they do not stop me from doing things or being with other people. 4: Severe: These feelings stop me from doing things or being with other people.	☐
1.10 URINARY PROBLEMS Over the past week, have you had trouble with urine control? For example, an urgent need to urinate, a need to urinate too often, or urine accidents? 0: Normal: No urine control problems. 1: Slight: I need to urinate often or urgently. However, these problems do not cause difficulties with my daily activities. 2: Mild: Urine problems cause some difficulties with my daily activities. However, I do not have urine accidents. 3: Moderate: Urine problems cause a lot of difficulties with my daily activities, including urine accidents. 4: Severe: I cannot control my urine and use a protective garment or have a bladder tube.	☐

	SCORE
1.11 CONSTIPATION PROBLEMS Over the past week have you had constipation troubles that cause you difficulty moving your bowels? 0: Normal: No constipation. 1: Slight: I have been constipated. I use extra effort to move my bowels. However, this problem does not disturb my activities or my being comfortable. 2: Mild: Constipation causes me to have some troubles doing things or being comfortable. 3: Moderate: Constipation causes me to have a lot of trouble doing things or being comfortable. However, it does not stop me from doing anything. 4: Severe: I usually need physical help from someone else to empty my bowels.	☐
1.12 LIGHT HEADEDNESS ON STANDING Over the past week, have you felt faint, dizzy or foggy when you stand up after sitting or lying down? 0: Normal: No dizzy or foggy feelings. 1: Slight: Dizzy or foggy feelings occur. However, they do not cause me troubles doing things. 2: Mild: Dizzy or foggy feelings cause me to hold on to something, but I do not need to sit or lie back down. 3: Moderate: Dizzy or foggy feelings cause me to sit or lie down to avoid fainting or falling. 4: Severe: Dizzy or foggy feelings cause me to fall or faint.	☐

	SCORE
1.13 FATIGUE Over the past week, have you usually felt fatigued? This feeling is <u>not</u> part of being sleepy or sad. 0: Normal: No fatigue. 1: Slight: Fatigue occurs. However it does not cause me troubles doing things or being with people. 2: Mild: Fatigue causes me some troubles doing things or being with people. 3: Moderate: Fatigue causes me a lot of troubles doing things or being with people. However, it does not stop me from doing anything. 4: Severe: Fatigue stops me from doing things or being with people.	☐

Part II: Motor Aspects of Experiences of Daily Living (M-EDL)

2.1 SPEECH Over the past week, have you had problems with your speech? 0: Normal: Not at all (no problems). 1: Slight: My speech is soft, slurred or uneven, but it does not cause others to ask me to repeat myself. 2: Mild: My speech causes people to ask me to occasionally repeat myself, but not everyday. 3: Moderate: My speech is unclear enough that others ask me to repeat myself every day even though most of my speech is understood. 4: Severe: Most or all of my speech cannot be understood.	☐

	SCORE
2.2 SALIVA AND DROOLING Over the past week, have you usually had too much saliva during when you are awake or when you sleep? 0: Normal: Not at all (no problems). 1: Slight: I have too much saliva, but do not drool. 2: Mild: I have some drooling during sleep, but none when I am awake. 3: Moderate: I have some drooling when I am awake, but I usually do not need tissues or a handkerchief. 4: Severe: I have so much drooling that I regularly need to use tissues or a handkerchief to protect my clothes.	☐
2.3 CHEWING AND SWALLOWING Over the past week, have you usually had problems swallowing pills or eating meals? Do you need your pills cut or crushed or your meals to be made soft, chopped or blended to avoid choking? 0: Normal: No problems. 1: Slight: I am aware of slowness in my chewing or increased effort at swallowing, but I do not choke or need to have my food specially prepared. 2: Mild: I need to have my pills cut or my food specially prepared because of chewing or swallowing problems, but I have not choked over the past week. 3: Moderate. I choked at least once in the past week. 4: Severe: Because of chewing and swallowing problems, I need a feeding tube.	☐

	SCORE
2.4 EATING TASKS Over the past week, have you usually had troubles handling your food and using eating utensils? For example, do you have trouble handling finger foods or using forks, knives, spoons, chopsticks? 0: Normal: Not at all (no problems). 1: Slight: I am slow, but I do not need any help handling my food and have not had food spills while eating. 2: Mild: I am slow with my eating and have occasional food spills. I may need help with a few tasks such as cutting meat. 3: Moderate: I need help with many eating tasks but can manage some alone. 4: Severe: I need help for most or all eating tasks.	☐
2.5 DRESSING Over the past week, have you usually had problems dressing? For example, are you slow or do you need help with buttoning, using zippers, putting on or taking off your clothes or jewelry? 0: Normal: Not at all (no problems). 1: Slight: I am slow but I do not need help. 2: Mild: I am slow and need help for a few dressing tasks (buttons, bracelets). 3: Moderate: I need help for many dressing tasks. 4: Severe: I need help for most or all dressing tasks.	☐

	SCORE
2.6 HYGIENE Over the past week, have you usually been slow or do you need help with washing, bathing, shaving, brushing teeth, combing your hair or with other personal hygiene? 0: Normal: Not at all (no problems). 1: Slight: I am slow but I do not need any help. 2: Mild: I need someone else to help me with some hygiene tasks. 3: Moderate: I need help for many hygiene tasks. 4: Severe: I need help for most or all of my hygiene tasks.	☐
2.7 HANDWRITING Over the past week, have people usually had trouble reading your handwriting? 0: Normal: Not at all (no problems). 1: Slight: My writing is slow, clumsy or uneven, but all words are clear. 2: Mild: Some words are unclear and difficult to read. 3: Moderate: Many words are unclear and difficult to read. 4: Severe: Most or all words cannot be read.	☐
2.8 DOING HOBBIES AND OTHER ACTIVITIES Over the past week, have you usually had trouble doing your hobbies or other things that you like to do? 0: Normal: Not at all (no problems). 1: Slight: I am a bit slow but do these activities easily. 2: Mild: I have some difficulty doing these activities. 3: Moderate: I have major problems doing these activities, but still do most. 4: Severe: I am unable to do most or all of these activities.	☐

