Exploring biomarkers for parkinsonian diseases and disease modification trials

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Declaration of originality

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Nirosen Vijiaratnam

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Abstract

Parkinsonian disorders encompass neurodegenerative conditions presenting with similar core clinical motor characteristics that are collectively termed parkinsonism though their associated features and progression vary. Modifying the course of these conditions remains a key goal considering their substantial impact on quality of life and survival. No treatments to date have achieved this. Demonstrating disease modification will require better identification of target populations by reducing cohort heterogeneity with precision approaches as well as selecting cases with maximal neuroprotective potential while also making changes to clinical trial designs and selecting more suitable measures for monitoring disease changes over time and to serve as more suitable endpoints. Quantifiable biomarkers with diagnostic specificity for each parkinsonian disorder that sufficiently predict progression at the time of trial recruitment while also being able to measure disease progression and therapeutic effects of interventions could potentially improve limitations of current trial approaches.

Insulin resistance is a characteristic of parkinsonian disorders and pre-clinical and early-stage clinical trials suggest this may be a promising target for achieving disease modification in these conditions. As part of this PhD, I embarked on recruiting and following up patients in clinical trials of Parkinson's disease and multiple system atrophy exploring the use of the glucagon like peptide-1 receptor agonist exenatide to achieve disease modification. In this thesis I explore biomarkers specific to insulin resistance in addition to more general disease state biomarkers which reflect axonal injury and dopaminergic denervation. I will present the potential for each of these biomarkers to be utilised in future trial analysis by exploring the complexities that can impact on their validity for use. Recommendations will also be proposed for potential future secondary outcome analysis of the current trials as well as overall better approaches for future clinical trial design by incorporation of the biomarkers studied.

Impact statement

Modifying the disease course of parkinsonian disorders remains an important unmet need despite decades of clinical trials with different agents aiming to achieve this. One way of improving likelihood of success is by identifying better biomarkers. A key limitation of previous studies is the presumption that credible biomarker findings in observational and natural history studies in early and prodromal disease stages are applicable in clinical trials enrolling patients with later disease stages.

This thesis explores a combination of biomarkers with the purpose of demonstrating their potential value for improving demonstration of disease modification in two actively recruiting clinical trials in Parkinson's disease (PD) and multiple system atrophy (MSA). This included peripheral insulin resistance markers and type 2 diabetes (T2DM) which was of interest as this pathway is a key target of GLP-1 receptor agonists which are being studied in the trials, the DAT-SPECT specific binding ratio which reflects dopaminergic denervation and neurofilament light chain levels (NfL) which reflect axonal damage.

The work firstly demonstrates that T2DM does not have an impact on disease severity and progression in atypical parkinsonian disorders unlike what has previously been noted in PD. Building on this, the work also notes that a smaller proportion of patients with the parkinsonian disorders have peripheral insulin resistance than what has previously been noted in other observational studies while also clarifying that there was no significant difference in the proportion of patients who have peripheral insulin resistance between the diseases unless cognitive impairment was present. Overall, these findings add value to potential future trial recruitment by firstly clarifying that T2DM status will need to be treated differently between disorders if utilized for enriching trial recruitment and secondly suggesting that peripheral insulin resistance markers are unlikely to be useful in tracking the diseases or treatment effects in the trials.

The work exploring the value of NfL suggests it may be a useful marker for distinguishing PD from atypical disorders while also predicting disease progression in PD and MSA. Contrasting findings were however noted between testing in a natural history study and the clinical trial in that NfL levels in PD were higher than healthy controls (HC) in the former but this was not the case in the latter. This highlights the limitations of analytical approaches and cohort characteristics and demonstrates the importance of not presuming that all findings in natural history studies would apply to trials and suggests better standardization of analysis approaches will be required.

A further study in the thesis explored the potential for utilising the widely used and available striatal DAT SPECT scan for demonstrating disease modification. This study demonstrated that this biomarker could still be useful at later disease stages though analysis approaches may need to be modified by focusing on the anterior putamen subregion and not limiting analysis to the less affected side which is currently the approach taken in most trials using this marker as an outcome measure. A key point that this work highlights is that findings in early and prodromal disease stages are not always applicable to later stages.

The work concludes by demonstrating that combining biomarkers such as NfL with simple clinical variables and genetic status would best predict progression in PD compared to just age and gender alone. This suggests that this combination could potentially be useful for balancing trial arms in future trials. The work also proposes that combining NfL with DAT-SBR may be a potential credible outcome measure though this needs to be explored in larger sample sizes and longitudinally.

Taken together, this thesis provides some useful preliminary findings on how biomarkers can be used in combination and makes suggestions for which biomarkers would be credible for use in the two trials explored as well as others more broadly. The work also highlights the importance of modifying analysis approaches depending on the cohorts being studied and makes suggestions for larger scale validation of the findings.

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List of abbreviations

AADC	L-aromatic amino acid decarboxylase
α-synuclein	alpha-synuclein
α -syn SAA	α-synuclein seed amplification assay
Aβ	Amyloid beta peptides
AD	Alzheimer's disease
APOE4	apolipoprotein E
APP	amyloid precursor protein
AUC	area under the curve
CTSD	cathepsin D
CBS	Corticobasal syndrome
CCL5	chemokine ligand 5
CNS	central nervous system
CNTN-1	contactin-1
CRP	C-reactive protein
DAT	dopamine transporter
DBM	Deformation-based morphometry
DJ-1	deglycase
DOPA	3,4-dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid

DLB	Dementia with lewy bodies
DNH	dorsal nigral hyperintensity
ET	Essential tremor
EVs	extracellular vesicles
GBA1	Glucosidase beta acid 1
Gcase	β-glucocerebrosidase
GAP-43	growth associated protein 43
GI	gastrointestinal
GFAP	glial fibrillary acidic protein
HbA1c	glycated hemoglobin
HC	healthy controls
5-HIAA	5-hydroxy-3-indoleacetic acid
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HVA	homovanillic acid
HY	Hoehn and Yahr
IMR	immunomagnetic reduction
Il	Interleukin
IRS-1	insulin-receptor substrate-1
IRS-1 p-Tyr	tyrosine-phosphorylated insulin receptor substrate-1
LN	lentiform nucleus

LRRK2	Leucine-rich repeat kinase 2
MCP-1	monocyte chemoattractant protein-1
miRNA	microRNA
MSA	Multiple system atrophy
NAA/Cr	N-acetyl aspartate/creatine
ncRNA	noncoding RNAs
NfL	neurofilament light chain
NFTs	neurofibrillary tangles
NLR	neutrophil-to-lymphocyte ratios
Ng	neurogranin
NMI	neuromelanin imaging
PD	Parkinson’s disease
PDCP	PD-related cognitive pattern
PDD	Parkinson’s disease dementia
PDRP	PD-related pattern
PET	positron Emission Tomography
PGC1	peroxisome proliferator-activated receptor γ coactivator 1
Pink-1	PTEN induced kinase 1
PIGD	postural instability and gait disorders
PLA	proximity Ligation Assay

PMCA	protein misfolding cyclic amplification
MRS	magnetic resonance spectroscopy
31P-MRS	Phosphorus based magnetic resonance spectroscopy
PPMI	Parkinson's progression markers initiative
PRKN	Parkin RBR E3 Ubiquitin Protein Ligase
pSer65Ub	phosphorylated ubiquitin residue at the serine 65
PSP	progressive supranuclear palsy
p-tau	phosphorylated tau
QSM	quantitative susceptibility mapping
RT-QuIC	real-time quaking-induced conversion
SBR	specific binding ratio
Ser-129p-α-syn	phosphorylated α-synuclein at serine-129
SCFA	short-chain fatty acids
SN	substantia Nigra
SNARE	soluble N-ethylmaleimide sensitive factor attachment protein
SNAP-25	synaptosomal-associated protein 25
sncRNA	small ncRNA
SNP	single nucleotide polymorphism
SPECT	single photon emission tomography
SWEDDS	scans without evidence of dopaminergic deficit

SWI	susceptibility- weighted imaging
t-tau	total tau
T2DM	Type 2 diabetes mellitus
TSPO	translocator protein
VAMP	vesicle-associated membrane proteins
VBM	voxel-based morphometry
VMAT2	vesicular monoamine transporter 2
VOI	volume of interest
YKL-40	chitinase-3-like protein 1

Publications arising from thesis

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Chapter One

Parkinsonian disorders and biomarkers

1.0 Summary of chapter

Biomarkers with high sensitivity and specificity for the diagnosis of Parkinson's disease are beginning to form the backbone for a proposed staging system for incorporation in Parkinson's disease clinical studies and trials. The routine use of specific biomarkers such as seed amplification assays of α-synuclein and tau should greatly aid in the accuracy of diagnosis during recruitment of patients with parkinsonian disorders into trials. There remain however further challenges in the pursuit of biomarkers for clinical trials of disease modifying agents namely: optimising the distinction between different disorders; the selection of subgroups most likely to benefit from a candidate disease modifying agent; as sensitive means of confirming target engagement; and in the early prediction of longer-term clinical benefit. The exploitation of a combination of biomarkers reflecting specific biological pathways or reflecting degrees of the degenerative process will therefore greatly add to our ability to plan trials and assess disease modifying properties of interventions. The choice of which biomarker(s) to use in the context of disease modifying clinical trials will depend on the intervention, the stage (at risk, premotor, motor, complex) of the population recruited and the aims of the trial. In this chapter I explore the range of biospecimen and imaging biomarkers that have been studied to date in different parkinsonian disorders and levels of evidence for use. The goal of the chapter is to understand different approaches and current limitations of biomarkers that will help in identifying appropriate choices and analysis approaches for disease modification clinical trials that I will be recruiting to.

1.1 Overview of Parkinsonian disorders

Parkinsonian disorders encompass neurodegenerative conditions presenting with similar core clinical motor characteristics that are collectively termed parkinsonism though their associated features and progression vary (1-3). Modifying the course of these conditions remains a key goal considering their substantial impact on quality of life and survival. No treatments to date have however, achieved this. Reasons for a lack of success include inadequate patient selection by pure reliance on clinical markers, failure of target engagement, inadequacies in trial design and the absence of objective measures of true disease progression (4). Achieving and demonstrating disease modification will require multipronged improvements. This includes better identification of target populations by reducing cohort heterogeneity with precision approaches as well as selecting cases with maximal neuroprotective potential. Making changes to clinical trial designs and selecting more suitable measures for monitoring disease changes over time and to serve as more suitable endpoints will also be critical (4, 5). Quantifiable biomarkers with diagnostic specificity for each parkinsonian disorder that sufficiently predict progression at the time of trial recruitment while also being able to measure disease progression and therapeutic effects of interventions could potentially improve several limitations of current trial approaches.

1.11 Clinical characteristics of Parkinsonian disorders

Parkinsonian disorders collectively represent the second largest group of neurodegenerative diseases (1). These conditions are characterised by a combination of parkinsonism (bradykinesia, extrapyramidal rigidity, and tremor) and non-motor features (autonomic dysfunction, cognitive decline, sleep alterations and neuropsychiatric symptoms). Red flags (specific symptoms or signs that argue for or against a diagnosis) are key in determining a specific diagnosis for the underlying disorder (6-10). (Table 1.1) No single red flag however provides definitive diagnostic certainty while variations in the prominence of these characteristics at follow-up assessments can further impact on

the diagnostic process. Prior to the development of a clear clinical phenotype only non-motor symptoms or subtle parkinsonism can be present, the prodromal disease stage. A clear diagnosis cannot be made at this stage despite the occurrence of neurodegeneration.

PD is by far the most common disorder diagnosed in cases presenting with parkinsonism. Typical PD manifests with asymmetrical rest tremor, extrapyramidal rigidity, and bradykinesia with decrement while patients tend to report a sizeable benefit from dopaminergic treatments. Presentations are however, heterogenous resulting in an average latency of 10 years from first symptom onset to diagnosis (11). Several recognisable subtypes with pathophysiological and prognostic differences exist (12). Presentations that are atypical for PD, the atypical parkinsonian disorders collectively represent approximately 20% of cases presenting with parkinsonism (2, 3). Neuronal degeneration is typically more aggressive and symptomatic therapy less effective than in PD thus leading to a steeper loss of function and shorter survival (6). The most common atypical disorder is progressive supranuclear palsy (PSP), followed by multiple system atrophy (MSA) and corticobasal syndrome (CBS) (3). Lewy body disease encompasses Parkinson disease dementia (PDD) and dementia with Lewy bodies (DLB) and is an atypical parkinsonian presentation with more prominent cognitive dysfunction.

Table 1.1 Clinical and Neuropathological characteristics of parkinsonian disorders

	PD	Lewy body dementia	MSA	PSP	CBS
Clinical features	• bradykinesia, rigidity, resting tremor, and postural and gait disturbances • non-motor manifestations (olfactory and autonomic dysfunction, sleep disorders, psychiatric symptoms, pain, depression, fatigue and cognitive impairment)	• dementia together with cognitive and alertness fluctuations, recurrent visual hallucinations, features of parkinsonism, and/or rapid eye movement sleep behaviour disorder • repeated falls, dysautonomia, other psychiatric manifestations (delusion, apathy, depression), and hypersensitivity to neuroleptic medications • distinction between PDD and DLB is based on 1 year rule of dementia onset	• either a combination of or predominant parkinsonism (MSA-P) poorly responsive to levodopa treatment or a cerebellar syndrome (MSA-C) • early dysautonomia (orthostatic hypotension, erectile dysfunction, constipation, urinary incontinence/retention, respiratory stridor, and sweat gland dysfunction)	• ocular motor dysfunction, postural instability, akinesia, and cognitive dysfunction • ocular motor dysfunction including vertical supranuclear gaze palsy, slow velocity of vertical saccades, and frequent macro square wave jerks or eyelid opening apraxia	• asymmetric limb rigidity, bradykinesia and dystonia • cortical features (limb apraxia, aphasia, alien limb phenomenon and stimulus-sensitive myoclonus • cognitive and behavioural changes
Clinical subtypes	Tremor dominant, postural instability, and gait disorder, diffuse malignant subtype	Parkinson's disease dementia, Dementia with Lewy bodies	MSA-parkinsonian, MSA-cerebellar	PSP-Richardson's syndrome, PSP-ocular motor, PSP-postural instability, PSP-parkinsonism (PSP-	Progressive non-fluent aphasia, speech apraxia, posterior cortical atrophy, behaviour variant frontotemporal lobal degeneration, PSP-like syndrome

				P), PSP-frontal, PSP-progressive gait freezing, PSP-corticobasal syndrome, PSP-speech/language disorder, PSP-primary lateral sclerosis, PSP-cerebellar ataxia (PSP-C)	
Neuropathological features	• intraneuronal fibrillar inclusions composed predominantly of misfolded a-synuclein protein within the cell body (Lewy bodies) and neuronal processes (Lewy neurites) • lewy pathology predominantly affecting the brainstem	• lewy pathology affecting the brainstem, limbic and neocortical regions • mixed AD pathology (amyloid and tau aggregates) is frequently observed	• fibrillar cytoplasmic inclusions composed of misfolded a-synuclein protein within the oligodendrocytes (Papp-Lantos bodies) • Loss of myelin, gliosis, axonal degeneration, and neuronal loss in the olivopontocerebellar and striatonigral regions	• neurofibrillary tangles composed of hyperphosphorylated 4-repeat tau protein, neuropil threads, star-shaped tufted astrocytes, oligodendroglial coiled bodies • gliosis primarily in the basal ganglia, brainstem and diencephalon	• hyperphosphorylated 4-repeat tau protein within the cell bodies in the form of swollen, achromatic (ballooned) neurons, and in glial cells as astrocytic plaques, gross asymmetric neuronal loss in frontoparietal lobes (Corticobasal degeneration-CBD) • other pathologies in addition to CBD-such as those seen in Alzheirmer's disease, PSP, Lewy bodies, and other tau-positive and tau-negative (e.g. TDP-43) forms of frontotemporal lobar degeneration

1.12 Diagnostic and prognostic challenges

An early and accurate diagnosis of degenerative parkinsonian syndromes is pivotal though this can be challenging in a proportion of cases. Making a diagnosis of idiopathic Parkinson's disease can be straightforward when classical features are present. Diagnostic misclassification can however occur in up to 25% in routine clinical practice and a meta-analysis of 29 clinico-pathological studies noted a diagnostic accuracy of 80·6% (13). Conditions commonly misdiagnosed as PD include non-Parkinsonian tremor and atypical parkinsonian disorders. Misdiagnoses are worse at earlier disease stages with error rates of up to 35% (14). The heavy reliance on 'red flags' for atypical disorders contributes to this as these features can develop late. Clinicians also rely on diagnostic tests to solidify the clinical diagnosis though their diagnostic yield is context dependent. Similar diagnostic issues occur with atypical disorders as a substantial mismatch between the clinical syndrome present and underlying pathology can occur (9, 10). Diagnostic criteria for individual parkinsonian conditions incorporating core, supportive and exclusionary features have been proposed by the International Parkinson and Movement Disorder Society to enhance diagnostic accuracy of PD (7), PSP (9) and MSA (10) while alternate criteria for CBS (15) and DLB (8) also exist. Although validation studies suggest improved diagnostic accuracy when applying these criteria, they are limited by minimal availability of *post mortem* validation studies and the prolonged clinical observation course needed to fulfil criteria (16-18).

Parkinsonian patients with clear features fulfilling a diagnosis exhibit remarkable clinical heterogeneity which relates to different pathophysiological mechanisms and this disease heterogeneity can influence disease progression (19, 20). Gathering such a heterogeneous group of patients as current trials do and expecting a uniform response to a particular intervention is therefore counterintuitive. Methods for refining patient selection include only recruiting patients at a specific disease stage though this approach is limited by disease stage fluctuations over progression which can make this unreliable. Developing better predictors of phenotype in addition

to diagnostic certainty will therefore be critical in providing patients with better prognostic certainty while also improving patient selection for disease modification clinical trials.

1.2 Biomarkers for parkinsonian disorders

1.21 Biomarker characteristics and development

A biomarker is a characteristic that is objectively measured and evaluated from any substance, structure, or process that can be measured in the body or its products as an indicator of normal biological or pathogenic processes, or pharmacologic responses to a therapeutic intervention (21). Ideal features include being readily quantifiable in accessible clinical samples (clinical assessments, biofluid (blood, CSF, urine) and tissue (skin, Oro-gastrointestinal mucosa)) while being reliable, quick, and inexpensive. The biochemical and imaging measurement techniques used to quantify biomarkers are summarised in Panel 1 and 2.

Panel 1

Fluid & tissue biomarker measurement techniques

- ELISA

  -target-specific antibodies bind to the sample proteins

  -secondary antibody linked to an enzyme recognises the matched antibodies

  -fluorescent reaction is created when exposed to a chemical substrate

  -amount of antigen present correlates to intensity of colour change

  -detection range inferior to other high-sensitivity techniques

- Luminex

  -beads conjugated with antibody against specific analyte present different colour codes

-high-throughput screening

-can measure up to 80 different proteins or RNA from a single microplate

- Mesoscale Discovery

  -high-throughput measurement of single or multiple targets

  -antibodies can be conjugated to generate electro chemiluminescent signals unlike ELISA

- Single Molecule Array

  -antibody-based ELISA and bead-based platform

  -antibody-coated bead binds to a single molecule and analysed separately

  -multiplexing of up to 11 analytes, high sensitivity, and wide detection range

- Proximity Extension Assay

  -DNA oligonucleotide tags linked to matched antibodies that both bind to target protein

  -antibodies come into proximity on binding, DNA duplex formed, sequence amplified

  -wide library of matched antibodies with high sensitivity and specificity for their targets

- SomaScan

  -Aptamers (short, single-stranded DNA or RNA molecules) bind target

  -quantified by microarrays or quantitative PCR

  -allows creation of library with high sensitivity for targets

- Single Molecule Counting

  -antibody–antigen sandwich complexes from either beads or plates

  -broken up and fluorescently labelled detection antibody counted by laser beam

  -allows for a high dynamic concentration range

- Mass spectrometry

  -measures mass-to-charge ratio of one or more molecules present

  -provide quantitative information about composition of complex protein samples

  -can also provide information about conformational properties

- Microscopy

  -used to examine to structure and formation of aggregates

  -approaches include fluorescence (aggregates labelled with fluorescent probes) microscopy and electron microscopy (resolve oligomer structure at higher resolution)

- Seed Amplification Assays

  -aggregation assays that detect protein aggregates

  -Sample sonication and incubation with recombinant protein monomer

  -aggregate seeds template and induce aggregation of the excess protein monomers

  -reaction monitored by a thioflavin readout, aggregation curve characteristics recorded

- Extracellular vesicles protein measurement

  -released by cells, content represent central nervous system processes

  -precipitation to increase concentration and neuronal enrichment with immune capture

  -protein quantification with electrochemiluminescence (e.g. Mesoscale discovery)

Biomarkers are classified according to their specific function. ***Susceptibility biomarkers*** indicate the potential for developing a disease in an individual who currently does not clinically exhibit the

disease. ***Diagnostic biomarkers*** confirm the presence of a disease or identify individuals with a subtype of the disease. Diagnostic biomarkers should be better at earlier diagnosis than a clinical examination. ***Biomarkers for prognosis*** identify the likelihood of progression or the development of a clinical event while ***predictive biomarkers*** are employed to identify individuals who are more likely to experience either a favourable or unfavourable outcome or treatment effect. Prognostic biomarkers should have the potential to serve as surrogate end points by correlating with or anticipating clinical progression while also showing superiority over clinical measurements. ***Therapeutic response biomarkers*** encompass biomarkers functioning as ***surrogate endpoints*** which support rational mechanistic/epidemiological/clinical data with the aim of providing evidence of an effect of a clinical benefit, ***monitoring biomarkers*** which aim to serially assess the status of a disease for evidence of exposure while also in specific cases demonstrating a pharmacodynamic response and ***safety measure biomarkers*** which indicate the likelihood, presence, or extent of toxicity.

A single biomarker may have strengths in one domain while not excelling in others. Specificity for biomarker category may also depend on the time of application with respect to the phase of a disease. Specificity may be more important than sensitivity when the biomarker is used for sample stratification in a clinical trial, while the predictive value of the test is more informative than its sensitivity and specificity and should always be interpreted in relation to the predictive value of the current goal standard. Minimal variation in the general population and a clear cut-off between patients and healthy controls is key. The magnitude of change relative to the statistical noise (effect size) should also be similar or greater for the biomarker measurement than for the clinical measurement while not being significantly impacted by unrelated conditions present. If the effect size of a biomarker is greater than the effect size of clinical outcome measures, this may reduce the sample size required. Ideally, short-term changes in the biomarker could anticipate long-term clinical outcomes. If the biomarker predicts or correlates with a clinical outcome, it may potentially be used as a surrogate trial end point. The ideal sample type would vary depending on the goal of the biomarker in question. An example of this is CSF. Despite being an ideal biofluid marker of ongoing

pathologic processes given its proximity to the central nervous system (CNS), CSF would be ideal for diagnostic and end-point purposes rather than monitoring given the invasive nature of specimen collection. In this context, other biofluids that can be obtained in a less invasive manner could be more suitable. Similar arguments can be made of imaging biomarkers in view of their labour intensity, significant duration, and high costs.

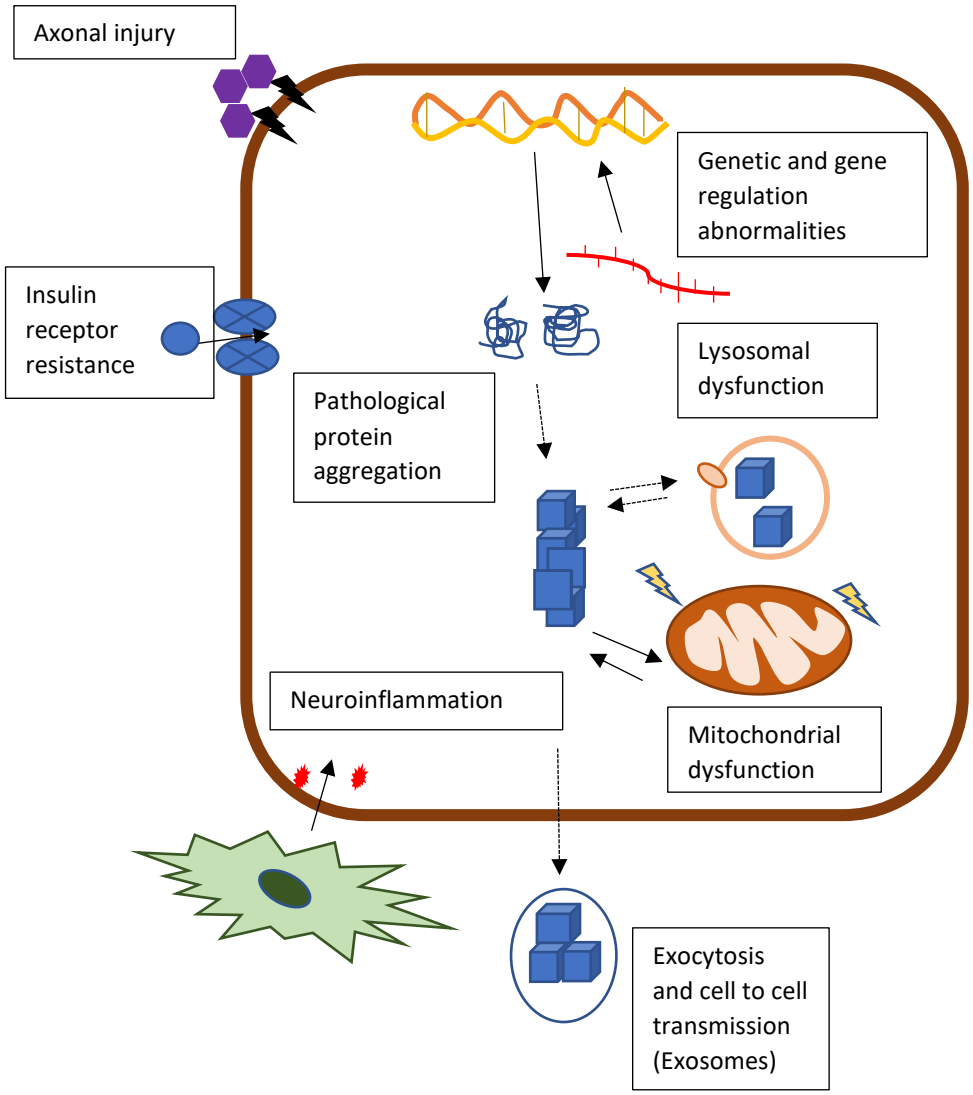
Developing a biomarker involves an initial exploratory study to identify candidate biomarkers and early assay development on patient and control biospecimens from standardized sources with harmonized protocols. This is followed by a validation phase which establishes the performance characteristics of the biomarker via key performance measures of sensitivity (proportion of true positives correctly identified by the biomarker among people with disease) and specificity (proportion of true negatives correctly identified by the biomarker among people without disease) to further generate receiver-operator curves (ROC) and area under the curves (AUCs), as well as determining likelihood ratios. This phase is also critical in determining reproducibility. The biomarker is then assessed in clinical utility and qualification phases which are crucial in minimizing overestimation by testing in large prospective clinical studies (22-26).

1.22 Fluid and tissue biomarkers for parkinsonian disorders

Parkinsonian disorders can largely be clustered into the underlying core protein abnormality that is occurring. PD, MSA and DLB share aggregation of pathological strains of α-synuclein (synucleinopathies), though variations in strain behaviour partly contribute to differences in presentations (1-3). PSP and CBS cases share primary tau aggregation (tauopathies) though alternative protein accumulation causes have been described (3). Neuropathological characteristics of these disorders are summarised in table 1.1. Core pathological protein aggregation can lead to and be influenced by common aberrancies in downstream cellular organelles and pathways (mitochondrial and lysosomal dysfunction, gene expression, insulin resistance, synaptic dysfunction)

with resultant neurodegeneration albeit with different emphasis and progression patterns in the different disorders. Measurement of these differences have been capitalised on for the development of fluid and tissue biomarkers. (Figure 1)

Figure 1 Neuronal cell body and mechanisms which are variably involved in the pathogenesis of parkinsonian disorders which can be targeted for biomarker measurement.



Alpha-synuclein

Alpha-synuclein (a-syn), a presynaptic amino acid is thought to regulate synaptic function and neurotransmitter release (27). A-syn undergoes posttranslational modifications (PTM), such as phosphorylation and conformational transformations, leading to the formation of phosphorylated (p-a-syn), oligomeric (o-a-syn) and fibrillary (f-a-syn) forms which aggregate and lead to neuronal toxicity (28). Lewy bodies (LBs) are the histopathological hallmark of PD and DLB and misfolded a-syn, particularly phosphorylated a-syn at serine-129 (Ser-129p-a-syn) represents their main component (29, 30). Accumulation of intra-neuronal Lewy bodies occurs progressively (31) with wide distributions noted in PD and DLB (32). LBs are considered a marker of neuronal degeneration in these conditions considering neuronal loss corresponds to their accumulation. Although some evidence suggests a protective role for LBs, o-a-syn and f-a-syn are thought to be cytotoxic (33). In MSA, f-a-syn mainly accumulates within oligodendroglial aggregates known as glial cytoplasmic inclusions (GCI) (34). These structures also compose a different pattern of p-a-syn to LBs (35).

Total a-syn levels

The different forms of a-syn are secreted into extracellular spaces and can be measured in body fluids (e.g., CSF, blood components, saliva, and tears) as well as peripheral tissue (e.g., skin, colon). As CSF provides a window to brain neuropathological changes, a-syn species levels have been most extensively studied there. Despite being consistently detected in CSF, total a-syn (t-a-syn) levels vary significantly within populations (HC, synucleinopathies) between studies (36-40). A decrease in t-a-syn levels in PD patients has been noted compared to HC and other neurodegenerative diseases in several cross-sectional studies (36-39) and corroborated in meta-analyses (40-43). Despite this, correlation between t-a-syn levels and disease severity, predicting progression and monitoring change over time or its reliability for distinguishing PD and other parkinsonian conditions has been inconsistent (37, 40). These variations relate to preanalytical and analytical factors, clinical heterogeneity of cohorts studied, and differences in the distribution of modified a-syn forms

collectively measured (44-46). Total CSF a-syn levels therefore remain unreliable for biomarker purposes.

Phosphorylated a-syn levels

Studies exploring PTM of a-syn have largely focused on Ser-129p-a-syn due to it being the predominant modified form in LBs (47) and its correlation with increasing overall pathology formation in the brain (48-54). Several studies have suggested CSF Ser-129p-a-syn levels are significantly elevated in PD compared to HC (40, 49, 51, 53, 55) with one study demonstrating reasonable diagnostic marker properties (49). Contradictory findings of no significant difference being noted in some studies however brings analytical factors into question (48, 54, 56, 57). Increased CSF Ser-129p-a-syn levels have been noted in MSA and PSP patients while reduced levels have been seen in DLB patients compared to HCs (48). No significant difference between PD patients and other synucleinopathies (48, 56) or tauopathies (48, 58) have however been noted. Although levels were increased in MSA compared to PSP and DLB patients in one study (48), these findings were contradicted in a separate study which demonstrated reduced Ser-129p-a-syn levels in MSA and PSP patients in comparison to PD patients and HC while no differences between MSA and PSP disease groups were noted (49).

Correlations between Ser-129p-a-syn and disease severity has also been inconsistent. Decreased Ser-129p-a-syn was associated with PD severity in some studies (49, 51, 52) while no association was noted in another (52). Decreasing levels over 2-years have also been noted though not statistically significant (48, 59). Total, Ser-129p-a-syn can also be measured in serum and plasma though concentrations vary (44, 50, 60-63). Ser-129p-a-syn levels were slightly increased in PD plasma compared to HC in one study though no significant differences were noted in oligomeric Ser-129p-a-syn levels (60). In a follow-up study, Ser-129p-a-syn levels remained unchanged over a span of 20 years after PD symptom onset (61) though plasma levels were associated with motor severity and progression but not cognitive decline in a separate 3.5-year follow-up study (64). A recent study

exploring a novel approach in diluted serum was able to demonstrate an excellent AUC of 0.92 for distinguishing PD from HC though interpretation was limited by the small sample size (65). Increased levels of several PTM a-Syn forms has also been noted in RBCs of PD patients compared HC (66) as has Ser-129p-a-syn in the cytosolic fraction of RBC (67). Lower levels have also been noted in PD patients with cognitive impairment compared to those without (68). Higher RBC Ser-129p-a-syn have been noted in MSA patients compared to HC while MSA-P patients had higher levels than MSA-C patients in one study (69).

Despite some promising findings, Ser-129p-a-syn levels in biofluids remains an unreliable biomarker largely due to substantial variations between studies by virtue of differences in detection sensitivities of assays available (70). The relatively higher levels of a-syn in RBCs compared to the other biological fluids potentially makes it easier to detect higher levels of modified forms of a-syn thus making it a valuable source for a-syn biomarkers. Further studies are however needed to better profile different species in large cohorts.

Outcomes are broadly similar for Ser-129p-α-syn in skin (71-76), though a predilection for autonomic compared to somatosensory nerve fibre deposition, greater overall deposition and widespread distribution (77) as well as a proximal to distal gradient can distinguish PD from MSA-P (78) and could therefore be considered for this critical diagnostic purpose. A rostro-caudal pSer129-α-syn deposition gradient in the gastrointestinal (GI) tract of PD patients has also been noted (79) with comparative reductions in PD compared to HC potentially reflecting ongoing neurodegeneration in the myenteric plexus (80). The finding of α-synuclein and pSer129-α-syn immunoreactivity in the GI tract of patients without neurodegenerative disease however raises the possibility of this being a reactive physiological phenomenon (81). While measurement in GI tissue is unlikely to play a role in diagnosing PD, disentangling reactive from pathological components warrants further study considering that deposition may occur here earlier and therefore might guide earlier treatment in pre-motor presentations of PD.

Ratios of PTM to total a-syn may be a more credible diagnostic and prognostic biomarker then individual metrics. Elevated Ser-129p-a-syn to total a-Syn ratios have been noted in PD compared to HC (49, 54). Ratios have also been shown to increase with disease progression in PD patients in two follow-up studies (51, 52). Ser-129p-a-syn to total a-syn ratios could also differentiate between PD and other synucleinopathies (49, 51, 54, 82-84) while also differentiating MSA and PSP patients (40).

Phosphotyrosine 39 a-syn (pY39 a-Syn) levels have been explored as a potential CSF biomarker based on c-Abl kinase studies demonstrating phosphorylation of a-Syn at this point in PD (85). In a small proof of concept study, no significant differences were noted between PD and HC. The ratio of pY39 a-syn to total Y39 a-syn levels was however significantly increased in PD patients (86). CSF pY39 a-syn levels are however low and the presence of minute amounts of unlabelled peptide standards could therefore complicate accurate estimation of levels therefore making interpretation of these findings unreliable. Further studies with larger cohorts are therefore needed. Promising findings of higher levels of nitration at Tyr125/136 residues and lower levels of nitration at Y39 and higher Tyr125/136 to Tyr39 ratios of a-syn in PD compared to HC in serum was also noted in one study (87) though no subsequent studies have confirmed these findings.

Oligomeric a-syn levels

Oligomeric a-syn has been detected in CSF (82, 83, 88, 89), blood constituents (plasma and RBCs) (89-94) and other biofluids (95, 96). Oligomeric a-syn levels in CSF are increased in PD compared to controls (43, 55, 82, 83, 88, 89, 95) as are plasma (90, 91) and RBC (93, 97) levels. Several blood based studies however contradict this by finding no level difference (89, 92, 94) while a meta-analysis concluded that the sensitivity and specificity of CSF assays are unsatisfactory (40). The ratio of o-a-syn to t-a-syn levels in CSF however improves diagnostic accuracy thus arguing for its potential use as a PD diagnostic biomarker (56, 82-84). Oligomeric a-syn levels are associated with PD severity and progression (52-54, 59). Despite contradictory evidence in one study (98), the prognostic value of o-a-syn levels in PD maybe a worthwhile consideration for future studies.

An electrochemiluminescence immunoassay of o-a-syn levels in the membrane fraction of RBCs noted increased levels in PD patients compared to HC but no significant changes in the cytosolic fraction (67). Levels of o-a-syn species in saliva were also increased in PD in addition to a higher ratio of o-a-syn to t-a-syn being noted compared to controls (95, 96, 99). Larger cohort replication studies in independent laboratories are however needed.

Two studies quantified oligomeric phosphorylated species of a-syn in CSF and plasma though differentiation between oligomers, fibrils and other aggregated forms were not possible with the ELISA technique used thus limiting further interpretation (48, 60). Phosphorylated aggregated forms of a-syn have been explored in one study where levels varied among the PD, DLB, PSP, MSA and HC, though levels appeared to best distinguish MSA from all other groups (100).

Challenges associated with measuring oligomeric forms of a-syn such as their susceptibility to dissociate (101) and lack of a standardised commercial kit approach make their current use as biomarkers unviable (52, 54, 59).

Exosome a-syn levels

Cell-to-cell propagation of a-syn partly underlies pathology spreading and progression in PD (102, 103). Exosomes are extracellular vesicles (EVs) containing a mixture of physiological and pathological proteins (104-107) which are transferred from cell to cell (e.g. neurons, microglia) for communication in health and disease (108). Exosomes can cross the blood-brain barrier and changes in exosome a-syn levels have been noted in CSF (109), plasma/serum (110-117), and saliva in PD and other synucleinopathies (118). Interaction between a-syn and exosomes accelerates a-syn aggregation (119) while exosomal a-syn species can seed aggregation and pathology formation in vitro. These findings along with the ability to isolate EVs from biological fluids have prompted studies evaluating their potential for biomarker use. Lower levels of EV-bound a-syn and the presence of seeding-competent a-syn species in the CSF of PD and DLB patients was reported in one study (109). Different strategies have been employed to isolate and quantify brain derived EVs by mitigating the

impact of RBC contamination (46, 110). This includes the use of antibodies directed against the neural L1 cell adhesion molecule (L1CAM) amongst others (115, 120, 121).

Isolation of CNS-specific L1CAM-derived EVs from plasma and serum showed that a-syn is present at higher levels in PD patients compared to HC (111-116) though this was contradicted by a separate study showing decreased levels (117). Levels of aSyn in L1CAM-positive EVs are also higher in individuals with REM sleep behaviour disorder (RBD) which is characterised by a lack of atonia in this sleep phase when it should be present (113, 114) and levels remain high in individuals who progress to develop PD (113). This is of particular interest as the presence of RBD is a strong predictor of the future development of synucleinopathies (122). A weak correlation with PD disease severity (110) and more severe motor progression at 22 months has also been observed (113). Levels currently lack value for monitoring disease progression in PD possibly due to combined measurement of brain- and peripheral nerve-derived EVs (118, 120).

Several studies have explored pathological a-syn species in relation to total a-syn in plasma/serum exosomes from PD patients and HC (113, 117, 120, 123). Oligomeric and phosphorylated a-syn species have been noted inside and on the membrane surface of plasma exosomes (123). Lower levels of oligomeric aSyn levels were reported in PD patients in one study with differences seen between PD patients with and without tremor (117) though this has been contradicted by others demonstrating higher levels (113, 120). Increased neuronal exosomal Ser-129p-a-syn levels have been noted in a subgroup of PD patients though this did not correlate with disease severity (114). In another study, unmodified and Ser-129p-a-syn species were found on the membrane surface and inside exosomes (123). Lower levels of total a-syn and higher ratios of a-syn oligomer/total a-syn and Ser-129p-a-syn /total aSyn in plasma exosomes and higher levels of o-a-syn and o-a-syn/t-a-syn in saliva has been noted in PD patients (123) though there was no correlation with disease severity.

EVs have also been noted in urine and saliva (118, 124, 125) though EV derived a-syn has only been noted in saliva (118, 125). Salivary levels of a-syn oligomers along with the ratio of o-a-syn/t-a-syn is

higher in PD compared to controls (118, 125). Levels are not associated with age and disease duration (118, 125). The value of EV a-syn for distinguishing parkinsonian conditions is currently limited to one study finding the ratio of a-syn levels in oligodendroglial exosomes compared to neuronal exosomes differentiating PD from MSA patients (116).

Follow-up studies in larger cohorts are needed to improve the sensitivity and specificity and to validate EV potential as biomarkers for differentiating between PD and other synucleinopathies. The incorporation of other protein biomarkers could potentially improve the diagnostic potential of EVs (112, 118, 124-126).

a-syn seed amplification

Seed amplification assays (SAA), such as real-time quaking-induced conversion (RT-QuIC) and Protein misfolding cyclic amplification (PMCA), are arguably the most important achievement in the field of biomarkers to date and will likely be the most useful diagnostic biomarker for trials. These techniques can amplify and detect minute amounts of aggregated α-synuclein in CSF (127-130). Studies comparing brain and CSF samples have demonstrated excellent performance for distinguishing PD from HC (sensitivity and specificity (90%–100%)) (129, 131-137) with comparable results for both seeding methods (129, 134) across laboratories (129). Assays can also distinguish PD from non-synuclein disorders such as PSP and CBS (137), though accuracy for distinguishing MSA from these conditions is poor (sensitivity 4%–82%) and studies exploring α-syn SAA to distinguish MSA from PD have reported variable findings (128, 130, 138-140). Similarly, α-syn SAA distinguishes DLB from HC well but its ability to distinguish DLB from PD is poor (141). As differences in α-synuclein strains and therefore biochemical, morphological, and structural properties of the final α-syn SAA reaction products partly underlie PD and MSA phenotypic heterogeneity, different outcomes may be explained by the fact that different chemical environments (SAA reaction mixes) can differentially influence formation and growth of different strains. Protocols optimized for PD may not therefore work so well

for MSA detection (137, 142). Considering strain similarities between PD and DLB, alternate biomarkers may need to be considered for this purpose.

In attempts to avoid lumbar puncture, α-syn SAA has been explored in samples obtained through less invasive approaches. Increased α-synuclein *skin* seeding activity has been observed in PD (*post mortem* and living) patients with excellent distinction from non-neurodegenerative cases (143) while aggregation rates using RT QuIC correlate with cognitive and motor status (135). Seeding on skin was also noted in a small number of *post mortem* MSA cases with variable sensitivity and specificity in different body regions thus highlighting this important consideration in future studies (144). Similarly, seeding activity in *submandibular gland* tissue of PD patients has been noted though sensitivity (73.2% vs 100%) and specificity (78.6% vs 94%) for distinguishing PD from HCs varies between studies (145, 146) while preliminary findings in saliva are also promising (147). Higher seeding activity has been noted in olfactory mucosa (OM) as well in MSA and PD patients (138, 148, 149) though one study suggested no MSA-C cases demonstrated seeding activity unlike 90% of MSA-P cases who did (149). OM seeding activity was also associated with some motor features in this study. A recent report demonstrating excellent ability of *serum* immunoprecipitation-based RT-QuIC for distinguishing PD from HC may herald a new approach towards diagnosing PD through a simple blood test, though lower detection rates in MSA, likely due to technical factors, will still need to be overcome (150). Similarly, the demonstration of seeding activity from pathological α-synuclein derived from plasma EVs is also promising (151). The use of less invasive samples will be ideal for trial recruitment but will require demonstration of comparability with the high sensitivity and specificity achieved with CSF although recent meta-analyses suggest comparability between CSF and skin for diagnostic purposes in PD (139, 152)).

Recent studies have demonstrated correlation with PD disease severity and progression though specific kinetic cut-offs remain elusive (129, 153). The potential role for a-syn SAAs for predicting the development of PD in prodromal stages also appears promising (128, 153, 154). RT-QuIC correlates

with worse MSA progression though this was not the case for PD in this study (128). This study also demonstrated the potential for RT-QuIC to predict different synucleinopathy pathologies from iRBD thus emphasising its strength as an early diagnostic biomarker (128). Current limitations of this technique include a lack of quantification of aggregates considering current approaches focus on kinetic parameters which measure aggregation rates. Utility for monitoring disease progression and severity would necessitate quantification of aggregate.

Current use recommendation: Seed amplification either in CSF or skin maybe the best approach for diagnosing synucleinopthies. Some work is required to improve assays for MSA diagnosis. Ratios of PTM of a-syn to total levels maybe best at tracking disease.

Alzheimer disease (AD) like biomarkers

Tau protein is a component of cytoskeletal microtubules. Six different isoforms with either three (3R) or four (4R) carboxy-terminal tandem repeat sequences exist in humans (155). Hyperphosphorylation causes loss of its affinity for microtubules and makes it resistant to proteolysis resulting in accumulation and development of neuronal filamentous inclusions, Neurofibrillary tangles (NFT) (155). Tau pathology is characteristic of PSP and CBS with aggregates mainly containing 4R-tau (156, 157). In PSP aggregation of predominantly 4R-tau occurs in NFT, oligodendrocytic coils, and astrocytic tufts firstly in the midbrain and basal nuclei and subsequently in the cerebral cortex (3). In contrast, 4R-tau pathology in CBD appears more in astrocytic plaques and to a lesser extent in neural inclusions and threads in grey and white matter (3). Considerable clinical and neuropathological overlap between diseases and within disease subtypes occurs.

Amyloid beta peptide (Aβ) on the other hand is cleaved from the amyloid precursor protein (APP) into the isoforms Aβ42 and Aβ40 which can form extracellular amyloid plaques (158, 159). Amyloid plaques are abundant in the CNS alongside NFTs and characterise Alzheimer's disease (AD). Tau and

AD pathology can coexist with Lewy body's in synucleinopathies considering a synergistic and bidirectional link between a-syn and amyloid as well as tau phosphorylation (160). Their presence in PD correlates with an acceleration of cognitive decline (161, 162) while amyloid and phosphorylated tau burdens also reflect higher cortical and striatal loads and a clinical cognitive abnormality 'hierarchy'- DLB > PDD > PD (163).

Biomarkers reflecting tau and amyloid pathology can be measured in CSF and blood and include levels of total tau (t-tau), phosphorylated tau (p-tau) and amyloid peptide isoforms (Aβ42 and Aβ40). CSF t-tau alone is poor at distinguishing primary tauopathies (164-166). CSF t-tau is however higher in tauopathies than PD, with the highest values noted in CBS. As Aβ42 is decreased in DLB and CBS patients, its combination with either t-tau or p-tau could potentially discriminate DLB or CBS from other parkinsonian disorders. A further study found higher CSF t-tau and p-tau levels in CBS patients compared to PSP and PD with t-tau demonstrating the best sensitivity (75%) and specificity (90.9%) for discriminating CBS from PD (167). Elevated t-tau and decreased Aβ42 levels in CBS have also been noted in a separate study in comparison to PD, PSP and MSA patients as well as HCs (168).

Different disease specific tau fragments in brain extracts and CSF (169) have also been explored as potential biomarkers. In PSP and CBS lower CSF levels of N-244 tau fragments have been noted compared to AD and HCs (170). N-244 levels do not however correlate with CSF p-tau levels. This in association with decreased CSF p-tau levels in primary tauopathies suggests reduced tau synthesis and/or secretion into the CSF of PSP and CBS patients and potentially different tau pathophysiology in PSP and CBS compared to AD (170). Preliminary evidence suggests ultrasensitive tau SAA may identify/exclude patients with tauopathies from PD at trial recruitment (171) though a combined assay with α-synuclein would be more ideal.

The combination of reduced Aβ42 and increased t-tau and p-tau levels is collectively termed an AD-like profile considering its specificity for diagnosing the condition (172). This profile is noted in up to 40% of DLB and CBS cases in contrast to no PSP cases (173). AD profiles have been investigated in

synucleinopathies, due to the frequent observation in PD and DLB of AD-like pathology which more specifically correlates with dementia than a-syn pathology (174). AD-like CSF profiles occur in larger proportions of cases with prominent cognitive dysfunction (PDD and DLB) (175-177). CSF core AD biomarkers may therefore be useful for differentiating DLB from other parkinsonian disorders. DLB patients have the lowest levels of CSF Aβ42 amongst parkinsonian conditions (173, 178). Moreover, when considering DLB patients also have higher t-tau concentrations compared to PDD and PD patients (176, 179), the combination of Aβ42 and t-tau maybe better at discriminating DLB and PDD (179). PD patients with lower CSF Aβ42 levels at disease onset have earlier appearance of cognitive impairment and more rapid conversion to PD related dementia (83, 180, 181). The measurement of CSF amyloid could therefore be of prognostic value in parkinsonian syndromes by reflecting brain amyloid content even prior to apparent clinical cognitive impairment (182).

Aβ42 can also be measured in blood. These levels do not seem to correlate with cerebral Aβ pathology (183) potentially owing to high amounts of the Aβ peptides in plasma being reflective of extra-cerebral sources (184). Ultrasensitive immunoassay technologies such as immunomagnetic reduction (IMR) have however improved correlation between plasma levels and cerebral amyloidosis (185). Findings of studies exploring blood levels of Aβ42 and correlations with cognitive function in PD have however been inconsistent (186-188) although a lower Aβ42 plasma level was associated with the postural instability gait difficulty (PIGD) subtype of PD in one study (189), interpretation of this finding is confounded by the fact that this phenotype predisposes to development of cognitive impairment in PD.

Exploration of total tau protein levels in blood has proven to be challenging with variable findings of increases or no differences being noted compared to HCs (187, 188) potentially due to rapid changes in blood concentrations making this approach unreliable (190). Higher t-tau levels seem to however be related with lower cognitive performance (191). Plasma levels of total and phosphorylated tau are significantly increased in all parkinsonian disease groups compared to controls with highest

levels being noted in patients with frontotemporal dementia (FTD). The combination with Aβ42 could potentially differentiate FTD from PD/APS with high accuracy (186).

Aβ42 and tau can also be detected in EVs. While also not of diagnostic value, elevated levels in combination with elevated a-syn (192, 193) and lower serine phosphorylated insulin receptor substrate (IRS-p312) which is a marker of neuronal insulin resistance in blood EVs (194), predicts worse motor and cognitive dysfunction progression phenotypes well. Larger replication studies of Aβ and tau in EVs are needed to better assess their validity for predicting cognitive dysfunction in PD before adoption for widespread use.