	SCORE
2.9 TURNING IN BED Over the past week, do you usually have trouble turning over in bed? 0: Normal: Not at all (no problems). 1: Slight: I have a bit of trouble turning, but I do not need any help. 2: Mild I have a lot of trouble turning and need occasional help from someone else. 3: Moderate: To turn over I often need help from someone else. 4: Severe: I am unable to turn over without help from someone else.	☐
2.10 TREMOR Over the past week, have you usually had shaking or tremor? 0: Normal: Not at all. I have no shaking or tremor. 1: Slight: Shaking or tremor occurs but does not cause problems with any activities. 2: Mild: Shaking or tremor causes problems with only a few activities. 3: Moderate: Shaking or tremor causes problems with many of my daily activities. 4: Severe: Shaking or tremor causes problems with most or all activities.	☐
2.11 GETTING OUT OF BED, A CAR, OR A DEEP CHAIR Over the past week, have you usually had trouble getting out of bed, a car seat, or a deep chair? 0: Normal: Not at all (no problems). 1: Slight: I am slow or awkward, but I usually can do it on my first try. 2: Mild: I need more than one try to get up or need occasional help. 3: Moderate: I sometimes need help to get up, but most times I can still do it on my own. 4: Severe: I need help most or all of the time.	☐

	SCORE
2.12 WALKING AND BALANCE Over the past week, have you usually had problems with balance and walking? 0: Normal: Not at all (no problems). 1: Slight: I am slightly slow or may drag a leg. I never use a walking aid. 2: Mild: I occasionally use a walking aid, but I do not need any help from another person. 3: Moderate: I usually use a walking aid (cane, walker) to walk safely without falling. However, I do not usually need the support of another person. 4: Severe: I usually use the support of another person to walk safely without falling.	☐
2.13 FREEZING Over the past week, on your usual day when walking, do you suddenly stop or freeze as if your feet are stuck to the floor. 0: Normal: Not at all (no problems). 1: Slight: I briefly freeze but I can easily start walking again. I do not need help from someone else or a walking aid (cane or walker) because of freezing. 2: Mild: I freeze and have trouble starting to walk again, but I do not need someone's help or a walking aid (cane or walker) because of freezing. 3: Moderate: When I freeze I have a lot of trouble starting to walk again and, because of freezing, I sometimes need to use a walking aid or need someone else's help. 4: Severe: Because of freezing, most or all of the time, I need to use a walking aid or someone's help.	☐

This completes the questionnaire. We may have asked about problems you do not even have, and may have mentioned problems that you may never develop at all. Not all patients develop all these problems, but because they can occur, it is important to ask all the questions to every patient. Thank you for your time and attention in completing this questionnaire.

Part III: Motor Examination

Overview: This portion of the scale assesses the motor signs of PD. In administering Part III of the MDS-UPDRS the examiner should comply with the following guidelines:

At the top of the form, mark whether the patient is on medication for treating the symptoms of Parkinson's disease and, if on levodopa, the time since the last dose.

Also, if the patient is receiving medication for treating the symptoms of Parkinson's Disease, mark the patient's clinical state using the following definitions:

ON is the typical functional state when patients are receiving medication and have a good response.
OFF is the typical functional state when patients have a poor response in spite of taking medications.

The investigator should "rate what you see". Admittedly, concurrent medical problems such as stroke, paralysis, arthritis, contracture, and orthopedic problems such as hip or knee replacement and scoliosis may interfere with individual items in the motor examination. In situations where it is absolutely impossible to test (e.g., amputations, plegia, limb in a cast), use the notation "**UR**" for Unable to Rate. Otherwise, rate the performance of each task as the patient performs in the context of co-morbidities.

All items must have an integer rating (no half points, no missing ratings).

Specific instructions are provided for the testing of each item. These should be followed in all instances. The investigator demonstrates while describing tasks the patient is to perform and rates function immediately thereafter. For Global Spontaneous Movement and Rest Tremor items (3.14 and 3.17), these items have been placed purposefully at the end of the scale because clinical information pertinent to the score will be obtained throughout the entire examination.

At the end of the rating, indicate if dyskinesia (chorea or dystonia) was present at the time of the examination, and if so, whether these movements interfered with the motor examination.

3a Is the patient on medication for treating the symptoms of Parkinson's Disease? ☐ No ☐ Yes

3b If the patient is receiving medication for treating the symptoms of Parkinson's Disease, mark the patient's clinical state using the following definitions:

☐ ON: On is the typical functional state when patients are receiving medication and have a good response.

☐ OFF: Off is the typical functional state when patients have a poor response in spite of taking medications.

3c Is the patient on Levodopa ? ☐ No ☐ Yes

3.C1 If yes, minutes since last levodopa dose: _____

	SCORE
3.1 SPEECH Instructions to examiner: Listen to the patient's free-flowing speech and engage in conversation if necessary. Suggested topics: ask about the patient's work, hobbies, exercise, or how he got to the doctor's office. Evaluate volume, modulation (prosody) and clarity, including slurring, palilalia (repetition of syllables) and tachyphemia (rapid speech, running syllables together). 0: Normal: No speech problems. 1: Slight: Loss of modulation, diction or volume, but still all words easy to understand. 2: Mild: Loss of modulation, diction, or volume, with a few words unclear, but the overall sentences easy to follow. 3: Moderate: Speech is difficult to understand to the point that some, but not most, sentences are poorly understood. 4: Severe: Most speech is difficult to understand or unintelligible.	☐
3.2 FACIAL EXPRESSION Instructions to examiner: Observe the patient sitting at rest for 10 seconds, without talking and also while talking. Observe eye-blink frequency, masked facies or loss of facial expression, spontaneous smiling and parting of lips. 0: Normal: Normal facial expression. 1: Slight: Minimal masked facies manifested only by decreased frequency of blinking. 2: Mild: In addition to decreased eye-blink frequency, Masked facies present in the lower face as well, namely fewer movements around the mouth, such as less spontaneous smiling, but lips not parted. 3: Moderate: Masked facies with lips parted some of the time when the mouth is at rest. 4: Severe: Masked facies with lips parted most of the time when the mouth is at rest.	☐

3.3 RIGIDITY

Instructions to examiner: Rigidity is judged on slow passive movement of major joints with the patient in a relaxed position and the examiner manipulating the limbs and neck. First, test without an activation maneuver. Test and rate neck and each limb separately. For arms, test the wrist and elbow joints simultaneously. For legs, test the hip and knee joints simultaneously. If no rigidity is detected, use an activation maneuver such as tapping fingers, fist opening/closing, or heel tapping in a limb not being tested. Explain to the patient to go as limp as possible as you test for rigidity.

0: Normal: No rigidity.

1: Slight: Rigidity only detected with activation maneuver.

2: Mild: Rigidity detected without the activation maneuver, but full range of motion is easily achieved.

3: Moderate: Rigidity detected without the activation maneuver; full range of motion is achieved with effort.

4: Severe: Rigidity detected without the activation maneuver and full range of motion not achieved.

SCORE

☐ Neck

☐ RUE

☐ LUE

☐ RLE

☐ LLE

3.4 FINGER TAPPING

Instructions to examiner: Each hand is tested separately. Demonstrate the task, but do not continue to perform the task while the patient is being tested. Instruct the patient to tap the index finger on the thumb 10 times as quickly AND as big as possible. Rate each side separately, evaluating speed, amplitude, hesitations, halts and decrementing amplitude.

0: Normal: No problems.

1: Slight: Any of the following: a) the regular rhythm is broken with one or two interruptions or hesitations of the tapping movement; b) slight slowing; c) the amplitude decrements near the end of the 10 taps.

2: Mild: Any of the following: a) 3 to 5 interruptions during tapping; b) mild slowing; c) the amplitude decrements midway in the 10-tap sequence.

3: Moderate: Any of the following: a) more than 5 interruptions during tapping or at least one longer arrest (freeze) in ongoing movement; b) moderate slowing; c) the amplitude decrements starting after the 1st tap.

4: Severe: Cannot or can only barely perform the task because of slowing, interruptions or decrements.

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SCORE

3.5 HAND MOVEMENTS

Instructions to examiner: Test each hand separately. Demonstrate the task, but do not continue to perform the task while the patient is being tested. Instruct the patient to make a tight fist with the arm bent at the elbow so that the palm faces the examiner. Have the patient open the hand 10 times as fully AND as quickly as possible. If the patient fails to make a tight fist or to open the hand fully, remind him/her to do so. Rate each side separately, evaluating speed, amplitude, hesitations, halts and decrementing amplitude.

0: Normal: No problem.

1: Slight: Any of the following: a) the regular rhythm is broken with one or two interruptions or hesitations of the movement; b) slight slowing; c) the amplitude decrements near the end of the task.

2: Mild: Any of the following: a) 3 to 5 interruptions during the movements; b) mild slowing; c) the amplitude decrements midway in the task.

3: Moderate: Any of the following: a) more than 5 interruptions during the movement or at least one longer arrest (freeze) in ongoing movement; b) moderate slowing; c) the amplitude decrements starting after the 1st open-and-close sequence.

4: Severe: Cannot or can only barely perform the task because of slowing, interruptions or decrements.

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3.6 PRONATION-SUPINATION MOVEMENTS OF HANDS

Instructions to examiner: Test each hand separately. Demonstrate the task, but do not continue to perform the task while the patient is being tested. Instruct the patient to extend the arm out in front of his/her body with the palms down; then to turn the palm up and down alternately 10 times as fast and as fully as possible. Rate each side separately, evaluating speed, amplitude, hesitations, halts and decrementing amplitude.

0: Normal: No problems.

1: Slight: Any of the following: a) the regular rhythm is broken with one or two interruptions or hesitations of the movement; b) slight slowing; c) the amplitude decrements near the end of the sequence.

2: Mild: Any of the following: a) 3 to 5 interruptions during the movements; b) mild slowing; c) the amplitude decrements midway in the sequence.

3: Moderate: Any of the following: a) more than 5 interruptions during the movement or at least one longer arrest (freeze) in ongoing movement; b) moderate slowing c) the amplitude decrements starting after the 1st supination-pronation sequence.

4: Severe: Cannot or can only barely perform the task because of slowing, interruptions or decrements.

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SCORE

3.7 TOE TAPPING

Instructions to examiner: Have the patient sit in a straight-backed chair with arms, both feet on the floor. Test each foot separately. Demonstrate the task, but do not continue to perform the task while the patient is being tested. Instruct the patient to place the heel on the ground in a comfortable position and then tap the toes 10 times as big and as fast as possible. Rate each side separately, evaluating speed, amplitude, hesitations, halts and decrementing amplitude.

0: Normal: No problem.

1: Slight: Any of the following: a) the regular rhythm is broken with one or two interruptions or hesitations of the tapping movement; b) slight slowing; c) amplitude decrements near the end of the ten taps.

2: Mild: Any of the following: a) 3 to 5 interruptions during the tapping movements; b) mild slowing; c) amplitude decrements midway in the task.