Measurement of other phosphorylated tau species (P-tau181, P-tau217, and P-tau231) in CSF and plasma can discriminate AD patients from cognitively unimpaired subjects and reflect cognitive measures and progression (195). P-tau181 levels have been studied in PD and their ability to predict disease severity and cognitive decline has been mixed and therefore cannot currently be recommended for trial use (196-198). Other tau species require further exploration in PD cohorts. Other amyloid-beta species (Aβ38, Aβ40) and their ratio has also been explored for discriminating DLB from AD though their performance is only modest (sensitivity 78%, specificity 67%) (199, 200).

Taken together there are only a limited number of current studies with small sample sizes and cross-sectional design exploring AD core peptides in blood in PD. Larger studies using ultrasensitive assay methods are needed to better assess the validity of Aβ and tau particularly for detecting and tracking cognitive dysfunction in PD. Future investigations should also include Aβ42/Aβ40 ratios to correct for interindividual differences.

Current use recommendation: CSF t-tau/Aβ42 ratios are useful for distinguishing tauopathies from synucleinopathies. Aβ42/Aβ40 ratios could be useful at predicting cognitive decline in PD.

Neuroinflammation

CNS immune cells are involved in supportive functions. Microglia are monocytic cells that phagocytose cellular rubble and protein aggregates, while astrocytes mainly support neuronal and synaptic activities. When activated, these cells produce inflammatory mediators (cytokines and chemokines) and deleterious molecules including reactive oxygen species (ROS) and nitric oxide (NO)(201). Neuroinflammatory processes can lead to neurodegeneration (202). Evidence for this comes firstly from the fact that aggregates of misfolded proteins preferentially concentrate in glial cells (GCI in MSA, tufted astrocytes in PSP and astrocytic plaques in CBS) thus implicating them in the neurodegenerative process (34, 157). Furthermore, a-syn pathology modulates immune responses through the expression of Human leucocyte antigen-DR molecules leading to glial activation. Activated glial cells further stimulate astrocytes and microglia thereby amplifying inflammatory responses and acting on dopaminergic neurons by promoting a-syn misfolding. This feed-forward mechanism contributes to the maintenance of inflammation and progression of degeneration (203).

YKL-40 (also known as Chitinase-3-like protein 1) and MCP-1 (monocyte chemoattractant protein-1, also called CCL2) are two most studied glial activation biomarkers detectable in CSF. CSF YKL levels were not found to be elevated in MSA compared to HC in a meta-analysis (204) nor was serum levels in a separate study (205) though levels seem to be higher compared to PD (206, 207) despite one study contradicting this (208). Higher levels were noted in a PSP study compared to PD (208). CSF levels were also raised in CBS compared to PD but similar to PSP (205). A-syn stimulates pericytes to produce MCP-1 and other inflammatory markers (209). Accordingly, higher CSF MCP-1 concentrations in conjunction with increased levels of pro-inflammatory cytokines and activated T-lymphocytes have been noted in PD (210). The combination of nine CSF biomarkers including YKL-40, MCP-1, t-tau, p-tau, Aβ42, a-syn, NfL and amyloid precursor protein soluble metabolites was able to discriminate PD from APS with a high accuracy (AUC = 0.95, 91% sensitivity and specificity) (208). Glial fibrillary acidic protein (GFAP) is another marker of glial cell activation. Although a meta-

analysis concluded CSF levels were elevated in MSA compared to HC findings are limited by the small number of studies included (211). Contradictory findings in studies comparing MSA and PD overall lead to current findings being inconclusive for recommending this biomarker (212, 213). CSF GFAP was also elevated in one PSP study compared to HC and this will require replication in larger cohorts (214). CSF GFAP levels are thought to be normal in CBS (215). Astrocytes play a critical role in brain cholesterol metabolism. Side metabolites of this process include 24S-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC). CSF levels of 24OHC and 27OHC are increased in CBS patients and this correlates with higher tau levels (216).

Peripheral measurement of lymphocyte profiles analysed by flow cytometry has also been explored as biomarkers. A-syn triggers responses in helper and cytotoxic T cells (217). T helper (CD4 + T) cells regulate the immune system via cytokines (218). CD4 + T cells can adopt pro- (T helper (Th) 1) and anti-inflammatory (Th2 or T regulatory (Treg)) phenotypes. A decline of CD4 + cells with a shift toward Th1 and a corresponding decrease in Th2 and Treg has been noted in PD representing a shift towards a proinflammatory state (219-221). Subtypes of Tregs are also diminished in PD (222) though no difference in the total number of T lymphocytes has been noted despite an increased proportion of activated cells (210). A shift from naive to activated Treg cells (CD45RA + to CD45RO +) resulting in higher levels of CD45RO + cells is associated with more severe motor and cognitive impairment in PD (223, 224). The proportion of Tregs expressing CD49d, coding for a molecule that enables migration into the central nervous system, is increased in PD and linked to lower motor impairment, suggesting a protective influence (225).

The neutrophil-to-lymphocyte ratio (NLR) is increasingly reported as an easily determinable and cost-effective biomarker. NLR is an indicator of the inflammatory status in infectious and neurodegenerative disorders (226, 227). Elevated NLR has been noted in PD compared to healthy controls in a meta-analysis (228). Late-onset PD may have a higher NLR than early onset PD (229).

NLR is negatively associated with striatal-binding ratios in DaTSCAN and positively associated with motor impairment though the latter is confined to tremor dominant cases (228, 230).

Taken together, lymphocyte profiles and NLR represent an interesting approach as biomarkers for disease progression of PD. While the NLR is supported by its easy accessibility and association with nigrostriatal degeneration, lymphocyte profiles could potentially play a role in the identification of subtypes within PD. Further prospective studies with clearly defined a priori hypotheses and sufficient power are needed to conclusively assess the value of lymphocyte profiles as biomarkers. Their role as biomarkers in atypical disorders is less clear.

The altered composition of lymphocytes leads to and is in turn influenced by an altered composition of cytokines thus leading to their investigation as potential biomarkers. Elevated levels of CRP and hs-CRP have been noted in patients with PD compared to HC (229, 231, 232) while higher CRP levels are associated with reduced survival (233). Interleukins comprise a group of more than 50 cytokines which exert pro- and anti-inflammatory effects (234). Increases in Il-6 and Il-10 levels between PD and healthy controls have been noted in a meta-analysis though with only a small-to-intermediate effect size (235). These findings however conflict with other studies (210, 220). A positive association was observed between Il-6 and depression in a 2-year follow-up (236). Il-10 is positively associated with autonomic and mood symptoms (237, 238). Data for TNF-α are inconclusive with increased (239-241), decreased (242), or similar levels (243) compared to HC being noted. Associations have however been noted with disease stage, fatigue, depression, and anxiety (244). Chemokine ligand 5 (CCL5, RANTES) levels were elevated in PD compared to controls with a positive correlation with Hoehn and Yahr stage, motor impairment, and disease duration (245, 246). In a large prospective study, elevated proinflammatory and low anti-inflammatory cytokine levels were associated with unfavourable motor deficits and cognitive outcomes (247) though these findings were contradicted by a trial (248).

Current use recommendation: Individual inflammatory markers have low diagnostic and prognostic biomarker value in parkinsonian conditions. Studies that combine several pro- and anti-inflammatory markers showed promising results both in diagnostic performance and as predictors of individual disease progression (247, 249) and can potentially be considered for measuring treatment response in studies exploring agents which have an impact on the immune system.

Genetics and Gene regulation

The relationship between genetic risk factors for PD, and the pathophysiological processes underlying PD are under renewed scrutiny based on the use of α-syn SAA in CSF. People with Leucine-rich repeat kinase 2 (*LRRK2*) mutations may develop typical PD, positive α-syn SAA in CSF and typical PD pathology at post mortem (250), while the phenotype, pathophysiology and α-syn SAA findings and post mortem pathology can also be completely different despite the same *LRRK2* mutation (251). The far lower rates of positivity of the CSF α-syn SAA among *LRRK2* mutation carriers, questions whether to include *LRRK2* mutation carriers within trials targeting alpha synuclein specifically, and potentially other broad interventions being considered for PD neurodegeneration (252). Nevertheless, there is great interest in targeting *LRRK2* as a means of influencing disease progression in PD, and genetic status may be of greater relevance for these interventions than other biomarkers.

Of relevance to this point, molecular dysfunction of pathways downstream from *LRRK2* also occur and these are being explored as biomarkers in trials targeting this enzyme. pS1292-LRRK2 levels are higher in urinary EVs in idiopathic PD and correlate with motor severity (253). Furthermore, CSF EV pS1292-LRRK2 levels are ten-fold higher than urinary EV levels suggesting relevance for CNS activity (254). Genetic variability may therefore be considered for selecting patients for precision medicine interventions as well as for helping balancing trial arms for progression or adjusting for baseline differences in longitudinal analysis. pS1292-LRRK2 levels or other downstream molecular abnormalities (whole-blood pS935 LRRK2 levels, peripheral blood mononuclear cell pT73 Rab10 levels,

urine di-22:6-bis (monoacylglycerol) phosphate, and CSF total LRRK2) may become useful tools for measuring target engagement and therapeutic response to agents specifically targeting these pathways as has been demonstrated in a recent early stage *LRRK2* inhibitor trial (255).

Other genetic factors can also determine phenotypic severity and progression. PD patients with the A53T alpha synuclein mutation experience worse autonomic and cognitive deterioration (256) while apolipoprotein E gene (*APOE4*) and Glucosidase beta acid 1 (*GBA1*) PD patients have accelerated cognitive (257-261) and motor deterioration (262) though this may be constrained to specific mutations/polymorphisms (263-265). Polygenic risk scores for predicting rate of progression appear promising although need replication (266, 267).

The influence of genetic factors in atypical parkinsonian disorders is more limited to tauopathies. The H1 haplotype and its sub-haplotypes (H1c, H1d, H1g and H1o) are a shared in PSP and CBD (268) and increases the risk of developing PSP (269). The haplotype increases 4R- tau expression (270, 271) and potentially influences the degree of methylation at the microtubule-associated protein tau (MAPT) locus (272). Similarly, *APOE* genotype influences the risk of 4R- tauopathies. The *APOE ε2* allele is associated with a higher tau burden and the *APOE ε2/ε2* genotype is a risk factor for PSP (273). Apolipoprotein E interacts with the lipoprotein receptor LRP1 which has been implicated in the cellular uptake and spread of tau, potentially indicating a mechanism for the association (274). Several genome-wide association studies have identified single nucleotide polymorphisms (SNP) in a number of genes, and these appeared to be shared in patients with CBS. At the current stage, these findings are unlikely to be of diagnostic or prognostic value (275). A recent finding in pathologically confirmed PSP cases of a genetic variation at the *LRRK2* locus being associated with survival may be useful but requires replication (276).

Noncoding RNA (ncRNA) are RNA species that are not translated into a protein but contribute to the regulation of gene expression. Small ncRNA (sncRNA) are shorter nucleotides consisting of miRNA (90% of detected sncRNA), piRNA, and siRNA. SncRNA have potential for biomarker use though

challenges in differential blood component composition (277) and issues with detection and quantification methods need to be overcome (278). Several PD studies have explored the potential for sncRNA use as biomarkers. In one study, samples from individuals in a discovery cohort and donors in an independent validation cohort suggested that miR-6836-3p and miR-6777-3p were upregulated in PD, while a trend toward downregulation of miR-493-5p, miR-487b-3p, and miR-15b-5p was noted compared to controls while levels were able to predict PD progression (279). Comparisons with previous studies are limited to one study suggesting a similar regulation direction effect of miR-15b-5p (280) though the known involvement of these miRNAs in biological processes associated with PD pathogenesis such as mitochondrial function, oxidative stress, and protein degradation) lends strength to the findings. The authors' demonstration of age-dependence of sncRNA expression will need to be factored into future study interpretation while the immune system cell origin of the miRNAs could be useful in guiding use as biomarkers for specific drug effect mechanisms. Other miRNAs have also been noted to be deregulated in other cohorts (277, 278, 281) though small cohort sizes make these findings less certain, and replication will be critical.

Unique CSF miRNA profiles have been noted in MSA patients compared to controls (increased (miR-184, miR-218-5p, and miR-7-5p) and decreased (miR-19a, miR-19b, miR-24, and miR-34c) as well as PD patients (higher miR-184 and miR-7-5p and lower miR-106b-5p and miR-let-7b-5p in MSA). These findings however lack sufficient accuracy for distinguishing MSA from PD (282, 283). A variety of differentially expressed miRNAs have also been noted in MSA patients compared to HCs and PD patients in blood (284-289). A panel consisting of five miRNAs (miR-31, miR-141, miR-181c, miR-193a-3p and miR-214) in serum also accurately differentiated MSA from PD in one study (AUC 0.95) (286) while expression levels of plasma miR-671-5p, miR-19b-3p and miR-24-3p were different between MSA subtypes (284).

MiRNAs can regulate the abundance of tau isoforms. MiR-132, is down-regulated in PSP brains and its target protein PTBP2 is increased (290). Increased miR-147a and -518e expression was also noted

in the forebrain of PSP patients in a separate study (291). In addition, this study confirmed that target genes for miR-147a and miR-518e are repressed in PSP though other findings in this study contradict the previous study findings. A separate study examining CSF miRNAs in PSP patients compared to HC noted up- regulation of miR-204-3p, -873-3p and -6840-5p which target genes associated with molecules related to the ubiquitin–proteasome system and autophagy (292). A single CSF-miRNA (miR-106b-5p) was found to be sufficient in one study for discriminating PD and PSP (miR-106b-3p) unlike PD versus MSA where combinations yielded better results likely due to different underlying pathologies (α-Syn vs tau) (283).

While the roles of sncRNAs in disease regulation is clear, the weak correlation with disease state and progression makes their use as clinically useful biomarkers for parkinsonian conditions less clear. Current studies are small, lack uniform sampling, quantification, and analysis approaches and this will need to be improved while the use of samples with less variability maybe worthwhile (293).

Current use recommendation: Genetic status could be useful for patient selection for trials examining their downstream pathways (*LRRK2*). Genetic status which predicts progression (*GBA/ APOe4*) could also be useful for balancing trial arms.

Lysosomal dysfunction

The autophagic-lysosomal pathway (ALP) is critical for a-syn clearance, with its impairment contributing to the accumulation of oligomeric and fibrillary a-syn (294). Heterozygous mutations in the Glucocerebrosidase (*GBA*) gene which encodes the lysosomal enzyme β-glucocerebrosidase (GCase) predisposes to PD, DLB and possibly MSA while also influencing their natural course (295-297). PD *GBA*-carrier patients develop more severe phenotypes with reduced survival rates (298). Impaired GCase activity leads to lysosomal dysfunction therefore negatively impacting on misfolded protein-related responses (299, 300). Other lysosomal enzymes such as cathepsin D (CTSD) are also

directly involved in the degradation of aggregated a-syn (300). GCase and CTSD activity is significantly decreased in different regions of both *GBA*-carrier and non-carrier PD and DLB brains (301-303) and GCase activity is reduced in early PD patients (304). Lysosomal enzymes measured in CSF reflect derangement in brain activity (305).

Lower GCase activity and CTSD concentrations independent of *GBA* carrier status has been noted in PD patients (84, 306) though diagnostic accuracy is poor. Combining GCase activity with o-a-syn/t-a-syn ratios improves this (AUC = 0.87, 82% sensitivity, 71% specificity) (84). Combining GCase activity and other enzymes (CTSD and β-hexoxaminidase) also provides better diagnostic accuracy compared to each biomarker alone (AUC = 0.77, 71% sensitivity, 85% specificity vs), while combining a-syn and Aβ-42 with lysosomal enzymes further improves this (AUC = 0.83, 75% specificity, 84% sensitivity) (306). Decreased CSF GCase activity has been noted in DLB patients but not in those with AD and FTD (307), further suggesting selective involvement in synucleinopathies. The ability of CSF GCase activity in discriminating synucleinopathies from one another and from other degenerative parkinsonisms remains to be explored.

CSF GCase levels correlate with cognitive impairment (308) while activity also seems to predict subsequent development of dementia regardless of genetic status in PD (309) . CSF GCase levels may therefore usefully allow enrichment of clinical trial arms testing agents targeting this enzyme (even in the absence of a *GBA1* mutation) as well as a method for confirming target engagement. Blood GCase activity is also reduced compared to HC though prediction of progression has not been explored (310, 311). GCase activity is being used as an exploratory outcome in recent disease modification trials in conjunction with its downstream hydrolytic product glucosylceramide. Glucosylceramide can distinguish *GBA*-PD from idiopathic PD and HC and be measured in both plasma and peripheral blood mononuclear cells and therefore used as a biomarker for target engagement in clinical trials targeting *GBA*-PD (312, 313).

Other CSF lysosomal biomarkers have been minimally studied. Lysosomal-associated membrane proteins 1 and 2 were decreased in PD; increased in PSP; and lysosomal-associated membrane proteins 1 and 2, microtubule-associated protein 1 light chain 3 and lysozyme were increased in CBD. A panel of these proteins discriminated between controls, PD and 4-repeat tauopathies (314).

Current use recommendation: CSF GCase/ blood Glucosylceramide levels could allow enrichment of clinical trial arms testing agents targeting this enzyme (even in the absence of a *GBA1* mutation) as well as provide a method for confirming target engagement in PD.

Mitochondrial dysfunction

Mitochondrial dysfunction and oxidative phosphorylation failure contributes to the pathogenesis of parkinsonian syndromes (315). The existence of inherited autosomal recessive parkinsonism due to the mutations of Parkin (*PRKN*), PTEN induced kinase 1 (*Pink-1*) and the *DJ-1* gene which encode proteins that mediate mitophagy supports this link (316, 317). The *DJ-1* gene encodes a multifunctional protein which is expressed in the brain and with particular abundance in astrocytes. Despite mixed findings (318, 319), a recent study noted decreased CSF DJ-1 levels in sporadic PD when compared to AD and healthy controls (36). CSF DJ-1 has been minimally explored for distinguishing parkinsonian syndromes. In one study, no significant differences were found between PD and APS (PSP, MSA and CBS), though a trend towards higher levels was observed in MSA (320). This has been confirmed in a separate study with MSA patients having higher levels than PD patients and control subjects. A good accuracy for discriminating MSA from PD (AUC = 0.84, 78% sensitivity and specificity) was noted and this improved by combining this with tau proteins (AUC = 0.92, 82% sensitivity, 81% specificity) (321).

Other less well studied mitochondrial biomarkers include phosphorylated ubiquitin at the serine 65 residue (pSer65Ub) which occurs by virtue of loss of the mitochondrial membrane potential triggering the stabilization of Pink1 at the outer mitochondrial membrane (322). While increased pSer65Ub levels have been observed in PD *post mortem* brains, lower levels have been identified in

familial PD with *Pink1/Parkin* mutations (323, 324). Explorations of this marker in biofluid samples will be of interest possibly as confirmation of target engagement and longitudinally to assess progression rates of disease in these PD subtypes. Similarly, the peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC-1α) has been of interest due to its role as a regulator of mitochondrial function (325). The PGC-1α reference gene and PGC-1α levels are downregulated in human brain and blood leukocytes in PD compared to control patients and this negatively correlates with disease severity (326-328). Interventions targeting mitochondrial processes might usefully measure peripheral levels of PGC-1α.

CoQ10 is a cellular antioxidant involved in transport electrons from complexes I and II to complex III in the mitochondrial electron transport chain. Hydroxbenzoate polyprenyltransferase (CoQ2) is an enzyme involved in CoQ10 biosynthesis and is encoded by the *COQ2* gene (329). Several mutations in *COQ2* are associated with increased susceptibility to MSA while CoQ10 levels and activity are decreased in MSA patients both carrying and not carrying mutations (330, 331). CSF levels are also lower compared to PD and PSP patients but do not differ between MSA-P and MSA-C cases (332). Blood levels are lower in MSA patients compared to HCs and levels are weakly correlated with clinical severity (333, 334). Reliability of this marker will need to be explored in larger validation studies with consideration of confounders such as diet and medication intake. Homocysteine is an amino acid involved in the modulation of oxidative stress and mitochondrial dysfunction and may serve as an indicator of neurodegeneration by virtue of being correlated with brain atrophy (335, 336). Serum levels are increased in MSA compared to HC and PD though this may be confined to males (337, 338). Levels are also associated with worse motor and non-motor symptoms in one Chinese study (339). Elevated homocysteine levels have also been noted in PSP patients compared to HC, though this has not been explored further (340).

A concern for the use of mitochondrial blood-based biomarkers is that they do not recapitulate neuronal mitochondrial dysfunction. Genetic mutations leading to mitochondrial dysfunction in PD

often show tissue-specific expression patterns and therefore peripheral blood changes may lack interpretability (341, 342). This is supported by a recent study showing negligible diagnostic value for well-established biomarkers of mitochondrial disease such as Fibroblast growth factor 21 and Growth differentiation factor 15 in reflecting mitochondrial dysfunction in PD patients (323).

Current use recommendation: Limited evidence mitochondrial biomarker use potentially confined to exploring if they can track treatment effects and target engagement in trials testing agents which specifically target mitochondria.

Insulin resistance

Type 2 diabetes mellitus (T2DM) is a risk factor for developing PD (343, 344) and its coexistence with PD results in more severe motor features and the development of cognitive impairment (343, 345-347). This link is in part explained by disruptions in physiological insulin signalling (insulin resistance) (348) which contributes to neurodegenerative processes (349). Insulin resistance occurs centrally in the nervous system and in peripheral organs including muscles and the pancreas with several potential biomarkers reflecting this.

Central insulin resistance is reflected by the measurement of abnormalities in insulin signalling. The effects of insulin are mediated by insulin-receptor substrate-1 (IRS-1). Tyrosine IRS-1 phosphorylation evokes insulin responses while serine phosphorylation primarily deactivates IRS-1 and attenuates insulin signalling (229) through activated downstream Akt and mTOR signalling (348, 350). Elevated IRS-1 phosphorylation at serine positions 616 (IRS-1 p-S616) and 312 (IRS-1 p-S312) were associated with attenuated insulin signalling in *post mortem* studies thus supporting their use as biomarkers of neuronal insulin resistance (351, 352). This pattern has subsequently been confirmed in plasma samples measuring EV levels of these proteins in PD patients (353, 354). A further study however demonstrated decreased Tyrosine-phosphorylated insulin receptor substrate-

1 (IRS-1 p-Tyr) was more predictive of a diagnosis of PD compared to healthy controls while also predicting cognitive impairment and motor symptom severity (194). This finding is further complemented by a sub analysis of the Exenatide PD2 study showing IRS-1 p-Tyr was associated with motor benefits noted in the trial while increases in the downstream marker p-Akt S473 could potentially serve as potential treatment response marker (353).

Several studies have provided evidence for the presence of peripheral insulin resistance in PD. Elevated plasma glucose concentrations predicted a lower risk for PD in one study (355) while insulin resistance as defined by a Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) value ≥ 2.0 or a glycated hemoglobin (HbA1c) concentration ≥ 5.7% was present in almost 60% of PD patients in a separate study though body mass index was the main driver of this observation (356). A small study using the gold standard to measure insulin sensitivity, the hyperinsulinemic-euglycemic clamp found slightly higher hepatic insulin resistance in eight untreated de-novo PD patients compared to matched controls. A separate study exploring plasma fasting glucose, fasting plasma insulin (FPI), glycated haemoglobin and fasting plasma amylin which is known to interact with protein aggregation in neurodegenerative disease (357, 358) noted no difference between PD and HCs except for lower FPI levels in PD patients (359). The HOMA-IR however moderately correlated with non-motor symptoms, though no association with cognition or motor symptoms was noted (360). Overall, this study suggests some dysregulation without clear evidence of access presence of peripheral insulin resistance in PD patients. This study however contradicts several other studies that suggest abnormal range HbA1C levels which can also reflect insulin resistance could predict motor and cognitive severity and progression in PD (361-364). Elevated HbA1c levels within a non-diabetic range (5.4–5.8%) are associated with impaired insulin secretion, even without escalating insulin resistance though levels ≥5.9% have been associated with substantial reductions in insulin secretion, insulin sensitivity and β-cell dysfunction (365). The discrepant predictive findings between glycated haemoglobin levels and other peripheral insulin markers could potentially be explained by the differential correlation between the spectrum of case glucose intolerance as well as BMI (366,

367). Whether peripheral and central insulin markers correlate well will also need to be explored further prior to use in clinical trials.

Central insulin resistance also occurs in MSA, CBS and PSP though their link with T2DM is unclear (226-228). Higher IRS-1pS312 and IRS-1pS616 have been noted in a postmortem MSA study while IRS-1pS312 levels correlated positively with disease duration thus pointing towards an association between the degree of insulin resistance in the brain and disease severity (352). Investigating this further in EVs would be useful while also exploring for potential differences between PD, MSA and other atypical parkinsonian conditions. Insulin-like growth factor-1 (IGF-1) is a hormone which modulates brain development, oligodendrogenesis, and myelination as well as playing a role in synaptic neurotransmission and neuroinflammation (368). Abnormally high levels in blood or CSF suggests problems with insulin signalling and possibly the presence of insulin resistance. Increased serum levels have been noted in MSA compared to HCs (369) as well as PD (370). IN PD and PSP, serum IGF-1 levels negatively correlated with UPDRS part III a measure of motor symptom severity while IGF-1 levels positively correlate with MSA disease duration and motor severity (370). More prevalent impaired fasting glycemia was also noted in the synucleinopathies compared to PSP. Future studies systematically clarifying the value of peripheral insulin resistance markers for distinguishing parkinsonian conditions and for use as therapeutic monitoring biomarkers in these conditions for agents targeting insulin resistance would be worthwhile.