3: Moderate: Any of the following: a) more than 5 interruptions during the tapping movements or at least one longer arrest (freeze) in ongoing movement; b) moderate slowing; c) amplitude decrements after the first tap.

4: Severe: Cannot or can only barely perform the task because of slowing, interruptions or decrements.

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3.8 LEG AGILITY

Instructions to examiner: Have the patient sit in a straight-backed chair with arms. The patient should have both feet comfortably on the floor. Test each leg separately. Demonstrate the task, but do not continue to perform the task while the patient is being tested. Instruct the patient to place the foot on the ground in a comfortable position and then raise and stomp the foot on the ground 10 times as high and as fast as possible. Rate each side separately, evaluating speed, amplitude, hesitations, halts and decrementing amplitude.

0: Normal: No problems.

1: Slight: Any of the following: a) the regular rhythm is broken with one or two interruptions or hesitations of the movement; b) slight slowing; c) amplitude decrements near the end of the task.

2: Mild: Any of the following: a) 3 to 5 interruptions during the movements; b) mild slowness; c) amplitude decrements midway in the task.

3: Moderate: Any of the following: a) more than 5 interruptions during the movement or at least one longer arrest (freeze) in ongoing movement; b) moderate slowing in speed; c) amplitude decrements after the first tap.

4: Severe: Cannot or can only barely perform the task because of slowing, interruptions or decrements.

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	SCORE
3.9 ARISING FROM CHAIR Instructions to examiner: Have the patient sit in a straight-backed chair with arms, with both feet on the floor and sitting back in the chair (if the patient is not too short). Ask the patient to cross his/her arms across the chest and then to stand up. If the patient is not successful, repeat this attempt a maximum up to two more times. If still unsuccessful, allow the patient to move forward in the chair to arise with arms folded across the chest. Allow only one attempt in this situation. If unsuccessful, allow the patient to push off using his/her hands on the arms of the chair. Allow a maximum of three trials of pushing off. If still not successful, assist the patient to arise. After the patient stands up, observe the posture for item 3.13. 0: Normal: No problems. Able to arise quickly without hesitation. 1: Slight: Arising is slower than normal; or may need more than one attempt; or may need to move forward in the chair to arise. No need to use the arms of the chair. 2: Mild: Pushes self up from arms of chair without difficulty. 3: Moderate: Needs to push off, but tends to fall back; or may have to try more than one time using arms of chair, but can get up without help. 4: Severe: Unable to arise without help.	☐
3.10 GAIT Instructions to examiner: Testing gait is best performed by having the patient walking away from and towards the examiner so that both right and left sides of the body can be easily observed simultaneously. The patient should walk at least 10 meters (30 feet), then turn around and return to the examiner. This item measures multiple behaviors: stride amplitude, stride speed, height of foot lift, heel strike during walking, turning, and arm swing, but not freezing. Assess also for “freezing of gait” (next item 3.11) while patient is walking. Observe posture for item 3.13. 0: Normal: No problems. 1: Slight: Independent walking with minor gait impairment. 2: Mild: Independent walking but with substantial gait impairment. 3: Moderate: Requires an assistance device for safe walking (walking stick, walker) but not a person. 4: Severe: Cannot walk at all or only with another person’s assistance.	☐

	SCORE
3.11 FREEZING OF GAIT Instructions to examiner: While assessing gait, also assess for the presence of any gait freezing episodes. Observe for start hesitation and stuttering movements especially when turning and reaching the end of the task. To the extent that safety permits, patients may NOT use sensory tricks during the assessment. 0: Normal: No freezing. 1: Slight: Freezes on starting, turning or walking through doorway with a single halt during any of these events, but then continues smoothly without freezing during straight walking. 2: Mild: Freezes on starting, turning or walking through doorway with more than one halt during any of these activities, but continues smoothly without freezing during straight walking. 3: Moderate: Freezes once during straight walking. 4: Severe: Freezes multiple times during straight walking.	☐
3.12 POSTURAL STABILITY Instructions to examiner: The test examines the response to sudden body displacement produced by a quick, forceful pull on the shoulders while the patient is standing erect with eyes open and feet comfortably apart and parallel to each other. Test retropulsion. Stand behind the patient and instruct the patient on what is about to happen. Explain that s/he is allowed to take a step backwards to avoid falling. There should be a solid wall behind the examiner, at least 1-2 meters away to allow for the observation of the number of retropulsive steps. The first pull is an instructional demonstration and is purposely milder and not rated. The second time the shoulders are pulled briskly and forcefully towards the examiner with enough force to displace the center of gravity so that patient MUST take a step backwards. The examiner needs to be ready to catch the patient, but must stand sufficiently back so as to allow enough room for the patient to take several steps to recover independently. Do not allow the patient to flex the body abnormally forward in anticipation of the pull. Observe for the number of steps backwards or falling. Up to and including two steps for recovery is considered normal, so abnormal ratings begin with three steps. If the patient fails to understand the test, the examiner can repeat the test so that the rating is based on an assessment that the examiner feels reflects the patient's limitations rather than misunderstanding or lack of preparedness. Observe standing posture for item 3.13 0: Normal: No problems: Recovers with one or two steps. 1: Slight: 3-5 steps, but subject recovers unaided. 2: Mild: More than 5 steps, but subject recovers unaided. 3: Moderate: Stands safely, but with absence of postural response; falls if not caught by examiner. 4: Severe: Very unstable, tends to lose balance spontaneously or with just a gentle pull on the shoulders.	☐