Current use recommendation: Using IR biomarkers to track treatment effects and target engagement in trials testing agents which specifically target insulin resistance in PD. Potentially balancing trial arms based on diabetes status in PD considering ability to reflect disease severity and predict progression.

Microbiome

The gut microbiome (GM) interacts with the host's health and its disruption is associated with PD (371-373) via a microbiota–gut–brain axis (374). Changes in specific microbial species abundance, intermediary metabolites involved in metabolic homeostasis such as short-chain fatty acids (SCFA) (375) and inflammatory cytokines (376) as well as host derived epigenetics which influence microbial DNA expression (377) have all been noted in PD. It is unlikely that these markers would ever be used to diagnose PD considering the more specific and easily accessible markers currently being explored though they could potentially be utilized to predict progression (378). Some preliminary evidence to support this is the relative abundance of species such as *Enterobacteriaceae* in the more severe PIGD phenotype (379) or reduced abundance of *Prevotella taxa* influencing more rapid progression (378). Similarly, underrepresentation of species producing SCFAs seems to contribute to faster progression (380, 381). Disentangling if this is mainly influenced by microbes or metabolites will be important for therapeutics and biomarker selection. The strength for predicting progression however appears moderate and progression prediction therefore likely best achieved in combination with other biomarkers.

Increased abundance of pro-inflammatory bacteria such as *Bacteroides* and lower anti-inflammatory bacteria such as *Ruminococcaceae* and *Coprobacillaceae* has been reported in MSA compared to HC (382-384). Other studies have suggested that a combination of six genera was predictive of MSA, while others have shown that a combined model of five genera was able to differentiate between MSA subtypes (AUC 0.89) (385). Similarly, constructing a set of 25 gut microbial gene markers to discriminate MSA from PD patients with an AUC of 0.83 (386). Sample sizes are small in these studies and a separate study comparing PD, with HC, MSA and PSP patients found minimal differences in atypical disorders compared to PD with the exception of reduced *Prevotellaceae* in MSA and reduced *Streptococcaceae* in PSP patients (387).

Current use recommendation: A range of therapeutic interventions related to GM are currently being considered including probiotics, prebiotics, antibiotics, and faecal microbiota transplantations (388). Gut microbiome biomarkers will therefore be increasingly employed to predict treatment response and better target treatment. The use of dietary and enema interventions resulting in improved motor symptoms as well as changes in the abundance of the bacterial taxa, *Ruminococcaceae* is one such example (389).

Synaptic degeneration

Disruptions to vesicle-mediated trafficking and secretory pathways with downstream effects on neurotransmitter levels and signalling as well as synaptic plasticity are key features of synucleinopathies in particular DLB (390). Proteins at different levels of this process have been explored for biomarker use. The principal components of the soluble N-ethylmaleimide sensitive factor attachment protein (SNARE) complex (synaptosomal-associated protein 25 (SNAP-25), the syntaxins 1A and 1B, syntaxin-binding protein-1, and the vesicle-associated membrane proteins (VAMP-1, VAMP-2)) which mediate vesicle fission have been measured in CSF and serum EVs though the confounding effects of levodopa on their levels alongside lack of correlation with disease severity, limit their potential usefulness (391, 392). These markers may however be used to improve the diagnostic accuracy of PD markers with one study demonstrating improvements when oligomeric α-synuclein measurement was combined with VAMP-2 in serum EVs (391).

β-Synuclein and GAP-43 which potentiate dopamine release at synaptic vesicles (390, 393) have been quantified in the serum and CSF of PD patients though lack of specificity in discriminating from controls (394, 395), inconsistent findings between studies (396-398) and weak correlations with disease severity (397) make future use unlikely. Lower CSF concentrations of the secretory granule proteins (VGF and secretogranin-2 (SCG2)) and the dense core vesicle protein PDYN has been noted in preliminary PD studies though the usefulness of these proteins appears to be in distinguishing PD from DLB as well as predicting cognitive decline (399, 400).

Synaptic plasticity is in part mediated by the postsynaptic protein, neurogranin (Ng) (401). Reduced phosphorylation of Ng and an interaction between Ng and α-synuclein occurs in PD (402). CSF Ng level differences in PD compared to HC has been inconsistent possibly in part due to dopaminergic therapies. CSF Ng levels do not seem to be different in atypical parkinsonian disorders compared to PD either thus limiting their diagnostic value (403, 404). Although correlation with motor disease stages has been consistently reported and in one study CSF Ng levels correlated with CSF α-synuclein levels, and cortical glucose metabolism (392, 405), consistent association with cognitive progression is lacking (397, 406-409).

Altered CSF levels of the synaptic plasticity markers Contactin-1 (CNTN-1) and the zinc transporter ZnT3 have been noted in PD though correlation with disease severity is less clear (410, 411) and needs clarification in larger studies. PD is characterised by the presence of excessive neuronal excitation and/or a loss of inhibition via elevated glutamate receptor and loss of GABA-ergic interneurons (412). As such, CSF levels of the excitatory-inhibitory regulatory protein Neuronal pentraxin-2 (NPTX2) (400) and the glutamate receptor GluA3 (411) have been explored for biomarker use and appear to correlate with cognitive status and presynaptic integrity (413) though preliminary findings suggest greatest potential value for distinguishing PD from DLB. Combining markers (CSF Ng, NPTX2, total α-synuclein, and age (414) or CNTN-1, total α-synuclein, total tau, phosphorylated tau, and Aβ1-42 (410) seems best at achieving this distinction. This highlights a potential role for measuring panels of CSF proteins levels reflecting neurotransmitter secretion, synaptic plasticity, and autophagy (415).

Neurotransmitter metabolites may also serve as a proxy for neurodegeneration. Decreased levels of the dopamine metabolite homovanillic acid (HVA) has been consistently noted in PD (416-421). Repeated CSF measurements in the DATATOP (deprenyl and tocopherol antioxidative therapy of parkinsonism) study did not however suggest usefulness for monitoring disease progression though this may relate to technical limitations. Advances in liquid chromatography coupled with tandem mass spectrometry techniques allow for simultaneous analyses of dopaminergic (e.g., 3,4-

dihydroxyphenylalanine [DOPA], dopamine, 3,4-dihydroxyphenylacetic acid [DOPAC]), noradrenergic (e.g., 3,4-dihydroxyphenylglycol, 4-hydroxy-3-methoxyphenylglycol) and serotonergic (e.g., 5-hydroxy-3-indoleacetic acid [5-HIAA]) metabolites in CSF (420). This metabolite panel has been consistently reduced in PD and correlates with motor severity and DaT-SPECT uptake (422, 423). Whether this panel predicts or tracks non-motor (cognitive or autonomic) progression needs to be explored.

Current use recommendation: Limited evidence for trial use. Likely confined to using panels combining synaptic degeneration markers with other aspects such as autophagy to measure treatment response but requires further validation.

Axonal damage

Neuro-axonal damage represents a downstream event from the neurodegenerative process. The axonal cytoskeleton comprises neurofilaments, structural proteins which allow for the radial growth of axons. Large, myelinated axons of the white matter have the highest content of these proteins (424). Tau in contrast is predominantly expressed in thin unmyelinated axons of the cortex. Neurofilament subunits (light and heavy) and total-tau protein (t-tau) are released in the interstitial space of the CNS upon axonal injury, irrespective of the cause though sensitive for white matter axonal involvement (424).

CSF neurofilament light chain (NfL) concentration does not seem to be increased in PD (425) though sharp increases have been noted in PSP, MSA and CBS compared to HC and PD (165, 425-427). Levels in DLB and PDD are similar to those of age-matched healthy subjects (428) and lower than in PSP and MSA (165). Similar higher CSF levels of the heavy chain subunit of the protein has also been noted in PSP and MSA compared to PD (429). T-tau seems to be lower in PD however compared to healthy subjects (430) but increased in APS (431). CSF NfL alone can discriminate PD from atypical disorders with high accuracy (AUC = 0.93, sensitivity 85%, specificity 92%). CSF NfL has shown a high diagnostic accuracy in discriminating between PD and PSP (AUC = 0.82, 75% sensitivity, 83%

specificity), and between PD and MSA (AUC = 0.94-0.99) (432-434). NfL levels do not however discriminate between MSA, PSP, and CBS, and therefore its utility will mainly be in suggesting the possibility of an APS.

NfL concentrations in blood and CSF strongly correlate (435-438) thus making blood NfL a promising biomarker for neurodegeneration though correction for age is important (437, 439-441). CSF NfL levels also correlate with pathological changes in presynaptic putaminal dopamine transporter (DAT) density in different Parkinsonian syndromes reflecting nigrostriatal degeneration (442) while higher baseline serum NfL is associated with a greater reduction of putaminal DAT binding ratio over time (443). Blood NfL seems to be higher in more advanced PD patients compared to controls, though findings are less clear in early disease stages with contrary findings of raised and normal levels being noted (435, 437, 440, 444). This may however relate to disease stage differences and choice of healthy controls used. A meta-analysis showed no differences in blood NfL in PD patients not stratified by disease severity compared to controls (436). Blood NfL concentrations at baseline and at follow-up (mean of 6.4 years) were however found to be higher in de novo PD patients compared to controls in a recent study (445). The association of blood NfL concentrations with degrees of motor impairment in PD also varies between studies and may relate to the disease stage at which it is measured (55, 440, 444, 446). Blood NfL is however associated with the more severe PIGD-subtype of PD (447, 448) while higher baseline NfL levels seem to predict more severe motor impairment over the course of disease across disease stages (443). NfL levels are higher in PIGD compared to the tremor-dominant PD subtype after 2 years of follow-up, and within the PIGD group higher blood NfL is associated with worse global cognition and UPDRS III at baseline while also predicting motor and cognitive decline (448). Furthermore, NfL baseline levels are associated with greater worsening of PIGD scores over 8-year follow-up (449). Consistent inverse associations of blood NfL levels with cognitive scores have been reported across PD diagnostic, treatment and severity stages (55, 439-441, 445, 446, 450, 451) though this was contradicted in a smaller number of studies (443, 444). Cut-off values for a higher risk of cognitive decline have also been proposed

(14 to 19 pg/ml)(440, 450) though cohort-related age-adjusted NfL levels are likely be more reliable (452). Blood NfL also predicts progression to milestones (walking-aid, nursing-home living, reaching final Hoehn and Yahr stage 5 or death) thus making it a useful progression marker of overall function for trial use (444, 453).

Blood NfL is also higher in MSA, PSP and CBS compared to HCs (434, 454) and to PD (186, 435, 437, 440). Accuracy levels up to 91% (sensitivity = 86% and specificity = 85%) for distinguishing APS from PD at a cut-off value of 14.8 g/L has also been suggested (437). This is also the case when parkinsonian symptoms are inconclusive with higher NfL levels predicting an eventual diagnosis of an APS (435, 437). Serum NfL was also able to distinguish MSA-C from cerebellar ataxia presentations in a small study (455). Blood NfL levels also correlate with disease severity and predict progression in MSA (456). Similarly, blood and CSF Nfl also predicts worse disease severity and progression in PSP (457, 458) while also being able to track progression over a 1-year period (208, 432, 459).

A less well studied protein involved in CNS myelin formation is Myelin basic protein (MBP) which is bound to the cytosolic surface of the oligodendrocyte membrane (460). MBP levels are increased in MSA compared to HCs and PD (213, 461) and is of reasonable diagnostic value in early PD (213). Larger cohort validation of this will be critical as will exploration of potential for tracking disease progression.

Current use recommendation: Unlikely to be of diagnostic value despite clear difference between PD and atypical disorders. Useful prognostic/progression marker for balancing arms though clear cutoffs for this currently lacking. May potentially play a role for monitoring treatment response.

1.23 Imaging biomarkers for parkinsonian disorders

A range of imaging modalities have been explored for their biomarker potential. These include approaches that measure brain structure, sonographic measurement of nigral signal, spectroscopy to explore brain biochemical changes, functional imaging to measure connectivity changes and radionuclide imaging to assess pre and post synaptic dopaminergic and non-dopaminergic integrity as well as metabolic functional changes. (Panel 2) Each approach has its strengths and weaknesses and potential biomarker roles in trials will depend on the stage of disease being studied as well as practical considerations of availability and effect strengths alongside and in comparison, with, fluid biomarkers.

Panel 2

Biomarker Imaging Techniques

- Structural MRI

  -quantification of brain structural change using regions-of-interest or whole-brain approaches

  -commonly used sequences include T1, T2, T2*, R2* (R2* = 1/T2*)-weighted, susceptibility-weighted, proton-density-weighted, fluid-attenuated inversion recovery, and neuromelanin-sensitive approaches

- Proton Magnetic resonance spectroscopy

  -estimates relative concentrations of proton-containing metabolites in brain

  -metabolites commonly assessed include N-acetylaspartate, choline-containing compounds, myo-inositol, and creatine

- Functional MRI

-evaluates neuronal activity by measuring transient variations in blood flow and variation correlation in functionally connected regions.

-utilized under task-based or under resting-state conditions

- Radiotracer imaging

  -Measures pre and post synaptic receptor and transporter density as well as glucose metabolism and microglial activation using different radiotracers

  -provides information on nigrostriatal dopaminergic, serotonergic and cholinergic system integrity, regional tissue glucose metabolism and activity and status of microglial-mediated inflammation

- Transcranial Sonography

  -ultrasound echogenicity measurement of brain tissues or structures through intact cranium -limited by lack of bone window in some subjects, and inter technician variability

Structural MRI techniques

Structural MRI approaches comprise; T1-weighted structural imaging methods which measure cortical and subcortical volumetric changes and brain atrophy; neuromelanin-sensitive T1-weighted imaging which is sensitive to measuring neuromelanin-iron complexes; iron-sensitive MRI which captures iron deposition and dopaminergic cell loss; and diffusion imaging using either single-tensor or two-compartment diffusion modelling (free-water) which reflects neurodegeneration and/or neuroinflammation.

T1-based structural MRI

T1-based structural MRI methods comprise; cortical thickness measurement, voxel-based morphometry (VBM) and Deformation-based morphometry (DBM). Structural differences are not valuable in diagnosing PD. Although unique atrophy patterns varying at different disease stages for example in prefrontal and cingulate cortices and the caudate and thalamus have been noted in PD patients without dementia when compared to HC, lack of a clear linear change across disease stages makes current trial biomarker use for tracking progression not viable (462-468). Similarly, noting greater and more widespread atrophy in supplementary motor area, temporal, parietal, and occipital cortices in patients who already demonstrate cognitive impairment clinically is unlikely to be of specific value in trials (469). Ascertaining the precise role of ultra-high-field scanners (7 T and above) which can provide sub millimetric anatomical information and higher degrees of diagnostic detail compared with 3 T MRI (470) will be important. Planned future longitudinal studies will be critical for informing this (471).

Several MRI features have been reported in atypical disorders. These include bilateral hyperintense rims lining the dorsolateral borders of the putamen as well as putaminal hypointensity in addition to atrophy of the putamen, cerebellum, middle cerebellar peduncles (MCP), or pons in MSA-P cases and cruciform pontine and MCP hyperintensities on T2-weighted or fluid-attenuated inversion recovery (FLAIR) images in MSA-C cases (472). Atrophy of the midbrain and superior cerebellar peduncle (SCP) is typically noted in PSP compared to PD, MSA-P, CBS and controls (473-476). Other radiological PSP signs include midbrain atrophy compared to pons, midbrain tegmentum atrophy (477-479), and midbrain T2 hyperintensity in conjunction with midbrain tegmentum atrophy with relative preservation of the midbrain tectum and cerebral peduncles (472, 477). Asymmetrical atrophy in the frontoparietal lobe corresponding to the more prominent contralateral clinical deficit is typically observed in CBS as is corresponding grey matter degenerative change (473, 480). More prominent dorso-frontal and parietal atrophy and less prominent midbrain atrophy has also been reported compared to PSP (473, 481). These signs can distinguish atypical disorders from PD however they have low sensitivity values and are highly dependent on image acquisition factors

(472, 482, 483). Also, specificity tends to be reached in later disease stages, at a time when disease modification may be hardest to achieve.

Employing VBM can potentially improve on this. Putaminal volumes are reduced in MSA compared to PD with reasonable ability for distinction (specificity 92%, sensitivity 44%) (472, 484, 485) as is striatonigral and olivopontocerebellar atrophy (486-488). Specific cortical atrophy changes can distinguish MSA-P from PD (488, 489) and cortical and para-hippocampal thinning occurs in patients who experience cognitive abnormality (490, 491). Relatively greater grey matter atrophy in the right Crus II cerebellar region has however been observed in MSA-C cases compared to MSA-P (492). Although putaminal and infratentorial atrophy occurs in both subtypes to a similar degree supratentorial atrophy is more predominant in MSA-P while infratentorial atrophy is more prominent in MSA-C (493, 494).

Volume reductions have been noted in the brainstem, midbrain, and frontal grey matter in PSP compared to HC though lowest volume overlap on a case basis is noted for the midbrain (481). VBM studies also demonstrate degeneration in the pons, thalamus, and striatum, as well as widespread cortical atrophy in the frontal, prefrontal, insular, premotor and supplementary motor areas compared to controls (495, 496). White matter degeneration has also been noted in the pulvinar, thalamus, superior and inferior colliculi, and frontotemporal regions (495, 496). Pontine atrophy rates are slower in PSP compared to MSA-P while rates of frontal and midbrain atrophy is associated with executive and motor impairment respectively (497) making the midbrain atrophy rate a potential trial outcome measure (498). Vector machine classification comparing disease-specific regions-of-interest to the whole brain can also assist in predicting a PSP diagnosis (499). Pontine to midbrain area and MCP to SCP widths are larger in PSP compared to PD, MSA-P, and controls (128, 136). An index with these ratios- magnetic resonance parkinsonism index also distinguishes PSP from these groups well (479), while a revised version incorporating ventricular width showed superior sensitivity (100%) and specificity (94.3%) for differentiating PSP-parkinsonism from PD (500).

Midbrain atrophy cannot be solely relied upon however as it is not universally evident in all PSP subtypes thus making comparisons with atrophy of other regions important (501).

In CBS, VBM studies suggests more prominent atrophy in the superior parietal lobe compared to PSP and MSA-P (502). As there is significant pathological heterogeneity in CBS cases, neuroimaging patterns can be useful for predicting this (503). Frontotemporal atrophy is associated with frontotemporal lobar degeneration with transactive response DNA binding protein-43 kDa (TDP-43) pathology, whereas temporoparietal atrophy was related to AD pathology (504). Predominant atrophy of the premotor cortex and supplementary motor area is more suggestive of CBD and PSP pathology (504) while more severe global atrophy indicates CBD over PSP (505). The predominant clinical syndrome (cognitive versus extrapyramidal) also corresponds with regional atrophy patterns (475).

Current use recommendation: Indexes incorporating multiple features maybe useful for diagnosing atypical disorders and distinguishing between them. Atrophy rates should be explored further to determine if shorter term changes could replace longer clinical follow-up used in trials.

Neuromelanin and iron sensitive imaging

Neuromelanin imaging (NMI) demonstrates only moderate sensitivity and specificity for distinguishing PD from healthy controls (506-510) while signal differences are also suboptimal for distinguishing atypical parkinsonian conditions from PD (511, 512). In contrast however, NMI shows reduced signal across disease stages (disease duration of 1.5 to 10 years) with a ventrolateral to anteromedial substantia nigra (SN) progression pattern consistent with the neuropathological patterns of cell loss.

Iron-sensitive techniques including R2* relaxation imaging, susceptibility- weighted imaging (SWI), and quantitative susceptibility mapping (QSM) have similar ability to quantify nigral iron deposition

as NMI (513-515). The absence of dorsal nigral hyperintensity corresponding to the region of nigrosome-1 (DNH) on iron-sensitive sequences distinguishes PD from controls well (470, 516-519) regardless of disease duration (520). The value of iron sensitive imaging for distinguishing parkinsonian disorders is limited. Putaminal hypointensity on SWI can be quantified to distinguish MSA (513) though overlap of this finding in PSP limits its use (506). SWI differences in PSP in several other brain regions have also been reported though this requires replication (506).

Although striatal, nigral, globus pallidus and caudate R2* relaxation rate increased in two separate studies after 2 years in early-stage PD (514, 521), separate studies exploring R2* or QSM in de-novo patients (515) and patients with a disease duration < 1 year showed no longitudinal changes (520). The use of R2* as a progression marker becomes clearer however in later disease stages (520) with increased relaxation time in SN R2* mapping over 3 years correlating with motor severity in cases with an initial disease duration of 5 years (522) while faster progression in the SN pars compacta seems to occur after a disease duration > 5 years (520).

Current use recommendation: NMI and iron-sensitive imaging could potentially be usefully developed as progression biomarkers though values will need to be considered in the context of disease duration. The use of iron-sensitive modalities will be particularly advantageous in trials targeting iron.

Diffusion Imaging

MR diffusion techniques enable better distinction of atypical parkinsonian conditions in view of more widespread tissue integrity changes noted (516, 522). Diffusion tensor imaging (DTI) uses diffusion to estimate axonal organisation in the brain. DTI provides quantitative analysis of water molecules along the direction of axonal fascicles (523). Parameters derived from DTI data include mean diffusivity (MD) and fractional anisotropy (FA). MD reflects the average magnitude of molecular displacement by diffusion while FA reflect the directionality of molecular displacement. Increased MD therefore reflects overall structural disintegration while reduced FA is thought to

reflect loss of axonal integrity. Increased MD and reduced FA in the callosum, caudate nucleus and amygdala as well as several cortical regions with discrepantly lower abnormalities in the temporal lobe has been noted in DLB compared to controls (524-526). DLB cases have decreased pontine and left thalamic FA compared to AD (527) though more prominent pathological overlap between DLB and AD makes diffusion imaging abnormalities less reliable in advanced disease stages (526).

Higher putaminal MD has been noted in MSA-P compared to PD, MSA-C, and controls (528). Reduced FA and elevated apparent diffusion coefficient (ADC) values in the putamen, cerebellum and pons has also been observed in MSA-P compared to PD and HC (529). MSA cases also demonstrate increased MD in the MCP and pons compared to PSP (530) while differently elevated ADC values in the putamen, pons and cerebellum can help distinguish MSA-P and MSA-C (531).

Increased MD and decreased FA in the superior cerebellar peduncle (SCP), orbitofrontal white matter, thalamus, and motor areas, amongst other regions has been reported in PSP (496, 530, 532, 533) as has elevated ADC values in the putamen compared to HC (534). Increased MD in the SCP can also discriminate PSP from PD and MSA (530), and increased ADC values can distinguish PSP from PD (535). A FA score of regions involved in PSP can also differentiate PSP from PD/DLB (536). Lower FA values in the SCP, and SCP FA value hemispheric asymmetry can distinguish PSP-RS from PSP-parkinsonism (537, 538). Free-water values are also more elevated in PSP in the posterior SN compared to MSA, PD and HC (539).

Decreased FA in frontoparietal tracts, intraparietal associative fibres, and sensorimotor cortical projections has been noted in CBS (480) as has reduced FA and increased MD in frontal and motor cortical WM contralateral to the more symptomatic side (540). Compared to PSP, a more asymmetric, supratentorial posterior WM pattern occurs in CBS (540). Elevated MD and reduced FA in the posterior corpus callosum has also been noted compared to PD (541).

Diffusion imaging with a single tensor imaging of the substantia nigra (SN) is less useful in early stage PD (542-544) (545) and evidence in later disease (disease duration 10 years) is limited to one study

demonstrating more anterior and rostral SN involvement (544). The finding of diffusion abnormality of the nucleus basalis of Meynert predicting development of cognitive impairment could be explored for balancing arms in small trials or selecting phenotypes that are likely to respond to specific treatments though replication of this finding is important (546). Free water imaging studies have been more consistent with increased signal in the posterior SN being noted in early PD (547, 548). Free water in the posterior SN also increases over 4 years and change over 1 year can predict Hoehn and Yahr 4-year change (548). This increase continues in later disease stages (duration over 7 years) where longitudinal increases in free water occurs in the anterior but not posterior SN (549). This modality is promising as a progression biomarker though may require selecting the region of interest depending on disease stage. Free-water imaging of the basal ganglia, midbrain, and cerebellum and the application of automated Imaging Differentiation is promising for differentiating PD from atypical conditions (550). This approach was found to be superior to a conventional Magnetic Resonance Parkinsonism Index as well as plasma NfL levels for distinguishing PD from atypical conditions (551).