	SCORE
3.13 POSTURE Instructions to examiner: Posture is assessed with the patient standing erect after arising from a chair, during walking, and while being tested for postural reflexes. If you notice poor posture, tell the patient to stand up straight and see if the posture improves (see option 2 below). Rate the worst posture seen in these three observation points. Observe for flexion and side-to-side leaning. 0: Normal: No problems. 1: Slight: Not quite erect, but posture could be normal for older person. 2: Mild: Definite flexion, scoliosis or leaning to one side, but patient can correct posture to normal posture when asked to do so. 3: Moderate: Stooped posture, scoliosis or leaning to one side that cannot be corrected volitionally to a normal posture by the patient. 4: Severe: Flexion, scoliosis or leaning with extreme abnormality of posture.	☐
3.14 GLOBAL SPONTANEITY OF MOVEMENT (BODY BRADYKINESIA) Instructions to examiner: This global rating combines all observations on slowness, hesitancy, and small amplitude and poverty of movement in general, including a reduction of gesturing and of crossing the legs. This assessment is based on the examiner's global impression after observing for spontaneous gestures while sitting, and the nature of arising and walking. 0: Normal: No problems. 1: Slight: Slight global slowness and poverty of spontaneous movements. 2: Mild: Mild global slowness and poverty of spontaneous movements. 3: Moderate: Moderate global slowness and poverty of spontaneous movements. 4: Severe: Severe global slowness and poverty of spontaneous movements.	☐
3.15 POSTURAL TREMOR OF THE HANDS Instructions to examiner: All tremor, including re-emergent rest tremor, that is present in this posture is to be included in this rating. Rate each hand separately. Rate the highest amplitude seen. Instruct the patient to stretch the arms out in front of the body with palms down. The wrist should be straight and the fingers comfortably separated so that they do not touch each other. Observe this posture for 10 seconds. 0: Normal: No tremor. 1: Slight: Tremor is present but less than 1 cm in amplitude. 2: Mild: Tremor is at least 1 but less than 3 cm in amplitude. 3: Moderate: Tremor is at least 3 but less than 10 cm in amplitude. 4: Severe: Tremor is at least 10 cm in amplitude.	☐ R ☐ L

SCORE

3.16 KINETIC TREMOR OF THE HANDS

Instructions to examiner: This is tested by the finger-to-nose maneuver. With the arm starting from the outstretched position, have the patient perform at least three finger-to-nose maneuvers with each hand reaching as far as possible to touch the examiner's finger. The finger-to-nose maneuver should be performed slowly enough not to hide any tremor that could occur with very fast arm movements. Repeat with the other hand, rating each hand separately. The tremor can be present throughout the movement or as the tremor reaches either target (nose or finger). Rate the highest amplitude seen.

0: Normal: No tremor.

1: Slight: Tremor is present but less than 1 cm in amplitude.

2: Mild: Tremor is at least 1 but less than 3 cm in amplitude.

3: Moderate: Tremor is at least 3 but less than 10 cm in amplitude.

4: Severe: Tremor is at least 10 cm in amplitude.

☐ R

☐ L

3.17 REST TREMOR AMPLITUDE

Instructions to examiner: This and the next item have been placed purposefully at the end of the examination to allow the rater to gather observations on rest tremor that may appear at any time during the exam, including when quietly sitting, during walking and during activities when some body parts are moving but others are at rest. Score the maximum amplitude that is seen at any time as the final score. Rate only the amplitude and not the persistence or the intermittency of the tremor.
As part of this rating, the patient should sit quietly in a chair with the hands placed on the arms of the chair (not in the lap) and the feet comfortably supported on the floor for 10 seconds with no other directives. Rest tremor is assessed separately for all four limbs and also for the lip/jaw. Rate only the maximum amplitude that is seen at any time as the final rating.

Extremity ratings

0: Normal: No tremor.

1: Slight: ≤ 1 cm in maximal amplitude.

2: Mild: > 1 cm but < 3 cm in maximal amplitude.

3: Moderate: 3 - 10 cm in maximal amplitude.

4: Severe: > 10 cm in maximal amplitude.

☐ RUE

☐ LUE

☐ RLE

☐ LLE

Lip/Jaw ratings

0: Normal: No tremor.

1: Slight: ≤ 1 cm in maximal amplitude.

2: Mild: > 1 cm but ≤ 2 cm in maximal amplitude.

3: Moderate: > 2 cm but ≤ 3 cm in maximal amplitude.

4: Severe: > 3 cm in maximal amplitude.

☐ Lip/Jaw

3.18 CONSTANCY OF REST TREMOR

SCORE

Instructions to examiner: This item receives one rating for all rest tremor and focuses on the constancy of rest tremor during the examination period when different body parts are variously at rest. It is rated purposefully at the end of the examination so that several minutes of information can be coalesced into the rating.

0: Normal: No tremor.

1: Slight: Tremor at rest is present ≤ 25% of the entire examination period.

2: Mild: Tremor at rest is present 26-50% of the entire examination period.

3: Moderate: Tremor at rest is present 51-75% of the entire examination period.

4: Severe: Tremor at rest is present > 75% of the entire examination period.

DYSKINESIA IMPACT ON PART III RATINGS

A. Were dyskinesias (chorea or dystonia) present during examination? ☐ No ☐ Yes

B. If yes, did these movements interfere with your ratings? ☐ No ☐ Yes

HOEHN AND YAHR STAGE

0: Asymptomatic.

1: Unilateral involvement only.

2: Bilateral involvement without impairment of balance.

3: Mild to moderate involvement; some postural instability but physically independent; needs assistance to recover from pull test.

4: Severe disability; still able to walk or stand unassisted.

5: Wheelchair bound or bedridden unless aided.

☐

Part IV: Motor Complications

Overview and Instructions: In this section, the rater uses historical and objective information to assess two motor complications, dyskinesias and motor fluctuations that include OFF-state dystonia. Use all information from patient, caregiver, and the examination to answer the six questions that summarize function over the past week including today. As in the other sections, rate using only integers (no half points allowed) and leave no missing ratings. If the item cannot be rated, place UR for Unable to Rate. You will need to choose some answers based on percentages, and therefore you will need to establish how many hours generally are awake hours and use this figure as the denominator for "OFF" time and dyskinesias. For "OFF dystonia", the total "Off" time will be the denominator. Operational definitions for examiner's use.

Dyskinesias: Involuntary random movements
Words that patients often recognize for dyskinesias include "irregular jerking", "wiggling", "twitching". It is essential to stress to the patient the difference between dyskinesias and tremor, a common error when patients are assessing dyskinesias.

Dystonia: contorted posture, often with a twisting component:
Words that patients often recognize for dystonia include "spasms", "cramps", "posture".

Motor fluctuation: Variable response to medication:
Words that patients often recognize for motor fluctuation include "wearing out", "wearing off", "roller-coaster effect", "on-off", "uneven medication effects".

OFF: Typical functional state when patients have a poor response in spite of taking mediation or the typical functional response when patients are on NO treatment for parkinsonism. Words that patients often recognize include "low time", "bad time", "shaking time", "slow time", "time when my medications don't work."

ON: Typical functional state when patients are receiving medication and have a good response:
Words that patients often recognize include "good time", "walking time", "time when my medications work."

A. DYSKINESIAS [exclusive of OFF-state dystonia]

SCORE

4.1 TIME SPENT WITH DYSKINESIAS

Instructions to examiner: Determine the hours in the usual waking day and then the hours of dyskinesias. Calculate the percentage. If the patient has dyskinesias in the office, you can point them out as a reference to ensure that patients and caregivers understand what they are rating. You may also use your own acting skills to enact the dyskinetic movements you have seen in the patient before or show them dyskinetic movements typical of other patients. Exclude from this question early morning and nighttime painful dystonia.

Instructions to patient [and caregiver]: Over the past week, how many hours do you usually sleep on a daily basis, including nighttime sleep and daytime napping? Alright, if you sleep_____hrs, you are awake _____hrs. Out of those awake hours, how many hours in total do you have wiggling, twitching or jerking movements? Do not count the times when you have tremor, which is a regular back and forth shaking or times when you have painful foot cramps or spasms i nthe early morning or at nighttime. I will ask about those later. Concentrate only on these types of wiggling, jerking and irregular movements. Add up all the time during the waking day when these usually occur. How many hours_____(use this number for your calculations).

0: Normal:	No dyskinesias
1: Slight:	≤ 25% of waking day.
2: Mild:	26 - 50% of waking day.
3: Moderate:	51 - 75% of waking day.
4: Severe:	> 75% of waking day.

1. Total Hours Awake:
2. Total Hours with Dyskinesia:
3. % Dyskinesia = ((2/1)*100):

4.2 FUNCTIONAL IMPACT OF DYSKINESIAS

SCORE

Instructions to examiner: Determine the degree to which dyskinesias impact on the patient's daily function in terms of activities and social interactions. Use the patient's and caregiver's response to your question and your own observations during the office visit to arrive at the best answer.

Instructions to patient [and caregiver]: Over the past week, did you usually have trouble doing things or being with people when these jerking movements occurred? Did they stop you from doing things or from being with people?

0: Normal:	No dyskinesias or no impact by dyskinesias on activities or social interactions.
1: Slight:	Dyskinesias impact on a few activities, but the patient usually performs all activities and participates in all social interactions during dyskinetic periods.
2: Mild:	Dyskinesias impact on many activities, but the patient usually performs all activities and participates in all social interactions during dyskinetic periods.
3: Moderate:	Dyskinesias impact on activities to the point that the patient usually does not perform some activities or does not usually participate in some social activities during dyskinetic episodes.
4: Severe:	Dyskinesias impact on function to the point that the patient usually does not perform most activities or participate in most social interactions during dyskinetic episodes.

B. MOTOR FLUCTUATIONS

4.3 TIME SPENT IN THE OFF STATE

Instructions to examiner: Use the number of waking hours derived from 4.1 and determine the hours spent in the "OFF" state. Calculate the percentage. If the patient has an OFF period in the office, you can point to this state as a reference. You may also use your knowledge of the patient to describe a typical OFF period. Additionally you may use your own acting skills to enact an OFF period you have seen in the patient before or show them OFF function typical of other patients. Mark down the typical number of OFF hours, because you will need this number for completing 4.6.

Instructions to patient [and caregiver]: Some patients with Parkinson's disease have a good effect from their medications throughout their awake hours and we call that "ON" time. Other patients take their medications but still have some hours of low time, bad time, slow time or shaking time. Doctors call these low periods "OFF" time. Over the past week, you told me before that you are general awake_____hrs each day. Out of these awake hours, how many hours in total do you usually have this type of low level or OFF function_____(use this number for your calculations).

0: Normal:	No OFF time.
1: Slight:	≤ 25% of waking day.
2: Mild:	26 - 50% of waking day.
3: Moderate:	51 - 75% of waking day.
4: Severe:	> 75% of waking day.