While diffusion imaging maybe of diagnostic use for distinguishing parkinsonian disorders, combining it with other MR modalities potentially improves diagnostic performance. A study combining volumetric MRI, DTI and neuromelanin-sensitive imaging suggested that combining neuromelanin-based SN volumes with pontine FA values of the midbrain best distinguished PSP-RS from PD (552). Similarly, high discriminative accuracy between PD and MSA as well as PSP by use of observer-independent machine learning approaches with automated volumetry or automated voxel-based diffusivity or multimodal MRI combining several MR parameters has been reported (462, 464, 553).

Current use recommendation: Potential progression biomarker for PD. Distinguishing different atypical parkinsonian disorders from one another and improving their diagnostic phenotypes.

Proton Magnetic Resonance Spectroscopy

Proton magnetic resonance spectroscopy (MRS) reveals the metabolic status of the region sampled for a specific disease process. In PD, N-acetyl aspartate/creatine (NAA/Cr) ratios in the SN are reduced compared to controls and correlate with disease severity (554, 555). Lower ratios have also been noted in the lentiform nucleus (LN), temporoparietal and posterior cingulate cortices, as well as the pre-supplementary motor area (556-559) though correlation with disease severity is less clear (557, 558). NAA/Cr ratios are lower in the rostral SN in PD with an inverted pattern in atypical parkinsonian patients and HC (560). Reduced bilateral hippocampal NAA/Cr ratios have been noted in DLB compared to HC (561). Lower NAA/Cr ratios have also been noted in the posterior cingulate gyrus of PDD patients compared to cognitively normal PD patients (562).

NAA/Cr ratios are lower in the putamen in MSA-P, and pontine base in both MSA-P and MSA-C and can discriminate MSA-P from PD (563). Several other studies exploring metabolites did not however note differences between MSA-P and PD (556, 564). Cerebellar NAA/Cr and NAA/myo-inositol ratios are however reduced in MSA-C compared to PD, MSA-P, PSP-RS and HC while cerebellar myoinositol/Cr ratios are increased compared to HC (564).

In PSP, NAA/Cr ratios are reduced in the LN, brainstem, centrum semiovale, frontal, and precentral cortex, while NAA/choline is reduced in the LN compared to HC (565). More prominent reductions are also noted in the putamen compared to PD and MSA (566). Reduced cerebellar NAA/Cr and NAA/myo-inositol ratios have also been noted in PSP-RS cases compared to HC and PD patients (564). Reduced scyllo-inositol concentrations and scyllo-inositol/Cr ratios in the supplementary motor area have also been noted in PSP compared to controls (567).

NAA/Choline and NAA/Cr levels are reduced in in the frontoparietal cortex, LN, and centrum semiovale in CBS compared to HC while lower levels in the frontoparietal lobe can help distinguish CBS cases from PSP (568) . NAA/Cr is reduced in the frontal cortex and putamen compared to PD and MSA while more prominent putaminal NAA/Cr ratio asymmetry has also been noted (566).

Phosphorus based magnetic resonance spectroscopy (31P-MRS) has been of specific interest for a subset of potential interventions as it can assess mitochondrial function. In vivo Pi/ATP and PCr/ATP ratios reflect oxidative phosphorylation pathways (569). Reductions in ATP and PCr (570) and increased Pi/ATP ratios (571) in the putamen and midbrain of PD patients compared to controls have been reported while differences can also distinguish PD from PSP (AUC 0.93) (572). Longitudinal ratio improvement suggestive of target engagement was also noted in a recently completed disease modifying trial of ursodeoxycholic acid (573).

Current use recommendation: There is some preliminary level of evidence that MRS could serve to improve diagnostics of parkinsonian disorders though this may be best used in combination with conventional MRI by increasing specificity. Confirmation of target engagement could potentially be of value when studying drugs acting on specific pathways.

Functional MRI

Resting-state and task-based functional MRI reveal networks involved in motor, cognitive, and affective processes. Network impairments have been associated with motor and non-motor symptoms. Reduced resting-state connectivity between the striatum and thalamus, midbrain, pons, and cerebellum has been noted in PD as has greater change in connectivity in the posterior putamen which is followed by change in the anterior putamen and caudate (574). Connectivity changes between cortical and subcortical areas have also been reported in PD (574). Reduced resting-state functional connectivity within the basal ganglia network can differentiate PD cases from HC well (sensitivity 100%, specificity 89.5%) (575). Symptom specific changes have also been noted with elevated functional connectivity of the globus pallidus internus and putamen with the cerebellothalamic circuit being noted in tremor-dominant cases (576) and abnormal functional connectivity in the pedunculopontine nucleus and corticopontine-cerebellar being related with

freezing of gait (577). Hallucinations have also been associated with a characteristic pattern of functional connectivity changes within the default mode network and visual processing areas (578).

MSA patients display reduced cerebellar connectivity with multiple brain networks compared to PD (579) while PSP cases exhibit more extensive functional connectivity disruptions throughout the brain (580) as well as rostral midbrain tegmentum network changes (581). In CBS, decreased functional connectivity occurs in the right central operculum, middle temporal gyrus, and posterior insula, while increased connectivity occurs in the anterior cingulum, superior frontal gyrus, and bilateral caudate nuclei (582). Thalamic functional connectivity to multiple cortical and cerebellar regions is decreased in PSP and CBS while whole brain functional connectivity to the dentate nucleus differs between these conditions (583).

Longitudinal task-based functional MRI has shown a decline in activity in the putamen and primary motor cortex over 1 year in PD patients compared to HC (584). In MSA changes are exclusively extra striatal while in PSP, all regions previously described are less active at one year compared to baseline (584). Symptomatic treatment can alter functional connectivity with increased connectivity noted in the supplementary motor area (585) and within the basal ganglia network (575) after levodopa administration.

Current use recommendation: Although available evidence for this modality is overall promising, replication of diagnostic and progression findings are necessary. Also unclear if this technique would be able to provide additional value to other imaging biomarkers in trials.

PET/SPECT imaging

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are nuclear medicine imaging techniques which provide metabolic and functional information. They are combined with CT and MRI to provide detailed anatomical and metabolic information. Tracers are

used to mark various aspects of pre/post synaptic nigrostriatal pathology as well as reveal functional brain changes in different brain regions.

Neuropathology imaging

Radiolabelled probes for imaging pathological proteins (α-synuclein, tau, amyloid), pre- and post-synaptic integrity of dopaminergic and non-dopaminergic neurons and synaptic integrity overall have been developed and studied for biomarker purposes with variable success.

Alpha-Synuclein

Several radio labelled probes for imaging a-synuclein have been explored including phenothiazine, indolinone, indolinone-diene and chalcone analogues, and structural congeners (319) though no tracer is currently of diagnostic value for PD. Issues to overcome include developing tracers for intracellular targeting with ideal lipophilicity, and tracer selectivity for a-synuclein over amyloid and tau aggregates (586, 587). More recently, a newly developed α-synuclein Positron Emission Tomography (PET) tracer, [18F] ACI-12589 was shown to bind to basal ganglia and cerebellar white matter in a small cohort though this was confined to MSA patients (588).

Current use recommendation: Larger studies examining diagnostic accuracy of PD and MSA will be critical.

Amyloid

Cerebral amyloid deposition can be assessed with 11C-PIB PET as well as other 18F labelled radiotracers. Uptake accurately reflects amyloid deposition (589) with a gradient of increasing amyloidopathy noted in conditions (PD < PD-MCI < PDD < DLB) (590, 591). Elevated 11C-PIB binding has been observed in over 50% of DLB cases particularly in cortical association areas, frontal and

temporoparietal cortices, cingulum, and striatum, though no clear binding difference between PDD and PD has been noted (592-594). Greater 11C-PIB binding in DLB has been noted (595). An APOE4 allele status which is known to be related to the development of cognitive impairment in PD is associated with greater 11C-PIB binding in DLB, PDD and PD mild cognitive impairment (PD-MCI) (595). A corresponding association between elevated amyloid PET and global cognitive severity in LBD has also been noted though the relationship with dementia onset has been mixed (594-596) though one study demonstrated greater cognitive decline after 1 year in DLB patients with amyloid-positive scans (597). In PD, the presence of striatal and cortical amyloidopathy is associated with greater cognitive dysfunction (598). Greater 11C-PIB binding is also associated with lower CSF amyloid beta-42 peptide in DLB (599, 600). 11C-PIB retention is absent in MSA (601) though age related amyloid deposition quantified with 11C-PIB PET is noted in atypical parkinsonian conditions (602).

Current use recommendation: While amyloid deposition scans clearly have usefulness as a prognostic biomarker, their role will need to be consider in comparison with other reliable biofluid and/or clinical markers of cognitive progression.

Tau

Several PET radiotracers have been developed though challenges for use include a lack of specificity due to variable tau fibril affinities though the development of second-generation tracers may improve this (603). Elevated 18F-AV-1451 uptake in the putamen, pallidum, thalamus, midbrain, and cerebellar dentate nucleus has been observed in PSP compared to HC (604-607). These findings were largely replicated with a separate tracer-11C-PBB3 (608). 18F-AV-1451 tracer retention corelate poorly with PSP clinical severity (607, 609), and compares poorly to midbrain atrophy rate for

predicting PSP progression (607) though increased uptake in the globus pallidus, midbrain, and subthalamus distinguishes PSP from PD (609, 610). Midbrain uptake of a different tracer-18F-THK5351 however correlates with PSP clinical severity (611).

In CBS 18F-AV-1451 binding is asymmetrically elevated in the contralateral putamen, globus pallidus, and thalamus to the clinically affected side compared to HC (605) and binding patterns can distinguish from AD and PSP (612). 18F-AV-1451 retention however is less sensitive at detecting neuronal loss than MRI cortical atrophy and 18F-FDG-PET reductions (612) likely due to poor binding of straight tau filaments associated with PSP and CBS (604, 613).

Primary sensorimotor and visual cortex elevated 18F-AV-1451 binding has also been noted in DLB compared to AD (614). 18F-AV-1451 and 18F-THK5351 off-target binding to neuromelanin neurons has also been noted in PD, CBS, and PSP though their value as biomarkers needs to be explored further (613, 615). Differential selectivity for the different tau isoforms and off target binding to a-synuclein pathology has recently been noted (616). When clearly established, this will need to be factored into future biomarker use.

Current use recommendation: Tau tracer PET imaging maybe a credible diagnostic marker for tauopathies and patterns may distinguish between them though their future role will need to be clarified further considering contrasting findings with different tracers in addition to consideration against potential future credible wet biomarkers such as RT-Quic. Their potential role for monitoring disease progression and treatment response will need to be studied further.

Dopaminergic

A variety of radionuclide tracers are available to examine pre- and post-synaptic striatal dopaminergic function using positron emission tomography (PET) or single photon emission tomography (SPECT)

imaging. At the presynaptic level, molecular targets and their respective tracers include L-aromatic amino acid decarboxylase (AADC/tracer F-DOPA), vesicular monoamine transporter 2 (VMAT2/tracer [11C]-dihydrotetrabenazine) and the dopamine transporter (DAT/tracers CFT PET and 123I-CIT SPECT) density.

These markers are sensitive for the detection of dysfunction or loss of striatal dopaminergic terminals and enable the identification of parkinsonian syndromes with nigral neurodegeneration though do not reliably distinguish PD from atypical disorders. Visually assessing for the presence of nigrostriatal degeneration with this modality is increasingly used in trial recruitment (617) to exclude patients with clinical presentations in keeping with PD but with scans without evidence of dopaminergic deficit (SWEDDS) due to drug induced parkinsonism for example (618-620). Objective measurement of striatal uptake in comparison to other brain regions may however be more useful in trials recruiting patients with more established PD as these ratios can reflect motor and non-motor disease severity as well as progression though disease stages, hemispheric dominance of dopaminergic deficit and type of tracer used are important considerations (621). Striatal dopaminergic markers decline most prominently in the first years of motor disease before largely plateauing within 5 years of diagnosis (622-625). Quantification of dopaminergic markers in the midbrain/SN may be better markers beyond this point (626). The type of dopaminergic tracer used can potentially be critical for tracking progression in trials and measuring treatment response. VMAT2 imaging for example is less subject to compensatory changes in expression than DAT and F-DOPA (627). Quantitative dopaminergic assessments have been used in a number of recent disease modification trials though with overall negative findings to date.

Dopamine receptor expression can also be estimated at the postsynaptic level with PET ligands such as [^{11}C]-raclopride, [^{18}F]-fallypride or ^{123}I-IBZM SPECT (all of which bind to D2 receptors) or agents such as [^{11}C]NNC 112 which binds to D(1) receptors (628). Preservation of post-synaptic dopamine receptors is typical of PD whereas post synaptic receptor loss early in the disease is more likely

indicative of an atypical form of parkinsonism. Imaging results depend on the dose and timing of oral dopaminergic replacement and the usefulness of this type of imaging approach may perhaps be restricted to restorative approaches such as cell or gene therapy interventions (629).

Current use recommendation: Useful for excluding SWEDDS. Use as a trial outcome measure will need more study in particular in cases with established parkinsonism at later disease stages.

Non-dopaminergic

Radionuclide imaging studies of the serotonergic and cholinergic systems demonstrate associations with non-motor PD pathophysiology. Reduced binding on serotonergic imaging has been noted in individuals with early PD (disease duration less than 5 years) (630). Serotonergic denervation also correlates with increased dopamine turnover and reduced levodopa responses (631). In later disease stages (disease duration 5 to 10 or more years), serotonergic transporter binding remains reduced compared to controls (630) and the degree of serotonergic pathology is associated with cognitive decline (632). Cholinergic denervation also occurs in early PD (disease duration less than 3 years) but is more pronounced in PD with dementia (633). Noradrenergic activity, quantifiable by PET imaging is reduced in PD and is associated with the presence of RBD and cognitive impairment (634).

Current use recommendation: The utility of these markers in tracking progression is of interest but not yet sufficiently clear.

Synaptic density

Synaptic density quantification irrespective of neurotransmitter type has also been of interest in PD. Tracers quantifying the concentration of the synaptic vesicle 2A protein (18F-UCB-H or 11C-UCB-J) reflect this and have been studied in several cohorts. Lower binding potential in both cortical and sub-

cortical regions have been noted in PD though this is most prominent in the SN (635). Correlation with clinical status has however been inconsistent though one study suggested more prominent and extensive reductions in PD dementia and DLB cases (636-638). Similarly, small cohort studies using 11C-UCB-J PET did not note binding changes over 2 years (636, 639).

Current use recommendation: Current evidence therefore does not support the use of this marker in clinical trials.

Neuroinflammation

The PET ligands 11C-PK11195, 11C-PBR28 and 18F-FEPPA which bind to the 18 kDa translocator protein (TSPO) on mitochondria in microglia have been used for imaging neuroinflammation with TSPO upregulation suggesting microglial activation (640). PD clinical severity and putaminal presynaptic dopaminergic integrity correlates with ^{11}C-PK11195 binding (641). Binding affinity can vary with TPSO genetic polymorphisms which needs appropriate adjustment in analyses (640, 642). Augmented 11C-PK11195 binding in the dorsolateral prefrontal cortex, putamen, pallidum, pons, and SN has been noted in MSA compared to controls (643). Likewise, changes in the basal ganglia, midbrain, frontal lobe, and cerebellum has been noted in PSP (644) while 11C-PK11195 uptake in the caudate nucleus, putamen, SN, pons, pre- and post-central gyrus and the frontal lobe has been noted in CBS (645).

Current use recommendation: Taken alone, TPSO patterns lack the ability to distinguish parkinsonian conditions though their future use may be as biomarkers of therapeutic response for interventions targeting neuroinflammation (646).

Metabolic and network imaging

Quantification of changes in regional cerebral blood flow can be performed using various radiotracers with cerebral perfusion SPECT. This demonstrates the metabolic status of brain tissue. Cerebral glucose metabolism can also be evaluated using 18Flabeled fluorodeoxyglucose [18F-FDG] where reduced tracer uptake indicates lower tissue glucose utilization.

Glucose metabolism

^{18}F-FDG-PET parieto-occipital hypometabolism is noted in PD (647, 648) while preserved glucose metabolism in the basal ganglia distinguishes PD from MSA and PSP (647). Inferior parietal and left caudate glucose hypometabolism in PD also correlates with motor and cognitive deficits (649).

A unique PD-related pattern (PDRP) characterised by elevated pallidothalamic and pontine metabolic activity with reduction in the supplementary motor area, premotor cortex, and parietal association areas has also been noted in cases prior to dopaminergic treatment (650) and can differentiate PD from atypical parkinsonism (651).

PDRP progresses in early PD (disease duration less than 2 years) over 24 months, suggesting potential progression marker use in the early stages (652) though a critical limitation is that acute dopaminergic treatment diminishes the pattern (653). A PD-related cognitive pattern (PDCP) characterised by a reduction in the medial frontal and parietal association regions, and metabolic increase in cerebellar cortex and dentate nuclei (654) has also been described. This pattern seems to occur years after the PDRP (652, 655), increases over time (652) and is higher in those with dementia (656). The PDCP also correlates with memory and executive performance (654) while its lack of change with dopaminergic treatment potentially supports its use as a marker of cognitive dysfunction (657).

Occipital and temporoparietal hypoperfusion can distinguish DLB from controls though only occipital hypoperfusion can differentiating DLB from AD (658). Perfusion is unable to distinguish PDD cases from DLB (659, 660). Hypoperfusion in the left occipital region in combination with episodic memory

deficits can however distinguish DLB from CBS patients (661). Asymmetric perfusion profiles have been noted in CBS with some ability to distinguish from PSP (662, 663). Hypoperfusion in the occipital cortex has also been noted in PD compared to controls (660, 664) while frontal hypoperfusion can occur longitudinally (665). In MSA-P, putaminal hypoperfusion is observed compared to PD (666) while cerebellar hypoperfusion is a feature of MSA-C patients compared to controls (667).

Current use recommendation: PDRP/PDCP patterns could potentially be used to demonstrate treatment response and shorten disease modification trials if shown in future studies to predict longer term outcomes. Disease specific patterns in atypical disorders are intuitive though unlikely to be of additional value to regional changes noted in conventional imaging. Demonstrating that these disease specific changes occur in early motor stages where clinical diagnostic certainty is less clear could potentially increase their value.

Cerebral Perfusion

Cerebral perfusion SPECT evaluates metabolic status by quantifying regional cerebral blood flow changes using radiotracers. Occipital and frontal hypoperfusion is a feature of PD (660, 668), and remained present after 1 year of follow-up in one study (669). Occipital hypoperfusion is also a feature of DLB and distinguishes the condition from HC, CBS, and AD (658, 670). Occipital hypoperfusion is also however noted in PDD cases (659, 660). Putamen hypoperfusion distinguishes MSA-P from PD (671) while cerebellum and pontine hypoperfusion is noted in MSA-C patients (667). Perfusion asymmetry is a characteristic of CBS (672, 673) and asymmetry in frontal and parietal cortices distinguishes it from PSP (673).

Current use recommendation: Perfusion SPECT findings are currently limited by small single site studies.

Transcranial Sonographic Imaging

Increased Substantia Nigra (SN) echogenicity likely due to accumulation of nigral iron is observed in PD (674-676) though a proportion of healthy controls and Essential Tremor patients also exhibit this (677). This sign can however differentiate PD from PSP and MSA with good sensitivity (91%) and specificity (82–96%) (674). Hyper-echogenicity remains unchanged over follow-up (678) and does not correlate with disease severity (676, 679) or presynaptic DAT loss (680) in PD thus limiting use as a progression marker. Frequencies of the sign in DLB and PDD are similar to PD (681). DLB and PDD can however be distinguished with a combination of transcranial sonography indices (sensitivity 96%, specificity 80%) (681). Although present in up to 80% of cases of CBS the hyperechogenic SN has not been validated in pathologically confirmed cohorts (682-685).

Increased echogenicity of the Lentiform Nucleus (LN) is noted in atypical parkinsonian disorders and can support the diagnosis in conjunction with a normo echogenic SN (683, 686). This profile is observed in MSA-P and PSP with good sensitivity (100%), though specificity is low (59%). When echogenicity of the SN is normal this predicts MSA-P well (sensitivity 90%, specificity 98%). Increased LN echogenicity alone is however poor at distinguishing parkinsonian conditions (685). LN hyperechogenicity in conjunction with ventricular enlargement of >10mm assessed on transcranial sonography can however indicate PSP (685).

Current use recommendation: Combination of sonographic markers for atypical disorders maybe proof to be useful though validation studies in larger cohorts are required.

1.24 Limitations of biomarkers

A framework for considering the definition and variability of a parkinsonian disorder according to a biomarker is beginning to emerge with several credible candidates likely to fulfil different roles.

When applying stringent criteria to scrutinise their validity, only a small number of current approaches appear to be credible options (summarised in table 1.2 and 1.3) The presence/absence of α-syn or tau SAA-CSF is potentially a major step forward. Several practical obstacles need to be considered however prior to the routine use/reliance on biomarkers in the clinical trial context. Firstly, tau essays will need further validation. Acquiring some biomarkers e.g. CSF requires an invasive procedure which may be unacceptable for some participants. Growing evidence of the equivalence of α-syn SAA-in skin to that seen with CSF could however overcome this limitation. The demonstration of equivalence of testing on even less invasive samples such as serum/plasma or within peripherally obtained EVs is therefore a priority. With greater demonstration of validity, routine testing of peripherally acquired biomarkers can become normal practice, for example the widespread availability of plasma NfL testing in healthcare laboratories.

Interpretation of discrepant results between studies attributable to preanalytical and analytical confounders, different techniques employed and a lack of factoring of different protein species measured (total α-synuclein vs oligomeric) needs careful critique. Similarly, imaging studies are affected by methodological discrepancies including different assumptions for correction of serial data as well as sample size, power, and study design caveats and the use of different outcome measures. Collaborative studies allowing analysis of larger sample sizes with adequate follow-up that employ standardized sampling and analysis methodology will improve these limitations, as demonstrated by the harmonisation of large numbers of samples processed in the Parkinson's progression markers initiative (PPMI).

The major limitation in biomarker discovery is undoubtedly difficulty with validation. Association between a change in a biological assay alongside a clinical state need not equal causation. For example, biological changes may represent healthy compensatory responses to a pathological process. Furthermore, even biomarkers that do reflect active processes of neurodegeneration may not have linear relationships over the course of disease particularly if production ultimately declines

because of widespread tissue death. While it is possible to use clinico-pathological data for validation, confirmation that a biomarker predicts slowing of disease progression necessarily requires the identification of an agent which achieves this according to our threshold whether that be clinical, patient reported, functional impairment or quality of life milestones which have inherent limitations.

To date, no single biomarker can yet be recommended to act as a surrogate for clinical disease progression in PD though some promising examples are emerging (Table 1.2). Combinations of fluid biomarkers invariably increase the strength of their individual predictive properties. While fluid and imaging biomarkers are often collected from the same trial participants, explorations of the utility of multiple fluid biomarkers as a panel alongside imaging in combination, are rare. Challenges for future trials will be in the choice of selection of suitable combinations of fluid and imaging biomarkers that complement each other. This will certainly need to be strongly guided by the biological action of the agent being tested and the stage of the disease of their participants being treated, though those biomarkers that appear to align with disease progression most closely should be prioritised. How much weight each biomarker in the panel will ultimately carry will become more easily evident following a positive clinical trial.

Table 1.2 Summary of biofluid/tissue biomarkers with replicable value

	PD	DLB	MSA	PSP	CBS
Diagnostic					
Skin Ser-129p- α -syn			+		
CSF Ser-129p: total α -syn	+				
CSF/Skin α -syn SAA	+	+	+		
CSF p-tau+AB42		+			+
Blood miRNA panel			+		

CSF Coq10			+		
CSF/blood NfL			+	+	+
Predicting progression					
CSF p-tau+AB42	+	+			
NLR	+				
Il6/10	+				
GBA mutations	+	+			
APOe4 status	+				
T2DM status	+				
HBA1C	+				
Gut microbiome profile	+				
Blood NfL	+				
Tracking progression					
CSF o-α-syn: total α-syn	+				
Blood NfL				+	
Therapeutic response					
LRRK2 pathway downstream molecules	+				
GCase/Glucocyserimide	+				
Mitochoncrial PGC-1α	+				
IRS-1pTyr/p-AKT s437	+				

Table 1.3 Summary of imaging biomarkers with replicable value

	PD	DLB	MSA	PSP	CBS
Diagnostic					
T1 VBM			+	+	+
MR diffusion			+	+	+
MR spectroscopy (NAA/CR +Pi/ATP)	+			+	
Tau PET				+	
FDG PET	+	+			
Predicting progression					
Amyloid PET		+			
Tracking progression					
VBM (pontine atrophy)				+	
DAT PET/SPECT	+				
Iron sensitive imaging	+				
Free water imaging	+				
Therapeutic response					
MR Spectrocopy Pi/ATP	+				
DAT PET/SPECT	+				

Legend for tables 1.2 &1.3:

+ indicates evidence for biomarker use in ≥3 studies when comparing disease group with HC.

+ in diagnostic biomarker category indicates evidence for biomarker use in ≥3 studies when comparing disease with HC in addition to at least one study demonstrating evidence for distinguishing the disease from another parkinsonian disorder.

+ in therapeutic response category indicates at least 1 clinical trial has explored the use of the biomarker for this purpose.