1. Total Hours Awake: _____
2. Total Hours OFF: _____
3. % OFF = ((2/1)*100): _____

	SCORE
4.4 FUNCTIONAL IMPACT OF FLUCTUATIONS Instructions to examiner: Determine the degree to which motor fluctuations impact on the patient's daily function in terms of activities and social interactions. This question concentrates on the difference between the ON state and the OFF state. If the patient has no OFF time, the rating must be 0, but if patients have very mild fluctuations, it is still possible to be rated 0 on this item if no impact on activities occurs. Use the patient's and caregiver's response to your question and your own observations during the office visit to arrive at the best answer. *Instructions to patient [and caregiver]: Think about when those low or "OFF" periods have occurred over the past week. Do you usually have more problems doing things or being with people than compared to the rest of the day when you feel your medications working? Are there some things you usually do during a good period that you have trouble with or stop doing during a low period?* 0: Normal: No fluctuations or No impact by fluctuations on performance of activities or social interactions. 1: Slight: Fluctuations impact on a few activities, but during OFF, the patient usually performs all activities and participates in all social interactions that typically occur during the ON state. 2: Mild: Fluctuations impact many activities, but during OFF, the patient still usually performs all activities and participates in all social interactions that typically occur during the ON state. 3: Moderate: Fluctuations impact on the performance of activities during OFF to the point that the patient usually does not perform some activities or participate in some social interactions that are performed during ON periods. 4: Severe: Fluctuations impact on function to the point that, during OFF, the patient usually does not perform most activities or participate in most social interactions that are performed during ON periods.	☐
4.5 COMPLEXITY OF MOTOR FLUCTUATIONS Instructions to examiner: Determine the usual predictability of OFF function whether due to dose, time of day, food intake or other factors. Use the information provided by the patients and caregiver and supplement with your own observations. You will ask if the patient can count on them always coming at a special time, mostly coming at a special time (in which case you will probe further to separate slight from mild), only sometimes coming at a special time or are they totally unpredictable? Narrowing down the percentage will allow you to find the correct answer. *Instructions to patient [and caregiver]: For some patients, the low or "OFF" periods happen at certain times during day or when they do activities like eating or exercising. Over the past week, do you usually know when your low periods will occur? In other words, do your low periods always come at a certain time? Do they mostly come at a certain time? Do they only sometimes come at a certain time? Are your low periods totally unpredictable?"* 0: Normal: No motor fluctuations. 1: Slight: OFF times are predictable all or almost all of the time (> 75%). 2: Mild: OFF times are predictable most of the time (51-75%). 3: Moderate: OFF times are predictable some of the time (26-50%). 4: Severe: OFF episodes are rarely predictable (≤ 25%).	☐

C. "OFF" DYSTONIA

4.6 PAINFUL OFF-STATE DYSTONIA

Instructions to examiner: For patients who have motor fluctuations, determine what proportion of the OFF episodes usually includes painful dystonia? You have already determined the number of hours of "OFF" time (4.3). Of these hours, determine how many are associated with dystonia and calculate the percentage. If there is no OFF time, mark 0.

Instructions to patient [and caregiver]: In one of the questions I asked earlier, you said you generally have_____hours of low or "OFF" time when your Parkinson's disease is under poor control. During these low or "OFF" periods, do you usually have painful cramps or spasms? Out of the total_____hrs of this low time, if you add up all the time in a day when these painful cramps come, how many hours would this make?

0: Normal: No dystonia OR NO OFF TIME.

1: Slight: ≤ 25% of time in OFF state.

2: Mild: 26-50% of time in OFF state.

3: Moderate: 51-75% of time in OFF state.

4: Severe: > 75% of time in OFF state.

1. Total Hours Off: _____
2. Total Off Hours w/Dystonia: _____
3. % Off Dystonia = ((2/1)*100): _____

Summary statement to patient: READ TO PATIENT

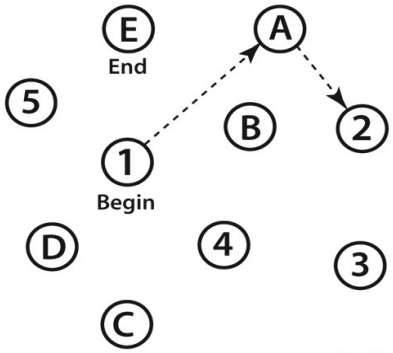
This completes my rating of your Parkinson's disease. I know the questions and tasks have taken several minutes, but I wanted to be complete and cover all possibilities. In doing so, I may have asked about problems you do not even have, and I may have mentioned problems that you may never develop at all. Not all patients develop all these problems, but because they can occur, it is important to ask all the questions to every patient. Thank you for your time and attention in completing this scale with me.

9.3 Montreal Cognitive Assessment

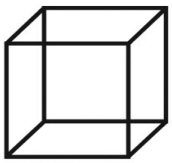
MONTREAL COGNITIVE ASSESSMENT (MOCA)
Version 7.1 Original Version

NAME :
Education : Date of birth :
Sex : DATE :

VISUOSPATIAL / EXECUTIVE — POINTS



Copy cube



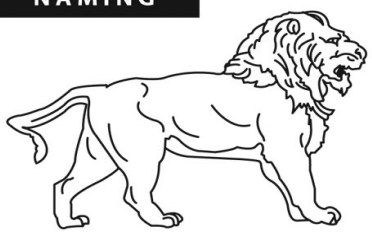
[ ] [ ]

Draw CLOCK (Ten past eleven)
(3 points)

[ ] Contour [ ] Numbers [ ] Hands

___/5

NAMING



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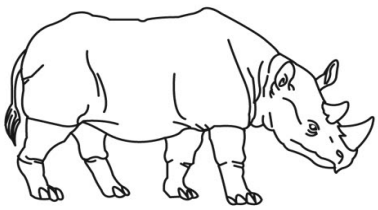
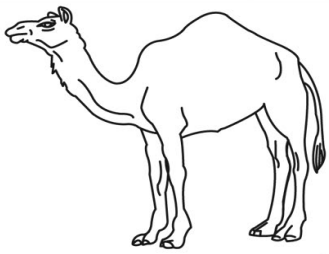


[ ]



[ ]

___/3

MEMORY Read list of words, subject must repeat them. Do 2 trials, even if 1st trial is successful. Do a recall after 5 minutes.