1.25 Conclusions and purpose of study

The identification of a better framework for the certainty of a parkinsonian diagnosis based on a biomarker such as positivity of α-syn SAA-CSF is a major step forwards, and less invasive equivalent alternatives will help even more. The further development of reliable biomarkers of neurodegeneration could further facilitate prognostication, identification of disease subtypes, conduct of clinical trials and identification of agents that may slow down or stop these processes. The precise role for biomarkers will depend on the mechanism of action of the agent in question, and the decision made regarding the stage of the illness at which the intervention is being applied. There is interest in recruiting people earlier in the neurodegenerative process, even prior to symptom onset, given that intuitively earlier intervention may provide a better chance of preventing irreversible cell death (687). Alongside trials in prodromal cohorts, there will remain a need to identify whether any disease modifying intervention has an impact on the 6-10 million people already struggling with symptoms, and in need of prevention of further decline.

The overarching goal of the work performed in this thesis is to inform on the best potential combination of biomarkers that could improve demonstration of disease modification in the exenatide PD3 and MSA trials. A challenge for these trials will be in selecting suitable combinations of fluid and imaging biomarkers that complement each other. This will need to be strongly guided by the biological action of the agent being tested and the stage of the disease of their participants being treated, though those biomarkers that appear to align with disease progression most closely should be prioritised. The three biomarkers selected for this work reflected different disease aspects with a predominant focus on determining if they would be useful for predicting and tracking progression. This included insulin resistance which was of interest as this pathway is a key target of GLP-1 receptor agonists which are being studied in the trials, and dopaminergic denervation and axonal injury which are general downstream features of the neurodegenerative process. Demonstrating improvements in each of these aspects in the trials and potentially utilizing them in combination with genetic status for

balancing trial arms could potentially provide important support for demonstrating disease modification in addition to addition to potential changes that maybe noted with clinical markers.

The aims of this study are therefore to;

1. Identify and recruit natural history and clinical trial cohort's representative of PD and atypical parkinsonian disorders (PSP, MSA) that would be suitable for studying disease modification and explore the various roles of biomarkers.
2. Explore if known risk factors of disease severity and progression which may represent a disease subtype in PD when present such as T2DM have similar implications for atypical parkinsonian disorders and if biomarkers reflecting the neurodegenerative process could provide additional evidence for sub-characterizing this cohort.
3. Explore the complexities of interpretation of biomarker utilization from the impact of clinical demographics and its impact on applying natural history study findings to clinical trial cohorts.
4. Study the additional value of combining biomarkers for diagnostic and prognostic purposes over utilizing individual biomarkers.

Each of these objectives will be presented and discussed in turn in the subsequent chapters of this thesis.

Chapter two

Materials and methods

2.0 Summary of chapter

Natural history cohorts of Parkinson's disease (the tracking Parkinson's study) and atypical parkinsonian disorders (PROSPECT) and the exenatide PD3 and exenatide MSA clinical trials were used for biomarker studies performed in this thesis. I was involved in recruitment, clinical assessment, and sample collection for the PROSPECT study and Exenatide PD3 and MSA trials. I designed the study and statistical plan and performed the analysis of data presented from the tracking Parkinson's study in this thesis. Each of these cohorts are detailed in this chapter.

2.1 The tracking Parkinson's study

2.11 General Outline

The tracking Parkinson's study is a prospective, observational, multicentre study which recruited 2000 patients from 1 February 2012 to 31 May 2014. Patients had a clinical diagnosis of PD that was corroborated by the Queen Square Brain Bank criteria and supported by neuroimaging when the diagnosis was not firmly established. Drug naïve and treated patients between 18 and 90 years were eligible. Recent onset cases who were diagnosed within the preceding 3.5 years were recruited. Exclusion criteria included severe comorbid illness that would not allow patient participation in clinic visits and features suggestive of other degenerative parkinsonian conditions such as progressive supranuclear palsy, or, parkinsonism attributable to cerebrovascular disease, and patients with drug-induced parkinsonism though drug-unmasked PD was allowed if justified by abnormal functional dopaminergic imaging with dopamine transporter (DAT) single photon emission computed tomography (SPECT) or fluorodopa (18F) positron emission tomography (F-DOPA PET). Seventy-two sites in the UK providing secondary care treatment for PD patients participated in the study with multicentre ethics committee and local research and development department approvals (REC Reference: 11/AL/0163).

2.12 Clinical Assessments

Clinical assessments were performed in the outpatient setting. Home visits were performed in remote settings. Study follow up visits were 6-monthly with more detailed observations at 0, 18, 36 and 54, 72 and 96 months. Standardized and validated scales, to document motor and non-motor features, quality of life and drug responsiveness. Clinicians determined their diagnostic certainty of PD at each visit (0-100%), while also noting clinical features they deemed to be atypical for PD. Detailed descriptions of each scale used are listed below.

- **The Movement Disorders Society Unified Parkinson's disease rating scale (MDS-UPDRS) Part 1** comprises part 1a which is completed by a rater by asking patients questions regarding their non-motor symptom burden and their impact on function and part 1b which comprises questions covering non-motor symptoms which patients complete themselves. Higher scores indicate increased severity. Total scores range from 0 to 52.
- **MDS-UPDRS Part 2** is a patient self-completed questionnaire covering motor aspects of experiences of daily living. Higher scores indicate increased severity. Total scores range from 0 to 52.
- **MDS-UPDRS Part 3** is a rater completed clinical examination of motor signs covering tremor, rigidity, bradykinesia, speech, gait, posture, and postural stability. The scale can be performed in the ON medication state and in the practically defined OFF medication state. Higher scores indicate increased severity. Total scores range from 0 to 72.
- **MDS-UPDRS Part 4** which measures motor fluctuations and dyskinesia and comprises questions which are rater completed as well as questions which patients complete.
- **Hoehn & Yahr (H&Y)** which is a rater completed scale of overall presentation and impact of motor symptoms and signs. Higher scores indicate increased severity. Scores range from 0 to 5.
- **Levodopa equivalent daily dosages (LED)** To facilitate comparisons between patients taking different regimes of conventional PD medications, a set of conversion factors have been used to convert each of the commonly used PD medications to a "Levodopa equivalent dose (LED)". The LED of each of their medications can then be summed for inter-patient / inter-group comparisons.
- **MoCA**. This scale is a validated global measure of cognitive ability. Higher scores indicate increased severity. Scores range from 0 to 30.
- **Verbal fluency** Patient asked to mention as many animals as they can over a span of 90 seconds.

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2.13 Sample collection

Blood samples were collected at all sites at study entry. This included an ethylene diamine tetra acetic acid (EDTA) sample for DNA extraction and an acid citrate dextrose (ACD) sample for cryopreservation of peripheral blood lymphocytes at the European Centre for Cell Cultures (ECACC) in Wiltshire, England to generate a long-term backup resource. All DNA samples were stored for analysis and distribution at the study's centralized laboratory at Cardiff University, Wales. In all PD patients the G2019S mutation at LRRK2 was genotyped using a Kompetitive Allele Specific Polymerase (KASP) assay (LGC Genomic solutions) and the GBA mutation carrier status established by DNA sequencing of all coding exons. The genes PARK2 and PINK1 were screened for mutations using DNA sequencing and multiplex ligation-dependent probe amplification (MLPA) (MRC Holland) in all young onset PD patients. Genotyping of all DNA samples using the Illumina Human Core Exome array is also planned. Serum samples were stored in 6 aliquots at study entry and every 18 months in recent onset patients for future proteomic analysis at –80◦ centigrade.

2.2 The prospect study

2.21 General Outline

The PROSPECT study is a natural history cohort consisting of PSP cases as defined by the NINDS-SPSP criteria (688), CBS cases based on the Armstrong criteria (15) and MSA cases following the revised Gilman criteria (689). Patients with progressive movement or cognitive disorders, thought likely to have atypical parkinsonian syndrome but not meeting any diagnostic criteria were also recruited as indeterminate (IDT) cases. Seven UK study sites (University College London [UCL], Oxford, Cambridge, Newcastle, Brighton, Newport, and Manchester) recruited PSP, CBS, and IDT cases. MSA cases were

recruited through 28 specialist centres in the UK, France, Spain, Germany, and Russia. Healthy Control participants were also recruited in this study and included a spouse or a friend of the cases. All participants are invited to register for *post mortem* brain donation. Participants were recruited from 1 September 2015 with recruitment expected to be completed in early 2024. Study-wide ethical approval was obtained from the UCL Queen Square Institute of Neurology research ethics committee and the MSA study also received approval from the Pavlov First Saint-Petersburg State Medical University Ethics Committee (№ 204 dated 26.02.2018) and HCB/2015/0798.

2.22 Clinical Assessments

Core and optional study assessments were performed at baseline, 6, 12, 24, and 36 months of follow-up, with brief assessments at 48 and 60 months. At each study visit, a neurological history and examination was performed. Clinicians determined their favoured diagnosis and the level of diagnostic certainty for the atypical syndrome at each visit (0-100%). Detailed descriptions of each scale used are listed below.

- **The Montreal Cognitive Assessment (MoCA**) scores range from 0-30, with higher scores indicating greater impairment.
- **The PSP Rating Scale (PSP-RS)** is performed by the rater with a combination of questions and examination measuring motor, and non-motor symptoms that aims to measure symptom burden and impact on function. The scale comprises sections for history, mental examination, bulbar examination, supranuclear ocular motor examination, limb examination of parkinsonism and dystonia and gait and midline examination. This scale was performed on PSP, CBS, and IDT cases in the study. Higher scores indicate increased severity. Scores range from 0 to 100.
- **The Unified Multiple System Atrophy Rating Scale (UMSARS)** focuses on all aspects of the disorder, including autonomic, cerebellar, and parkinsonism manifestations. USMARS has an

Activities of Daily Living score (UMSARS Part 1); a Motor Examination score (UMSARS Part 2); Autonomic examination score (UMSARS Part 3) and a global disability scale (UMSARS Part 4) Higher scores indicate increased severity. Scores range from 0 to 104. When total scores are reported these include Part 1 and 2 only. This was performed for MSA cases.

- **The MDS Unified Parkinson's Disease Rating Scale Part II** questionnaire measuring motor impairment (scores range from 0-52, with higher scores indicating greater impairment)

2.23 Sample collection

Blood (Serum and plasma) and CSF samples were collected at each visit for patients who consented. Extracted samples were stored in aliquots at study entry and follow-up visits for future proteomic analysis at –80◦ centigrade. A subset of cases had DNA extracted from blood samples. DNA was subsequently used for genotyping and single-nucleotide polymorphism imputation to obtain MAPT (OMIM 157140) H1/H1, APOE (OMIM 107741) ε4 allele, and TRIM11 (OMIM 607868) rs564309 minor allele group frequencies.

2.24 Neuroimaging

A subset of PROSPECT participants attended three scanning centres (UCL, Cambridge, and Oxford) and underwent baseline volumetric T1-weighted magnetic resonance imaging (MRI) on 3T scanners (Siemens, Prisma, or TRIO).

2.3 The exenatide PD3 Trial

2.31 General Outline

This is a simple parallel group multicentre phase 3, double-blind, randomised, placebo-controlled trial which includes a 96-week exposure period to exenatide to explore a potential disease modification effect. Participants were recruited through six UK specialist movement disorders clinics (National Hospital for Neurology and Neurosurgery (UCLH, London), King's College Hospital National Health Service (NHS) Foundation Trust (London), Oxford University Hospitals NHS Foundation Trust (Oxford), Derriford University Hospital (Plymouth), Salford Royal Hospital (Manchester) and Western General Hospital & Royal Infirmary of Edinburgh (Edinburgh). The trial began recruitment on 20 January 2020 and closed on 30 April 2022 after recruiting 194 patients. Follow-up assessments were completed in February 2024, and the analysis will be complete by July 2024. The trial received REC (initial date of approval 15/10/2019, REC reference no.19/SC/0447) and other regulatory approvals (EudraCT 2018-003028-35).

Patients screened had a clinical diagnosis of PD with the support of the Queen Square brain bank criteria. Other key inclusion criteria included a Hoehn and Yahr (H&Y) stage ≤2.5 in the ON medication state, age between 25 and 80, being on dopaminergic treatment for at least 4 weeks before enrolment and being able to administer trial medication. Key exclusion criteria were a suspicion of other causes for Parkinsonism, being unable to attend visits in the practically defined OFF medication state, a Body mass index (BMI) <18.5, having a known abnormality on CT or MRI brain imaging considered likely to compromise compliance with trial protocol, significant cognitive impairment MOCA <21, concurrent severe depression Patient Health Questionnaire score ≥16, having prior intracerebral surgical intervention, participation in previous disease modifying trials, previous exposure to exenatide, impaired renal function (creatinine clearance <50 mL/min), history of pancreatitis, type 1 or type 2 diabetes mellitus, severe gastrointestinal disease (e.g., gastroparesis), hyperlipidaemia (cholesterol or

triglyceride levels greater than 2 × the upper limit of normal), history or family history of medullary thyroid cancer or multiple endocrine neoplasia 2 syndrome, hypersensitivity to exenatide's excipients and being pregnant or breast feeding.

Detailed evaluations occurred at screening, baseline, 24, 48, 72 and 96 weeks. Participants also attended a 12 weekly visit in between to collect supplies of investigational medicinal product (IMP) and for safety checks. Participants were randomly allocated to receive either exenatide extended release 2 mg subcutaneous injection (Bydureon) once weekly for 96 weeks (n = 100) or placebo subcutaneous injection once weekly for 96 weeks (n = 100) based on a minimisation algorithm (with a random element incorporated) and balancing by research site, participants with greater (H&Y stage 2.5) or lesser (H&Y stage 2.0 or less) PD severity (in the ON medication state), and participation in sub studies (remote monitoring, imaging or not participating).

2.32 Clinical Assessments

Eligibility was confirmed at screening visits. The MDS-UPDRS part 3 and Timed Walk assessments were performed in the OFF and ON states during visits 2, 4, 6, 8, and 10. The MoCA, NMS scale, Unified Dyskinesia Rating Scale and PHQ-9 were also performed. Participants self-completed the MDS-UPDRS parts 1, 2 and 4, Parkinson's Disease Questionnaire-39, and EQ-5D-5L. Participants also completed the 3-day Hauser Diary prior to visits 2, 6 and 10. Detailed descriptions of each scale used are listed below.

- **The MDS-UPDRS part 3 motor OFF** The scale can be performed in the ON medication state and in the practically defined OFF medication state. This is defined as the score obtained in a patient who has withheld all short acting conventional PD medications for at least 8 hours and all long-acting conventional PD medications for at least 36 hours.

- **MDS-UPDRS part 1, 2, 3 and 4 ON medication scores**. Part 3 of the MDS-UPDRS as well as the other elements (Part 1, 2 and 4) of the scale will also be evaluated in the presence of conventional PD medication (ON state).
- **MoCA**
- **Timed Tests**: Participants will be asked to perform a Sit-stand-walk timed test in both the OFF medication and ON medication state. The timed Sit-stand-walk test will incorporate time taken from seated position to stand and walk 10 metres, turn, and return to original seated position.
- **Unified dyskinesia rating scale (UDysRS):** This is considered to be the most useful and objective way of quantifying dyskinesia severity. This will be assessed in the ON medication state. Higher scores indicate worse severity.
- **Patient Health Questionnaire-9 (PHQ-9):** This scale allows for self-quantification of Depression severity. This will be assessed in the ON medication state.
- **Non-motor symptom scale (NMSS):** This validated scale is a tool to collect data on the frequency and severity of 30 non-motor symptoms sometimes experienced by PD patients. This will be assessed in the ON medication state.
- **39 item Parkinson's disease questionnaire (PDQ-39):** This is the standard disease specific measure of quality of life in PD comprising 39 questions. It has been extensively validated in previous studies.
- **LED**
- **EQ-5D-5L:** This is a simple, 5 question form and visual analogue scale that allows calculation of quality adjusted life years (QALY) to enable health economic analyses to be performed.
- **Hauser diary** of PD state (Time- On, Off, Troublesome Dyskinesia, Non-troublesome dyskinesia, Asleep). Diary data allows quantification of the amount of time during a 3 day period that patients spend in the varying states of movement ability.

2.33 Sample Collection

A 10ml blood sample was also collected at visits 2, 4, 6, 8 and 10 and centrifuged plasma stored in 1 ml aliquots at -80^{0}C at all sites. Two 10ml blood samples were also taken at the screening visit in EDTA sample tubes for the extraction of inherited material by University College London Hospitals Neurogenetics Laboratory. Fasting bloods (glucose, insulin, c-peptide, triglycerides, and HbA1c) for potential insulin resistance analysis are collected at visits 2, 6, and 10.

At UCLH, consenting participants had 15mls of CSF sample taken via lumbar puncture at baseline (Visit 2) and this is repeated at week 96 (Visit 10) and stored. The lumbar punctures were carried out under local anaesthetic with the patient lying on their side, with their legs pulled up and their chin tucked in (sometimes the procedure was carried out whilst the patient was seated and leaning forwards). A hollow needle was carefully inserted into the base of the spine which contains the nerves coming from the spinal cord. CSF was slowly removed and centrifuged and stored in 1ml aliquots with the unique trial participant identification number. Patients were instructed to lie flat for at least 1 hour after the procedure to minimise post lumbar puncture headache. I performed all of the lumbar punctures for the trial.

2.34 Imaging

The DaTSCAN imaging sub study was performed at the UCLH site on all consenting sub study participants. Scans were performed prior to or during visit 2 and repeated on these participants at the time of the Visit 10 appointment (-14/+7 days). DaT imaging was performed three hours after the intravenous injection of Iodine-123 Ioflupane (Datscan TM, GE Healthcare, Chalfont St. Giles, UK.) using a SPECT/CT scan of 40 minutes duration.

2.35 Outcomes analysis plan

Primary outcome analysis will evaluate the impact of treatment allocation (exenatide or placebo) on the difference between MDS UPDRS part 3 OFF medication scores at 96 weeks follow-up adjusting for baseline. For the purposes of this thesis, only data from baseline assessments was analysed.

2.4 Exenatide MSA Trial

2.41 General Outline

This is a Phase IIa, open label, randomised, parallel group, single site trial of MSA patients. Fifty patients with early stage MSA were recruited and randomised to receive Exenatide injections, or to act as controls. Exenatide was given as a once weekly subcutaneous injection in addition to participant's regular medication. All patients continued to receive standard of care treatment for MSA.

Participants aged 30-80 years old with a diagnosis of Possible or Probable MSA of the parkinsonian subtype (MSA-P) or cerebellar subtype (MSA-C) according to The Gilman Criteria were enrolled (689). Other inclusion criteria were being less than five years from the time of documented MSA diagnosis or from the time of documented parkinsonian / ataxic neurological condition that later turned out to be MSA, being able to walk at least 10 metres with or without assistance, having an anticipated survival of at least three years in the opinion of the investigator, willing to adhere to the study drug regimen, agreeing to use effective contraception if of child bearing age. Exclusion criteria included females who were pregnant, planning pregnancy or breastfeeding or women of child-bearing potential who do not practice an acceptable method of birth control. In addition, patients with advanced disease as defined by speech impairment as assessed by a score of ≥ 3 on UMSARS question 1, swallowing impairment as assessed by a score of ≥ 3 on UMSARS question 2, impairment in ambulation as assessed by a score of > 3 on UMSARS question 7 and falling more frequently than once per week as assessed by a score of ≥ 3 on UMSARS question 8 were excluded. Participants with a

clinically significant or unstable medical or surgical condition, which in the opinion of the investigator might preclude safe completion of the study and those with an active malignant neoplasms or history of a malignant neoplasm in the last 5 years were excluded as were participants with movement disorders other than MSA. Concurrent dementia (MoCA <21), severe depression (Beck Depression Inventory-II ≥30), a history of deep brain stimulation surgery, prior exposure to the investigational product within 90 days, a BMI < 18.5, diabetes, end stage renal disease or severely impaired renal function (creatinine clearance <30ml/min), clinically significant cardiac disease, pancreatitis and/or alcoholism, severe gastrointestinal disease, ongoing treatment with sulphonylureas and known allergies to the IMP and excipients of the IMP were all exclusion criteria.

A random sequence for study arm allocation was computer generated by a randomisation service provider (SealedEnvelope.com). Stratified randomisation was performed with strata defined by MSA sub-type (MSA-P versus MSA-C). Blocking was used within strata to enable achievement of equal numbers in each group.

2.42 Clinical Assessments

Detailed assessments were performed at baseline and every 12 weeks for a total of 48 weeks. An additional assessment visit was also performed at 96 weeks.

Patients' medical history, physical (chest, heart, abdomen, skin, lymph nodes) and neurological examination as well as electrocardiogram and vital signs were assessed at screening and if clinically necessary at subsequent visits. The MoCA, BDI-II, UMSARS Part 1-4, The Composite Autonomic Symptom (COMPASS) Select, The COMPASS Change Scale, MSA Quality of Life (QoL) scale, and The Unified Dystonia Rating scale were performed at baseline, 12, 24, 36, 48 and 96 weeks. Detailed descriptions of these scales are listed below:

- **MoCA**

- **The Beck's Depression Inventory-II (BDI)** is a patient rated depression severity scale.

- **The Unified MSA Rating Scale (UMSARS)**

- **The Composite Autonomic Symptom (COMPASS) Select** is a subset of the full COMPASS consisting of 46 questions in five domains (orthostatic intolerance, secretomotor, bladder, constipation, and sleep), leading to a total score between 0 and 125, with a higher score indicating greater impairment

- **The COMPASS Change Scale is a derivation of COMPASS-select** in which the participants score their change in autonomic symptoms since their last exam. It comprises 26 retrospective questions in which participants scored how much their autonomic symptoms had changed. It is divided into change of 6 autonomic function domains (or 5 in women) [i.e. orthostatic intolerance, sexual failure (erectile dysfunction, male only), bladder disorder, secretomotor disorder, constipation, and sleep disorder] with a maximum score of 160 for men and 150 for women.

- **MSA-Quality of Life scale (MSA-QoL)** is a validated 40 item self-completed questionnaire in which a participant is asked to record the problems due to their MSA over the preceding 4 weeks.

- **Timed Motor Tests** include a hand tapping task to evaluate the number of hand taps that an individual can perform within 30 seconds and a timed, sit, stand, walk task.

- **The Unified Dystonia Rating scale** is an objective assessment of the severity of dystonia in each body region.

2.43 Sample Collection

Serum was collected at baseline in 50 patients and was collected at week 12, 24, 36, 48 and 96 and stored in aliquots at -80^{0}C for future biomarker analysis. Fasting bloods (glucose, insulin, c-peptide, triglycerides, and HbA1c) for potential insulin resistance analysis is also collected at these time points. CSF was collected and stored at baseline and 48 weeks similarly to the exenatide PD3 trial for biomarker analysis.

2.44 Outcomes analysis plan

The primary outcome will be the difference in total UMSARS scores (Parts I and II) at 48 weeks compared to baseline between exenatide treated patients and best medically treated patients. For the purposes of this thesis, only data from baseline assessments will be analysed.

2.5 General approach to data across studies

2.51 Data collection

Participants were given unique/anonymous trial/study IDs that was used in all records and correspondence. Data was collected at the time-points indicated for each study on specific paper case report forms (CRFs) which were designed by each study team. Following completion all information contained within them was entered onto custom designed electronic databases which were designed to capture all relevant clinical data from the paper CRF to allow formal statistical analysis. Training on paper CRF completion and storage was provided for all staff involved in the studies including myself and listed on a delegation of responsibilities log for the two clinical trials. Clinical trial team members were also taught how to perform the PD assessment scales including certification from the MDS on the use of the MDS-UPDRS and the UMSARS scale for MSA.

2.52 Data management

Data was entered in the approved database by delegated staff at participating sites and data was protected using established procedures. Researchers were provided training for the use of databases. Databases were password protected and only accessible to members of the teams. The servers are protected by firewalls and are patched and maintained according to best practice. The physical location of the servers is protected by CCTV and security door access. Each database used (MACRO, REDCAP, SEALED ENVELOPE) implemented data validations to assist data quality, including

range checks on individual items and consistency checks between multiple items. The process was also compliant with all necessary regulatory requirements including an audit trail to allow for date/time stamped corrections accompanied by justification/explanation for any data amendments. All data are handled in accordance with the Data Protection Act 2018 and the General Data Protection Regulation (2016).

2.53 General approach to data analysis

I performed all statistical analyses in this thesis with the guidance of my primary supervisor and where necessary with the input of a statistician linked to the natural history studies or clinical trials. Data was firstly requested from the team overseeing the relevant study with an outlined proposal for the planned study. Once the study was approved by the overseeing committee the data set requested was assessed by the data management team and inspected by the relevant statistician for integrity/accuracy. When received I firstly approached the dataset by plotting distributions to explore for potential extreme outliers. When an outlier is identified, potential causes are considered including data entry or measurement errors, sampling problems and unusual conditions, and natural variation. If the outlier was not felt to reflect the population being studied for specific reasons it was removed from further analysis. I then explored the distribution of the data for normality by inspection and with statistical tests. Selection of statistical tests to explore outlined hypotheses were then determined from this and are expanded on in subsequent chapters.

Chapter three

Peripheral insulin resistance as a biomarker of parkinsonian disorders

3.0 Summary of chapter

Insulin resistance (IR) occurs due to decreased sensitivity to metabolic actions of insulin. IR can occur in body tissues involved in glucose homeostasis resulting in peripheral insulin resistance as well as the CNS resulting in central insulin resistance. IR can occur as part of the natural ageing process and has been noted in patients with parkinsonian disorders. Central IR and T2DM results in more severe disease in PD. This relationship is less clear for peripheral IR in Parkinson's disease as is the impact of IR and T2DM on atypical disorders.

In this study, I aimed to explore some of these aspects across the spectrum of T2DM and peripheral IR and central IR in cohorts of patients with PD, MSA and PSP. I aimed to evaluate if peripheral insulin resistance is more prevalent in PD, MSA, PSP than HC and to explore its relationship with patient demographics and clinical severity. I also aimed to explore if co-morbid T2DM influences MSA and PSP severity and progression as it does in PD.

I noted a similar proportion of patients had peripheral IR in these conditions compared to healthy controls (HC). Peripheral IR markers were similar in all groups and were not associated with disease severity. The presence of T2DM did not impact on disease severity and progression in MSA and PSP patients either.

3.1 Introduction

Insulin maintains whole body glucose homeostasis (690) and controls cellular physiology relating to metabolic and mitogenic functions in tissue (691). Insulin resistance (IR) occurs due to decreased sensitivity to metabolic actions of insulin. This can impact on systemic glucose homeostasis and result in hyperglycaemia (peripheral IR) and subsequently type 2 diabetes (T2DM) based on predefined thresholds (692).

IR can occur as part of the natural ageing process and this is influenced by genetic variation, gender, and ethnicity amongst other factors (693, 694). IR can also occur in the central nervous system (CNS) with an impact on CNS cellular function. Both peripheral and central IR may contribute to neurological symptoms.

Blood glucose and insulin after an overnight fast represents the basal steady state of glucose homeostasis. Models of interaction dynamics predicting fasting steady-state concentrations for a range of possible combinations of insulin resistance have been explored and surrogate indices proposed. These indices are superior to just measuring fasting glucose or insulin. The Quantitative Insulin Sensitivity Check Index (QUICK1) appears to provide best estimates compared to other indices (695-697). The glycated haemoglobin (HbA1C) test is an alternative for measurement of insulin resistance. This measure reflects the mean of blood glucose levels over preceding weeks to months and has the added benefit of measurement without the need for fasting (365, 366).

Type 2 diabetes mellitus (T2DM) is a risk factor for developing PD (343, 344) and its coexistence with PD results in more severe motor features and the development of cognitive impairment (343, 345-347). These findings have been confirmed in the tracking Parkinson's cohort (347). This link is in part explained by IR (348) which contributes to neurodegenerative processes (349). Insulin resistance as suggested by increased expression of the key downstream messenger insulin receptor substrate-1

phosphorylated at serine residue 312 in neurons and oligodendrocytes in the putamen has also been noted in MSA *post mortem* brains while being diagnosed with T2DM in primary care has been linked to a future diagnosis of PSP. In addition to this tau pathology co-exists with brain insulin resistance in a range of primary tauopathies including CBD and PSP (352, 698, 699). The potential impact of coexistent T2DM on atypical parkinsonian disorders has not however been explored in clinical cohorts.

Although peripheral IR has been noted in almost 60% of non-diabetic PD patients (356), differences in comparison to HC has not been consistent nor has association with disease severity (357-359). Patients with abnormal HbA1C levels (low and high) not in the diabetic range, however, appear to have more severe motor and cognitive impairment and disease progression (361-364). Although the IR marker Insulin-like growth factor-1 (IGF-1) has been explored in MSA and PSP, and suggest peripheral insulin resistance exists in these cases, index markers and HbA1C have not been well studied in these disorders (369, 370).

These findings have been partly responsible for clinical trials of drugs targeting insulin resistance to achieve disease modification in parkinsonian disorders (700) including the exenatide PD3 and MSA trials. Demonstration of disease modification in these studies could potentially be informed by reliable and easily accessible biomarkers of insulin resistance. It is therefore important to better understand insulin resistance and its impact on parkinsonian disorders, and if biomarkers measuring IR reflect all parkinsonian diseases in a similar manner and could therefore play a useful role in trials.

In this study, I aimed to explore some of these aspects in PD, MSA and PSP patients.

A priori- I expected;

1) Peripheral insulin resistance to be present in PD, MSA and PSP in a similar proportion to HC.

2) Peripheral insulin resistance in all cohorts would be influenced by demographic factors in particular age and BMI.

3) Peripheral insulin resistance may or may not be associated with disease severity.

4) T2DM may impact on atypical disorders (PSP and MSA) similarly to PD considering the shared IR mechanisms previously noted.

Aims:

To evaluate if peripheral insulin resistance is more prevalent in PD, MSA, PSP than HC.

To assess if the presence of peripheral insulin resistance is related to age, gender, ethnicity, disease duration and BMI in these groups.

To evaluate if peripheral insulin resistance is associated with disease severity.

To determine if co-morbid T2DM influences MSA and PSP severity and progression as it does in PD.

3.2 Methods

3.2.1 Participants:

Participants in this study were divided into four diagnostic groups (HC, PD, MSA and PSP) from cases recruited into the Exenatide PD3 and MSA trials and the PROSPECT study. Characteristics of these studies are summarised in Chapter 2. All patients who had peripheral IR markers available at baseline from the exenatide PD3 and MSA trials were included for exploration of these biomarkers. Peripheral IR markers were performed at baseline assessments of HC and PSP patients who were newly recruited into the PROSPECT study.

Separately, patients were selected to determine if T2DM had an impact on the severity and progression of MSA and PSP from previously recruited cases in the PROSPECT study. All patients who provided information on their T2DM status at the baseline assessment were included.

3.2.2 Clinical assessments:

Patient age, gender, disease duration, and BMI was recorded at baseline for PD, MSA and PSP patients. Age, and gender were noted for HC. Ethnicity of participants was noted when available. As a range of ethnicities were reported across studies, I clustered participants into seven broad categories. These included white participants (White British, white English, white Scottish, white Irish, Canadian white, white other), Hispanic participants (Latino, Hispanic), South Asian participants (Indian, Bangladeshi, Pakistani), Other Asian participants (Other Asian, Chinese, Asian), Mixed participants (Other mixed background, White & Asian), African participants and Middle Eastern participants (Arab, Turkish).

In PD, motor (MDS-UPDRS part 3 OFF state scores), non-motor (NMSS total score) and cognitive (MoCA) scales were recorded. In MSA, the UMSARS total score and MoCA was noted. In PSP, the PSP Rating scale (PSP-RS) total score and MoCA were noted. The MoCA was also noted for HC.

3.2.3 Sample Collection and processing:

Sample collection methods are described in Chapter 2. Fasting glucose, fasting insulin and HbA1C levels were collected from blood test results at baseline visits of the exenatide PD3 and MSA trials and from a Prospect visit for HC and PSP patients.

3.2.4 Peripheral Insulin Resistance assessment:

I assessed insulin resistance status using fasting glucose, insulin and HbA1C levels. I further calculated a surrogate index of insulin resistance using fasting glucose and insulin levels-the Quantitative Insulin Sensitivity Check Index (QUICKI) (1/{log [fasting serum insulin (IU/mL)]+log [fasting serum glucose (mg/dL)]}) (701). This index was chosen over other approaches due to its thorough evaluation in multiple cohorts and better linear correlation with glucose clamp estimates then other indexes (695, 696). Although proposed cut-off values for defining IR vary between ethnic populations, BMI and disease states (metabolic syndrome, T2DM, non-alcoholic fatty liver disease), I adopted a value of <0.339 as this is the most widely proposed value in studies and online calculators (695, 696, 702). I

defined insulin resistance using a HBa1C level of >42 mmol/mol based on previous studies validating this cut-off and PD studies demonstrating this cut-off may predict progression (361, 362). Patients were also defined as obese if they had a BMI ≥30.

3.2.5 Statistical analysis:

Given non-normally distributed data, medians and interquartile ranges were reported for continuous variables while frequencies, and percentages were reported for categorical variables. When continuous variables were compared between more than two groups (diagnostic groups & ethnicity), the Kruskall-wallis test was used and Dunn's post-hoc test was then applied for individual group comparison. When continuous variables were compared between two groups (gender and IR groups) a Mann-Whitney U test was used. Chi-squared test was used for comparing categorical data. When more than two groups were assessed with the chi-squared test, adjusted Pearson residuals were assessed as a chi-squared post-hoc test of individual group comparison. A z-score test statistic of greater than or equal to 1.96 or less than -1.96 was given a significance at a $p<0.05$ level while greater than 2.576 or less than -2.576 was significant at the $p<0.01$ level.

Univariate and multivariate linear regression analysis was performed to investigate the association between T2DM status and baseline IR marker levels (HbA1C and QUICK index) and clinical measures of PD, MSA and PSP.

Associations between baseline T2DM status and change in motor, and cognitive outcomes over time (symptom duration from diagnosis as the time axis) were then investigated by linear mixed effects analysis, adjusted for age at symptom onset and gender in PSP and MSA. The mixed models had both a random intercept and a random slope. Where T2DM was significantly associated with the intercept this implies that a change in the biomarker would shift the progression line up and down but not alter the rate of change. Where T2DM was significantly associated with the slope, a change in the biomarker would alter the rate of change but not where the progression line is at a time of zero.

Cox proportional hazards regression was then used to investigate whether T2DM predicted the development of disease milestones (wheelchair use, residential home requirement, PEG recommendation, unintelligible speech) and mortality after adjustment for age at baseline, gender, and baseline overall clinical severity (UMSARS/PSP-RS). Progression and survival analysis was not performed for peripheral IR markers as these were largely part of ongoing clinical trials where follow-up data was not available for analysis. A $p<0.05$ indicated statistical significance. All statistical analysis and figures were generated using Stata V.17.1.

3.3 Results

Clinical characteristics of patients included for peripheral insulin resistance analysis are summarised in table 3.1. PSP patients were older than HC ($p=0.010$) as well as those with PD ($p<0.001$) and MSA ($p=0.040$). PSP patients also performed significantly worse on the MoCA compared to all other groups. The PSP cohort comprises smaller numbers than the other groups which may have an impact on precision of findings. PD patients had a longer disease duration than those with MSA ($p<0.001$) and PSP ($p<0.001$).

Table 3.1: Demographics of patient cohorts tested for peripheral and central insulin resistance markers

Peripheral IR cohort	HC (n=60)	PD (n=190)	MSA (n=50)	PSP (n=19)
Age, years	63.56 (56.52-69.74)	60.26 (55.54-68.66)	62.35 (58.29-69.27)	**70.78 (66.58-74.59)** */^/**
Gender, male (%)	25/60 (41.70)	135/190 (71.10)	24/50 (48.00)	7/12 (36.84)
Time since diagnosis, years		**4.06 (2.27-5.70)** **/!!	0.78 (0.38-1.49)	1.15 (0.54-1.89)
MOCA	28.00 (27.00-29.00)	28.00(27.00-29.00)	28.00 (26.00-29.00)	**21.00 (19.00-25.00)** */^/**
BMI	27.66 (25.00-28.70)	27.18 (4.64)	25.52 (23.35-28.08)	25.41 (23.25-29.32)
Obese (%)	14.5	16.3	20.0	17.6

*HC vs PSP p<0.05 **PD vs PSP p<0.01 ^MSA vs PSP p<0.01 !!PD vs MSA p<0.01

3.3.1 Comparison of peripheral insulin resistance markers between diagnostic groups

Fasting glucose was higher in PD compared to HC (p=0.0036) and MSA patients (p=0.0001) though HbA1c levels were lower in PD compared to HC (p=0.0020). (Table 3.2) Fasting insulin levels were higher in PSP patients compared to HC (p=0.0464) and PD patients (p=0.0035). PSP patients had overall lower QUICKI levels compared to other groups with a trend towards significance being noted compared to HC (p=0.0519) and MSA cases (p=0.0602) and a significant difference being noted compared to PD cases (p=0.0155). A higher proportion of PSP patients were classified as suffering from peripheral insulin resistance [QUICKI (adjusted residual 2.865)] based on the pre-defined cutoff.

Table 3.2: Summary of peripheral insulin resistance markers in different diagnostic groups

	HC (n=60)	PD (n=190)	MSA (n=50)	PSP (n=19)
Peripheral markers				
Glucose (mmol/L)	5.00 (4.70-5.30)	**5.30 (4.90-5.60)*/^**	4.80 (4.70-5.30)	5.10 (4.80-5.80)
Insulin (mIU/L)	7.55 (5.00-11.10)	6.80 (4.95-9.65)	8.10 (6.00-10.70)	**10.80 (8.10-13.40)"**
HBA1C (mmol/mol)	38.00 (36.00-40.00)	**36.60 (34.40-38.80)***	37.00 (35.00-39.00)	38.00 (37.00-41.00)
QUICK1 Index	0.36 (0.33-0.38)	0.35 (0.34-0.38)	0.35 (0.33-0.37)	**0.33 (0.32-0.34)"**
Presence of systemic insulin resistance				
QUICK <0.339 (%)	20/58 (34.48)	48/187 (25.7)	14/48 (29.2)	**11/18 (61.11)**
HBA1c >42 (%)	7/60 (11.67)	8/189 (4.2)	6/45 (13.3)	3/19 (15.79)

*PD vs HC p<0.01, ^PD vs MSA p<0.01, " PSP vs PD p<0.01

3.3.2 Relationship between demographics and insulin resistance markers

As demographics such as age, gender, and BMI have previously been shown to be associated with the occurrence of insulin resistance, I explored their relationships in each diagnostic group in my cohort. Univariate regression analysis was performed treating insulin resistance markers as the dependent variable and demographic factors as the independent variables. (Table 3.3) A significant positive association was noted between age and fasting glucose in the MSA group while a trend towards a positive association was noted in the PD group (Coefficient: 0.01 (SE 0.00), p=0.060). Age was not significantly associated with other markers of peripheral insulin resistance in all diagnostic groups. A significant positive relationship was noted between BMI and the QUICK index in HC, PD and PSP patients. A trend towards a positive relationship was noted in MSA patients. No significant relationship was noted between BMI and HbA1C levels.

As variations in the prevalence of insulin resistance in different gender and ethnic groups has been noted I explored this in my diagnostic cohorts. No HC gender differences in demographics or peripheral insulin resistance markers or proportion classified as experiencing insulin resistance was noted. (Table 3.4) Male MSA patients had higher BMI but no IR marker differences. Male PSP patient had higher fasting glucose compared to females though this was not noted in other diagnostic cohorts.

The proportion of patients who reported their ethnicity is summarised in table 3.5. Majority of patients reported white ancestry in all diagnostic groups. This was followed by south Asian and then other Asian ancestry. I examined if there was a difference in fasting glucose, insulin and HbA1c levels in each diagnostic group and did not note a significant difference (data not presented). I then explored if the proportion of patients who were suffering from insulin resistance differed between diagnostic groups and did not note a significant difference. (Table 3.6)

Table 3.3: Summary of univariate regression analysis between insulin resistance markers and demographic factors in different diagnostic groups

Coefficient (SE), p value		Fasting glucose	Fasting Insulin	QUICK	HbA1C
HC					
	Age, years	0.00 (0.01), 0.445	-0.04 (0.09), 0.628	0.00 (0.00), 0.181	0.03 (0.05), 0.497
	BMI	0.00 (0.02), 0.874	**1.06 (0.21), <0.001**	**-0.00 (0.00), <0.001**	0.11 (0.14), 0.433
	PD				
	Age, years	0.01 (0.00), 0.060	-0.02 (0.05), 0.687	-0.00 (0.00), 0.952	0.02 (0.02), 0.392
	Time since diagnosis, years	0.01 (0.01), 0.508	-0.21 (0.15), 0.155	0.00 (0.00), 0.357	0.01 (0.08), 0.877
	BMI	**0.03 (0.01), 0.009**	**0.38 (0.10), <0.001**	**-0.00 (0.00), <0.001**	-0.02 (0.05), 0.686
MSA					
	Age, years	**0.02 (0.01), 0.033**	0.21 (0.13), 0.125	-0.00 (0.00), 0.150	0.08 (0.06), 0.214

	Time since diagnosis, years	0.07 (0.08), 0.379	2.12 (1.21), 0.086	-0.01 (0.01), 0.461	-0.48 (0.56), 0.401
	BMI	0.01 (0.02), 0.395	0.39 (0.22), 0.085	-0.00 (0.00), 0.088	-0.03 (0.11), 0.817
	PSP				
	Age	-0.03 (0.03), 0.384	-0.26 (0.15), 0.102	0.00 (0.00), 0.099	0.15 (0.14), 0.311
	Time since diagnosis, years	-0.18 (0.19), 0.359	0.33 (1.05), 0.756	-0.00 (0.01), 0.757	0.12 (0.94), 0.901
	BMI	0.08 (0.04), 0.051	**0.53 (0.20), 0.019**	**-0.00 (0.00), 0.027**	**0.41 (0.18), 0.040**

Table 3.4 Comparison of demographic and insulin resistance markers in different gender

Coefficient (SE), p value	HC		PD		MSA		PSP	
	Male	Female	Male	Female	Male	Female	Male	Female
Age, years	65.32 (57.52-72.09)	62.91 (53.66-67.69)	61.08 (55.83-68.91)	59.19 (53.39-65.28)	61.18 (59.37-65.08)	64.31 (56.79-71.58)	70.23 (63.29-74.59)	70.79 (66.78-74.52)
BMI	25.86 (24.06-28.24)	24.47 (22.86-28.49)	25.78 (23.74-28.11)	24.53 (21.83-28.64)	27.08 (24.99-29.87)	**23.74 (22.31-25.59)****	26.49 (25.85-27.66)	28.38 (23.26-29.32)
Fasting Glucose, mmol/L	5.00 (4.80-5.30)	5.00 (4.70-5.30)	5.30 (4.90-5.60)	5.20 (4.80-5.50)	4.80 (4.60-5.30)	4.85 (4.70-5.20)	**5.80 (5.40-7.00)***	4.80 (4.70-5.20)

Fasting Insulin, mIU/L	8.40 (5.10-11.80)	7.10 (4.40-10.20)	6.75 (4.70-9.75)	6.85 (5.10-8.75)	7.20 (6.00-10.70)	8.40 (5.90-10.30)	10.80 (9.60-12.80)	11.25 (6.90-13.50)
HbA1C, mmol/mol	38.00 (36.00-40.00)	39.00 (35.00-41.00)	36.60 (35.00-38.80)	35.50 (34.40-39.00)	36.00 (34.00-38.00)	39.00 (35.00-41.00)	39.00 (37.00-43.00)	37.00 (36.00-39.50)
QUICK <0.339 (%)	15/25	23/33	41/126	7/48	16/22	18/26	1/6	6/12
HbA1C >42 (%)	2/25	5/35	3/134	3/55	2/23	2/22	**3/7***	0/12

Table 3.5 Summary of patients who reported ethnicity in each diagnostic group

	HC	PD	MSA	PSP
White	48/57	177/190	43/50	15/18
Hispanic	0/57	2/190	0/50	0/18
South Asian	4/57	5/190	4/50	3/18
Other Asian	4/57	3/190	1/50	0/18
African	0/57	1/190	0/50	0/18
Middle Eastern	1/57	0/190	2/50	0/18
Mixed	0/57	2/190	0/50	0/18

Table 3.6 Summary of proportion of patients defined as having peripheral insulin resistance in different ethnic groups across diagnostic cohorts

	HC		PD		MSA		PSP	
	QUICK <0.339	HbA1C>42	QUICK <0.339	HbA1C>42	QUICK<0.339	HbA1C>42	QUICK<0.339	HbA1C>42
White	16/46	5/48	45/162	5/176	11/41	1/42	8/14	2/15
Hispanic			1/1	0/2				
South Asian	2/4	¼	0/5	1/5	3/4	1/4	2/3	1/3
Other Asian	1/4	¼	2/3	0/3	0/1	0/1		
African			0/1	0/1				
Middle Eastern	0/1	0/1			0/2	1/2		
Mixed			0/1	0/2				

3.3.3 Association between peripheral IR and disease severity

To examine if motor and non-motor symptom severity was influenced by insulin peripheral resistance markers, I performed univariate regression analysis for IR markers (HbA1C, and QUICK index) treating the clinical score as the dependant variable and the marker as the independent variable. No significant associations were noted between peripheral insulin resistance markers and motor and non-motor test scores. (Table 3.7) I then explored this relationship adjusting for age and did not note a significant association either. I did not adjust for disease duration as it did not have an impact on IR markers. I did not adjust for BMI due to the possibility of collinearity with peripheral IR markers.

Table 3.7 Univariate and multivariate (age) regression analysis between motor and non-motor symptom severity and insulin resistance markers

	HbA1c		QUICK	
Coefficient (SE), p value	Univariate	Multivariate	Univariate	Multivariate
PD				
UPDRS 3 OFF	0.05 (0.32), 0.868	-0.02 (0.31), 0.950	12.86 (8.28), 0.122	12.44 (8.08), 0.125
NMSS	0.49 (0.61), 0.427	0.46 (0.61), 0.451	8.47 (16.07), 0.599	8.21 (16.07), 0.610
MOCA	0.02 (0.05), 0.630	0.03 (0.05), 0.515	1.53 (1.22), 0.213	1.58 (1.21), 0.192
PSP				
PSP-RS Total	0.01 (0.90), 0.988	-0.44 (0.85), 0.612	47.76 (117.85), 0.691	-31.73 (119.77), 0.795
MOCA	-0.14 (0.40), 0.726	0.00 (0.38), 0.997	-2.51 (51.81), 0.962	38.60 (49.95), 0.453
MSA				
UMSARS total	-0.59 (0.39), 0.144	-0.59 (0.41), 0.157	65.14 (35.50), 0.073	67.69 (36.67), 0.072
MOCA	-0.16 (0.09), 0.070	-0.15 (0.09), 0.114	10.50 (7.76), 0.183	8.54 (7.90), 0.285

3.34 Diabetes in atypical parkinsonian disorders

I explored if the presence of T2DM had an impact on the severity and progression of MSA and PSP in the PROSPECT study. This was not performed for PD in my work as it has previously been explored in a separate publication which demonstrated a significant impact on PD severity and progression.

3.341 PSP

In the PSP cohort, 18 patients were excluded as they did not report their diabetes status at baseline while 88 patients did not have DM and 17 did. No demographic, or clinical severity differences were noted between the groups (Table 3.8). Having diabetes did not significantly impact on the PSP-RS total score (Coefficient -0.81 (CI -6.76, 8.10), p=0.787) at baseline when adjusted for age, gender, and disease duration.

PSP-RS assessments were available in 86 patients at baseline, 47 at 6 months, 43 at 1 year, 19 at 2 years and 10 at 3 years in the PSP-NDM group and 17 patients at baseline, 8 at 6 months, 8 at 1 year, 6 2 years and 0 at 3 years in the PSP-DM group. Linear mixed effects analysis on baseline diabetes status with clinical outcomes in PSP patients over time adjusted for age at baseline and gender was then explored. Having T2DM did not significantly impact on PSP-RS total score progression (intercept: Coefficient –3.07, CI-14.33, 8.18, p=0.593 and slope: Coefficient 1.22, CI-1.49, 3.93, p=0.378). The co-occurrence of diabetes did not predict a shorter time to development of disease milestones (wheelchair use, residential home, PEG requirement, unintelligible speech) nor did it predict a shorter survival.

Table 3.8 Comparison of demographic and clinical measures of progressive supranuclear palsy cases with and without type 2 diabetes in the prospect study

Variable, mean (SD)	PSP-NDM	PSP-DM	p value
Age at baseline	71.19 (65.61-75.41)	72.67 (71.14-73.46)	0.2302
Gender, male/female	56/31	10/7	0.6640
Diagnosis duration	0.99 (0.53-2.00)	0.57 (0.22-2.18)	0.2514
PSP-RS Total	32.00 (24.00-41.00)	36.00 (20.00-46.00)	0.8415
MOCA	16.00 (15.00-18.00)	17.00 (14.00-18.50)	0.4928

3.342 MSA

In the MSA cohort screened, 73 patients who did not report their diabetes status were excluded. Of the remaining patients, 225 reported not having diabetes (MSA-NDM) and 21 reported having diabetes (MSA-DM). Fifteen cases (1 diabetic) had pathological diagnostic confirmation of MSA. No significant age and disease duration differences were noted between the groups. Gender differences are noted in table 3.7. When limited to those with UMSARS available, 101 patients did not have DM while 6 did have DM. Having diabetes did not significantly impact on the UMSARS total score at baseline when adjusted for age, gender and disease duration.