	FACE	VELVET	CHURCH	DAISY	RED
1st trial					
2nd trial					

No points

ATTENTION Read list of digits (1 digit/ sec.).
Subject has to repeat them in the forward order [ ] 2 1 8 5 4
Subject has to repeat them in the backward order [ ] 7 4 2
___/2

Read list of letters. The subject must tap with his hand at each letter A. No points if ≥ 2 errors
[ ] F B A C M N A A J K L B A F A K D E A A A J A M O F A A B
___/1

Serial 7 subtraction starting at 100 [ ] 93 [ ] 86 [ ] 79 [ ] 72 [ ] 65
4 or 5 correct subtractions: **3 pts**, 2 or 3 correct: **2 pts**, 1 correct: **1 pt**, 0 correct: **0 pt**
___/3

LANGUAGE Repeat : I only know that John is the one to help today. [ ]
The cat always hid under the couch when dogs were in the room. [ ]
___/2

Fluency / Name maximum number of words in one minute that begin with the letter F [ ] _____ (N ≥ 11 words)
___/1

ABSTRACTION Similarity between e.g. banana - orange = fruit [ ] train – bicycle [ ] watch - ruler
___/2

DELAYED RECALL

	Has to recall words **WITH NO CUE**	FACE [ ]	VELVET [ ]	CHURCH [ ]	DAISY [ ]	RED [ ]	Points for UNCUED recall only
Optional	Category cue						
	Multiple choice cue						

___/5

ORIENTATION [ ] Date [ ] Month [ ] Year [ ] Day [ ] Place [ ] City
___/6

 www.mocatest.org Normal ≥ 26 / 30

TOTAL ___/30
Add 1 point if ≤ 12 yr edu

Administered by: _____

9.4 PDQ-39 Questionnaire

PDQ-39 QUESTIONNAIRE

Please complete the following

Please tick one box for each question

	Due to having Parkinson's disease, how often during the last month have you....	Never	Occasionally	Sometimes	Often	Always or cannot do at all
1	Had difficulty doing the leisure activities which you would like to do?	☐	☐	☐	☐	☐
2	Had difficulty looking after your home, e.g. DIY, housework, cooking?	☐	☐	☐	☐	☐
3	Had difficulty carrying bags of shopping?	☐	☐	☐	☐	☐
4	Had problems walking half a mile?	☐	☐	☐	☐	☐
5	Had problems walking 100 yards?	☐	☐	☐	☐	☐
6	Had problems getting around the house as easily as you would like?	☐	☐	☐	☐	☐
7	Had difficulty getting around in public?	☐	☐	☐	☐	☐
8	Needed someone else to accompany you when you went out?	☐	☐	☐	☐	☐
9	Felt frightened or worried about falling over in public?	☐	☐	☐	☐	☐
10	Been confined to the house more than you would like?	☐	☐	☐	☐	☐
11	Had difficulty washing yourself?	☐	☐	☐	☐	☐
12	Had difficulty dressing yourself?	☐	☐	☐	☐	☐
13	Had problems doing up your shoe laces?	☐	☐	☐	☐	☐

*Please check that you have ticked **one box for each question** before going on to the next page*

Due to having Parkinson's disease, how often <u>during the last month</u> have you....		***Please tick <u>one</u> box for each question***				
		Never	**Occasionally**	**Sometimes**	**Often**	**Always** or cannot do at all
14	Had problems writing clearly?	☐	☐	☐	☐	☐
15	Had difficulty cutting up your food?	☐	☐	☐	☐	☐
16	Had difficulty holding a drink without spilling it?	☐	☐	☐	☐	☐
17	Felt depressed?	☐	☐	☐	☐	☐
18	Felt isolated and lonely?	☐	☐	☐	☐	☐
19	Felt weepy or tearful?	☐	☐	☐	☐	☐
20	Felt angry or bitter?	☐	☐	☐	☐	☐
21	Felt anxious?	☐	☐	☐	☐	☐
22	Felt worried about your future?	☐	☐	☐	☐	☐
23	Felt you had to conceal your Parkinson's from people?	☐	☐	☐	☐	☐
24	Avoided situations which involve eating or drinking in public?	☐	☐	☐	☐	☐
25	Felt embarrassed in public due to having Parkinson's disease?	☐	☐	☐	☐	☐
26	Felt worried by other people's reaction to you?	☐	☐	☐	☐	☐
27	Had problems with your close personal relationships?	☐	☐	☐	☐	☐
28	Lacked support in the ways you need from your spouse or partner?	☐	☐	☐	☐	☐
	If you do not have a spouse or partner tick here		☐			
29	Lacked support in the ways you need from your family or close friends?	☐	☐	☐	☐	☐

*Please check that you have ticked **one box for each question** before going on to the next page*

Due to having Parkinson's disease, how often <u>during the last month</u> have you....		***Please tick <u>one</u> box for each question***				
		Never	**Occasionally**	**Sometimes**	**Often**	**Always**
30	Unexpectedly fallen asleep during the day?	☐	☐	☐	☐	☐
31	Had problems with your concentration, e.g. when reading or watching TV?	☐	☐	☐	☐	☐
32	Felt your memory was bad?	☐	☐	☐	☐	☐
33	Had distressing dreams or hallucinations?	☐	☐	☐	☐	☐
34	Had difficulty with your speech?	☐	☐	☐	☐	☐
35	Felt unable to communicate with people properly?	☐	☐	☐	☐	☐
36	Felt ignored by people?	☐	☐	☐	☐	☐
37	Had painful muscle cramps or spasms?	☐	☐	☐	☐	☐
38	Had aches and pains in your joints or body?	☐	☐	☐	☐	☐
39	Felt unpleasantly hot or cold?	☐	☐	☐	☐	☐

*Please check that you have ticked **one box for each question** before going on to the next page*

Thank you for completing the PDQ 39 questionnaire