UMSARS assessments were available in 107 patients at baseline, 27 at 6 months, 67 at 1 year, 37 at 2 years and 2 at 3 years in the MSA-NDM group and 6 patients at baseline, 5 at 6 months, 4 at 1 year, 2 2 years and 1 at 3 years in the MSA-DM group. Linear mixed effects analysis on baseline diabetes status with clinical outcomes in MSA patients over time adjusted for age at baseline & gender was then explored. No significant effect of T2DM on the intercept (Coefficient 0.90 (CI -28.33,30.13), p=0.952)

and slope (Coefficient -1.35 (CI -6.52, 3.83), p=0.611) was noted. The co-occurrence of diabetes did not predict a shorter time to development of disease milestones (wheelchair use, residential home, PEG requirement, unintelligible speech) nor did it predict a shorter survival.

Table 3.9 Comparison of demographic and clinical measures of multiple system atrophy cases with and without type 2 diabetes in the prospect study

Variable, median (IQR)	MSA-NDM	MSA-DM	p value
Age at baseline	64.83 (59.19-71.24)	64.34 (59.50-69.80)	0.4949
Diagnosis duration at baseline	1.11 (0.58-2.35)	2.12 (1.11-2.90)	0.5393
Gender, male/female	100/125	4/17	0.0240
UMSARS Total	44.00 (32.50-53.00)	44.00 (28.00-51.00)	0.8340

3.4 Discussion

In this study I explored peripheral insulin resistance biomarkers in parkinsonian disorders and examined the potential impact of T2DM on atypical parkinsonian disorders. Insulin resistance was prevalent in a substantial proportion of patients in these conditions though this was not significantly different to HC with the exception of PSP. I did not note a significant relationship between peripheral IR markers and clinical severity in all the disorders nor did I see an influence on clinical severity and progression from T2DM in atypical parkinsonian disorders.

Fasting insulin levels have varied between studies exploring this in PD (356, 703). I noted normal levels with corresponding higher glucose levels in PD patients compared to HC and MSA cases. This could reflect an insufficient insulin response which has been reported in PD (704). The discrepant findings noted between fasting glucose and HbA1C levels noted in my PD cohort however requires further

consideration. Possible explanations include a mismatch in the two measurements due to glycaemic excursions not captured by the glucose measurement, technical measurement variability or assay standardization and/or physiologic mechanism apart from fluctuations in plasma glucose that need to be studied further (705).

The prevalence of IR has been minimally explored in PD and less so in atypical disorders. Studies have used the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index and HbA1C levels to demonstrate that up to two-thirds of PD patients suffer from IR with one study suggesting that levels were higher in PD than HC (356, 706). A separate study using the goal standard hyperinsulinemic-euglycemic clamp for measuring IR however did not note a difference between HC and PD patients albeit in a relatively small sample size (707). I noted a relatively smaller proportion in all groups with up to a third of cases demonstrating IR with the QUICK index cutoff and an even smaller proportion with the HbA1C level cutoff. Potential differences in cohort characteristics which strongly influence IR markers such as the proportion of patients being defined as obese could partly explain the difference (356, 708). The use of the QUICK index over the HOMA-IR index also potentially contributed to my findings though the QUICK index was chosen due to demonstrated superiority for detecting IR (695-697). The smaller proportion of IR detected with HBA1C cut-offs as opposed to the QUICK index cut-off may also reflect the later tool being better at detecting IR (709) though the HbA1C cutoff used in my work was chosen for its previously demonstrated ability to predict PD progression (361-364).

Despite a documented relationship between IR and cognitive impairment in a number of neurodegenerative diseases (710, 711), no correlation with cognitive status or decline has been noted in PD studies (356, 359) and this was also the case in my work. This may in part reflect the limited representation of cases with dementia which are more strongly linked to the presence of IR (712, 713). The higher prevalence of peripheral IR noted in the PSP group where the median MoCA was within the dementia range may further strengthen this argument. In addition to this, older age in this group may additionally have influenced the higher IR prevalence noted in this group (714).

I did not note a significant association between peripheral IR markers and motor and non-motor symptoms in PD and atypical parkinsonian disorders. This is broadly in keeping with other studies though one study noted a moderate correlation between the HOMA-IR and the NMSS in PD (356, 703). CNS IR may be more relevant in this regard with higher prevalence and better correlation with motor and cognitive severity being noted in PD (194, 353, 354). Peripheral and central IR do not always co-occur despite sharing initiators such as inflammation and oxidative stress (715, 716). This may in part be related to the saturable nature of the blood brain barrier and therefore a lack of a linear relationship between blood and CNS insulin levels, as well as the CNS not being prominently involved in the classic feedback loop controlling insulin release and glucose homeostasis. Future studies exploring this relationship in parkinsonian disorders will be important for determining the best biomarker to take forward in trials.

I did not note a significant impact from T2DM on disease severity and progression in PSP and MSA. This contrasts with PD where the co-occurrence of T2DM result in more severe motor and non-motor characteristics as well as more severe motor and cognitive progression (343, 345-347). T2DM and PD share several pathological processes encompassing neuroinflammation, lysosomal dysfunction, mitochondrial dysfunction and the development of central insulin resistance that leads to neurodegeneration (349). This process is in part mediated by hyperglycemia as demonstrated by the MARK-PD study and its downstream impact on alpha synuclein aggregation (717). Similar associations have not been clearly explored in atypical parkinsonian disorders. My findings will need to be explored further in larger natural history cohorts with *post mortem* diagnostic confirmation.

Limitations of my work include small sample sizes from a single centre for all cohorts apart from PD. Similarly, sample sizes and limited follow-up in the atypical studies aiming to establish the impact of T2DM on severity and progression limit the ability to interpret the negative findings in addition to the limited proportion of cases with brain bank diagnostic confirmation. Insulin sensitivity can vary by ethnicity (718). I did not note an ethnic difference in my work though this finding was limited by a

Caucasian majority being recruited into the study. Furthermore, I cannot exclude self-selection bias of patients at greater risk for IR due to diet, weight, or other lifestyle factors being recruited into the study. Given the high prevalence of pre-diabetes or undiagnosed diabetes as well as obesity in the population (719, 720), the relevance of my findings considering the lower proportion of cases in the obese range has to be assessed against this backdrop.

Peripheral IR is similarly prevalent in parkinsonian disorders though the presence of dementia may increase this. Considering a lack of difference to healthy controls and poor correlation with clinical severity, peripheral IR markers are unlikely to be useful biomarkers in clinical trials studying drugs targeting IR though their ability to predict and track disease progression and potential relationship with central insulin resistance will need to be clarified further prior to forming a more definitive conclusion.

Chapter four

Neurofilament light as a diagnostic, phenotypic and prognostic marker for parkinsonian disorders

4.0 Summary of chapter

Neurofilament light chain (NfL) is a biomarker which reflects axonal injury. Axonal degeneration occurs in parkinsonian disorders and broadly mirrors clinical severity differences between the conditions. Current studies are however limited by different findings of usefulness in PD and studies exploring its use are limited by sample sizes in MSA. Type 2 diabetes (T2DM) can result in worse disease severity and progression when it co-occurs in PD. This does not appear to be the case in atypical parkinsonian disorders based on my analysis in chapter 3. In addition, it remains unclear if part of the mechanism by which T2DM impacts on PD is by increased axonal degeneration.

In this chapter, I explored some of these aspects in the tracking PD and PROSPECT studies and aimed to see if findings would be replicable in the exenatide PD3 trial. I performed data collection for the PROSPECT MSA study and exenatide PD3 trial. I also performed sample NfL sample analysis for a subgroup of patients and all data cleaning and statistical analysis reported in the chapter. NfL's usefulness as a diagnostic biomarker in PD was mixed and dependent on the cohorts in which it was assessed. NfL was however an excellent biomarker for diagnosing MSA. Nfl weakly reflected disease severity in PD and MSA. Baseline NfL predicted different aspects of PD progression and weakly predicted MSA progression. Levels were higher in PD patients with coexistent T2DM when compared to those without T2DM supporting clinical differences previously noted and providing support that more severe axonal degeneration is occurring in this subtype of patients. This was not the case in PSP and MSA consistent with clinical findings in chapter 3.

NfL measured in blood is a useful diagnostic, disease severity and prognostic biomarker for parkinsonian disorders. This may be less clear in PD then atypical disorders. Caveats such as age and disease stages at which measurement is performed and clinical subtypes of disorders do however need to be considered when determining when it maybe usefully applied in clinical trials. Ultimately, however it would be important to explore if NfL provides additional value to disease specific biomarkers for diagnostic distinction or clinical markers which can predict progression well.

4.1 Background:

Neurofilament light chain (NfL) is a subunit of neurofilaments which are structural proteins that confer stability to neurons and are expressed abundantly in larger myelinated axons (721). NfL is constantly released into cerebrospinal fluid (CSF) and subsequently blood, with levels increasing in response to axonal injury thus making it a potentially useful biomarker for a range of CNS diseases (721). Despite being examined extensively in PD, studies to date have been inconsistent with some suggesting either increased levels or no differences compared to healthy controls. Studies in atypical disorders have been limited by small sample sizes though they suggest raised levels in MSA, PSP and CBS compared to HC and PD (208, 434, 435, 454, 721-723). Although Nfl lacks specificity for disease pathology, its association with axonal injury and the amount of neuronal damage occurring means that it may be useful in quantifying disease severity and/or predicting progression and survival in neurodegenerative diseases including PD and atypical parkinsonian disorders (441, 448, 454, 724, 725). This predictive ability has also previously been demonstrated in the PROSPECT PSP cohort (454).

Type 2 diabetes mellitus (T2DM) is a risk factor for developing PD (343, 344) and its coexistence with PD results in more severe motor features and the development of cognitive impairment (343, 345-347). These findings have been confirmed in the tracking Parkinson's cohort (347). Insulin resistance has also been noted in MSA *post mortem* brains. Being diagnosed with T2DM in primary care has been linked to a future diagnosis of PSP. Also, tau pathology co-exists with brain insulin resistance in a range of primary tauopathies including CBD and PSP (352, 698, 699). Coexistent T2DM in MSA and PSP does not however seem to significantly impact on disease severity and progression based on my analysis of PROSPECT cohorts in chapter 3. While NfL is higher in more severe PD phenotypes such as the postural instability and gait freezing subtype, levels and therefore degrees of axonal injury have not been explored in PD with coexistent T2DM which manifests with a more severe clinical phenotype, nor has it been explored in atypical disorders with coexistent T2DM.

In this study, I aimed to explore if NfL levels could predict severity and progression of PD and MSA while also being useful in providing biochemical evidence of more severe underlying axonal injury in this more severe subtype. A priori, I expected;

1) NfL levels would be elevated in PD and MSA compared to HC, but this would vary between cohorts assessed depending on when NfL was measured over the course of the disease.

2) NfL levels would variably predict disease severity and progression depending on the characteristic being assessed.

3) NfL levels would be elevated in atypical disorders (MSA and PSP) compared to PD regardless of disease stage being assessed.

5) NfL levels would reflect worse disease severity in patients with T2DM when it does impact on the clinical severity of the relevant parkinsonian disorder.

AIMS:

To evaluate if NfL is useful at measuring disease severity and progression within diseases and their subtypes.

To determine if co-morbid T2DM influences MSA and PSP severity and progression, and to determine if Nfl Levels reflect this in PD/ MSA/ PSP and are useful in distinguishing MSA, PSP, PD from healthy subjects.

To explore these aims and hypotheses, I analysed if baseline NfL levels were elevated in PD patients in the tracking study and in MSA patients in the PROSPECT study compared to HC. This was followed by an assessment of its relationship with symptom severity and progression. I then explored if NfL levels were elevated in cases of PD, MSA and PSP with T2DM versus those who were not (n-DM) and then examined if findings for using NfL to distinguish PD from HC and atypical disorders could be replicated in a clinical trial cohort with different disease characteristics.

4.2 Methods

4.2.1 Participants

Patient sub cohorts were selected from the tracking PD and PROSPECT studies and the exenatide PD3 trial to explore the different aims outlined. Sub-cohort characteristics are summarized in Figure 4.1.

Sub cohort 1 Diagnostic and prognostic value of NfL in PD and the impact of T2DM on Nfl levels:

The ability of serum NfL to distinguish PD patients from HC was performed in a subgroup from the tracking PD study and HC recruited through the PROSPECT study. HC were selected based on availability of serum samples for testing. No specific selection criteria were applied to HC. PD patients who had completed a minimum follow-up of 2.5 years (to provide a minimum valuable follow-up threshold for prognostic modelling), with available serum samples at baseline for analysis were selected. Further selection criteria were also applied to provide an equal representation of typical PD, with a high index of diagnostic certainty (>95%), and cases with atypical clinical features with a lower index of diagnostic certainty (<80%) at their 2.5-year clinical assessment.

Sub cohort 2 Diagnostic and prognostic value of NfL in MSA:

MSA and HC cases for this study were from the cross-sectional and longitudinal arms of the PROSPECT study. All patients with available plasma and CSF for testing were included. No other selection criteria were applied.

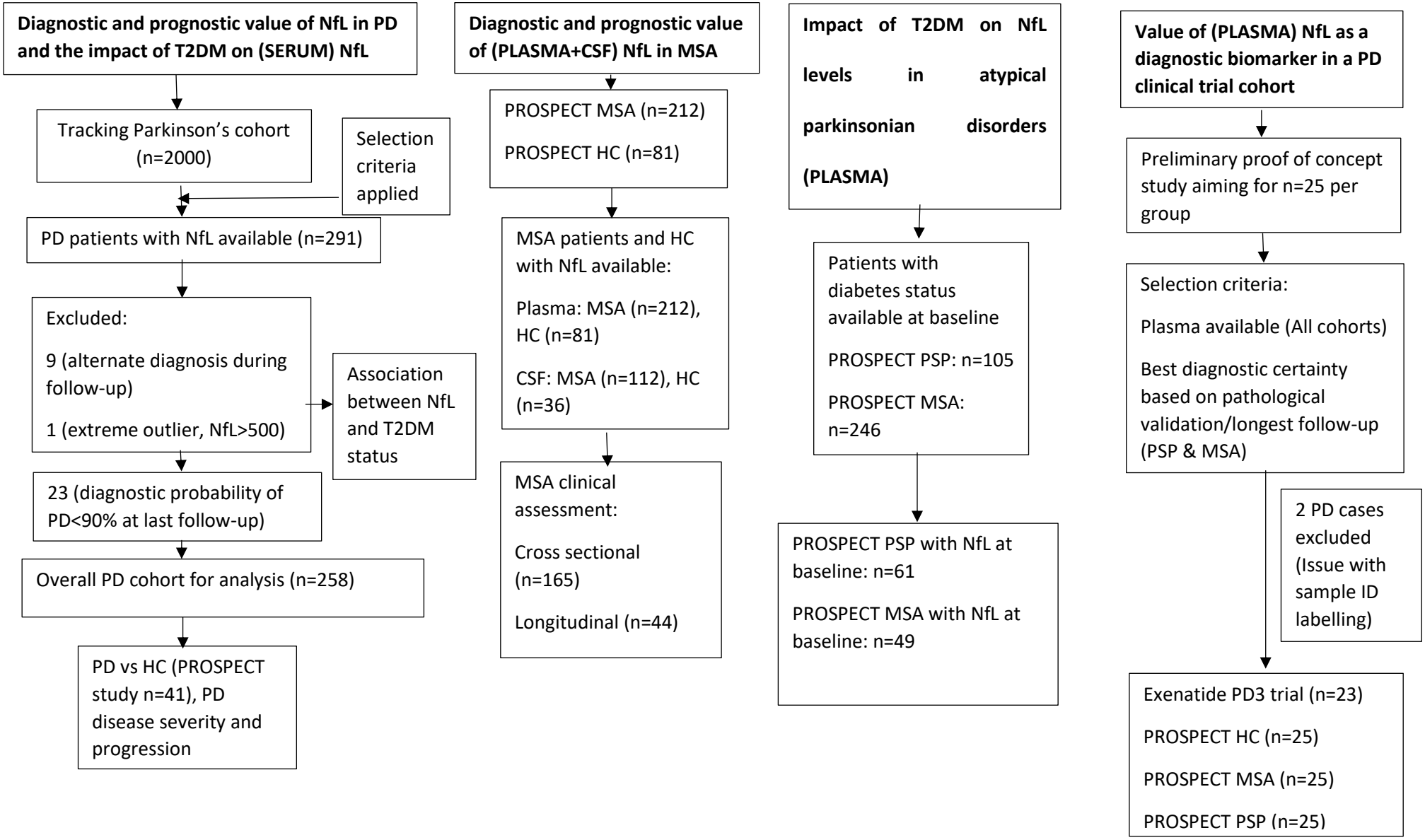
Sub cohort 3 Impact of T2DM on Nfl levels in atypical parkinsonian disorders:

Patients selected to determine if T2DM has an impact on the severity and progression of MSA and PSP were from the PROSPECT study. All patients who provided information on their T2DM status at the baseline assessment were included.

Sub cohort 4 Value of NfL as a diagnostic biomarker in a clinical trial cohort:

A comparison of NfL levels between PD and HC, MSA and PSP cases was performed on separately selected cases from the exenatide PD3 trial and PROSPECT study. A sample size of 25 was chosen as this study was designed to serve as a preliminary proof of concept study to inform if NfL findings from natural history studies would be applicable in a clinical trial cohort and therefore inform further testing on the entire trial cohort. The first 25 patients from the exenatide PD3 trial were selected. Twenty-five HC, MSA and PSP participants with available plasma samples were recruited from the PROSPECT study. MSA and PSP participants were additionally selected based on the best available diagnostic certainty either by maximal follow-up or pathological confirmation.

Figure 4.1 Summary of NfL study patient selection



4.2.2 Clinical assessments

Baseline demographics such as age, gender, disease duration and T2DM status were recorded. In this study, I included (for PD patients) selective motor (MDS-UPDRS 3 and Hoehn and Yahr stage - H&Y), and cognitive (MoCA, animal semantic fluency score - SF) measures. All patients recruited from the tracking PD study had been diagnosed within the preceding 3.5 years of study entry and a proportion underwent assessments every 18 months (although there were some interim visits at 6–12-month intervals which collected other information) with data available up to visit 10 (72 months). Clinicians determined their diagnostic certainty of PD at each visit (0-100%), while also noting clinical features they deemed to be atypical for PD. Patients who received an alternative diagnosis to PD during follow-up or who had a clinician diagnostic certainty of <90% at the last available visit were excluded from this analysis. All-cause mortality was also noted and studied as a relevant outcome.

For atypical parkinsonian disorder patients, in addition to demographics described, the UMSARS total scores were recorded in MSA cases and PSP-RS total scores were recorded for PSP cases at baseline and at follow-up visits where available. Patient reported development of wheelchair use, residential home admission, PEG recommendation and unintelligible speech was recorded as was the date at which this had developed. All-cause mortality was also noted.

4.2.3 Sample measurement

Sample collection methods are described in chapter 2. Serum (PD and PSP), plasma (MSA) and CSF (MSA+PSP) NfL concentrations were measured in duplicates using the 1-plex Single molecule array (Simoa) kit (NF-Light®, Quanterix) on a Simoa HD-1 Analyzer (Quanterix, Billerica, MA)(726) according to manufacturer's instructions. All blood and CSF samples from collaborating centers were tested in the UCL lab by the lab team blinded to clinical status, using one batch of reagents for each disease. I performed this analysis on samples used for the NfL as a diagnostic biomarker in a PD clinical trial cohort' under the supervision of the lab team. All NfL values were within the linear range of the assay.

For plasma, the mean intra-assay coefficient of variation (CV) of duplicate determinations for concentration was 4.5%. In the CSF, the mean intra-assay CV was 3.8%.

4.2.4 Statistical analysis

Given non-normally distributed data, median and interquartile ranges were described for continuous variables while frequencies and percentages were used to highlight proportions for demographic and clinical characteristics. Group differences (PD versus HC, MSA versus HC, PD versus PSP, DM status versus non-DM status) were compared using a Mann-Whitney U test for continuous data and chi-squared tests for categorical data. A Natural logarithm (Ln) transformation was performed to reduce right skewness for NfL levels as indicated by inspection of residuals.

The diagnostic accuracy of plasma NfL as a predictive marker of PD, PSP and MSA versus controls and one another was assessed via receiver operating characteristic (ROC) analysis with area under the curve (AUC) with a 95% confidence interval (CI) determined with age and gender as covariates.

Univariate and multivariable (adjusting for age, gender, and disease duration) linear regression analysis was performed to investigate the association between baseline NfL levels and clinical measures of PD and MSA overall as well as the association between NfL and diabetes status in each disease. Spearman rank test assessed correlation between the variables CSF and plasma NfL in the MSA study.

Associations between baseline serum NfL levels and change in motor and cognitive outcomes over time (disease duration from diagnosis as the time axis) were then investigated by linear mixed effects analysis, adjusted for age at diagnosis and gender in PD and MSA. The mixed models had both a random intercept and a random slope. Where NfL is significantly associated with the intercept this implies that a change in NfL would shift the progression line up and down but not alter the rate of change. Where NfL is significantly associated with the slope, a change in the biomarker would alter the rate of change but not where the progression line is at a time of zero.

Cox proportional hazards regression was then used to investigate whether the baseline NfL level predicted, postural instability, dementia, and mortality after adjustment for age, gender, and baseline MDS-UPDRS 3 in PD. In MSA cox proportional hazards regression was used to investigate whether the baseline NfL level predicted the development of wheelchair use, residential home requirement, PEG recommendation, unintelligible speech, and mortality after adjustment for age, gender, and baseline UMSARS total scores. A $p<0.05$ indicated statistical significance. All statistical analysis and figures were generated using Stata V.17.1.

4.3 Results

4.31 Group Data in the tracking PD cohort

Of the 2000 patients enrolled into the Tracking Parkinson's study, 291 were studied based on selection criteria described. The demographic (age, gender, disease duration from diagnosis) and baseline clinical characteristics (UPDRS 3, H&Y & MoCA) of this cohort was similar to the overall cohort. The purpose of this selection approach was to provide good representation of a subset of cases to model progression and to explore the possible use of baseline NfL to determine conversion to an atypical parkinsonian syndrome, in an early Parkinsonism cohort. The number of re-diagnosed cases was however low: including 3 cases of progressive supranuclear palsy (PSP), 1 case of multiple system atrophy (MSA) and 5 with other diagnosis (1 post-polio syndrome, 1 vascular parkinsonism, 1 parkinsonism with a scan without evidence of dopaminergic deficit, 1 essential tremor and 1 uncertain diagnosis). The demographic (age, gender, disease duration from diagnosis) and baseline clinical characteristics (MDS-UPDRS 3, H&Y & MOCA) of this cohort and HC from the PROSPECT study are summarised in Tables 4.1 and 4.2. Re-diagnosed cases in addition to a case which was deemed an extreme outlier (NfL 2124.12, no atypical clinical features or technical reasons to explain level) and

cases with a PD diagnostic certainty of <90% at the last available visit were excluded from further analysis. Progression analysis was performed on the remaining 258 patients (summarised in Figure 3.1). Of these cases, 252 were assessed at 18 months while 217, 128 and 60 were assessed at 36, 54 and 72 months respectively.

4.311 Evaluation of NfL as a diagnostic biomarker for PD

HC and PD patient characteristics are summarised in table 4.1. Serum NfL levels were significantly associated with increasing age but not gender in both groups. NfL was elevated in PD patients compared to controls at baseline. Logistic regression demonstrated a significant difference in NfL levels between HC and PD (Coefficient=2.67, P<0.001), adjusted for age and gender. ROC analysis indicated that serum NfL levels discriminated PD from HC with an AUC of 0.87, 95% CI: 0.82–0.94 in the tracking PD study.

Table 4.1 Comparison of healthy control (HC) and Parkinson's disease (PD) patient characteristics and NfL associations at baseline

Variable	HC (n=41)	PD (n=259)	HC vs PD (p-value)	HC Univariate, NfL vs variable indicated, Coefficient (CI)	pvalue	PD/HC Multivariate, Coefficient (CI)	P value
Age	68.00 (63.00-74.00)	68.97 (63.24-74.86)	0.482	**4.68 (2.56, 6.81)**	**<0.001**	**-0.16 (-0.23, -0.09)**	**<0.001**
Gender, male (%)	23 (56.1)	165 (63.7)	0.349	-0.00 (-0.16, 0.17)	0.960	-0.24 (-1.05, 0.09)	0.566
NfL	13.97 (11.71-19.36)	26.51 (19.77-34.99)	<0.001			**2.67 (1.87, 3.46)**	**<0.001**

Univariate coefficient values are reflective of linear regression analysis of NfL and the variable indicated in the HC group. Multivariate coefficient values are reflective of the prediction of case / control status by each variable in a multivariable model.

4.312 Evaluation of the relationship between NFL and PD clinical features

PD participant clinical features at baseline are summarised in Table 4.2. Serum NfL concentrations were associated with age (Coefficient = 5.86, p <0.001) but not gender or disease duration. Baseline MoCA and semantic fluency (SF) scores were significantly associated with serum NfL levels (MoCA Coefficient -0.60, p = 0.021; SF Coefficient -1.77, p= <0.001), indicating that serum NfL is associated with baseline markers of cognitive impairment. This remained significant for SF after adjustment for age, gender, and disease duration. NfL was not associated with measures of motor symptom severity measured by the H&Y and MDS-UPDRS 3 (Table 4.2).

Table 4.2 Evaluation of the relationship between NFL and clinical features of PD at baseline

Variables	Median (IQR) or total (%)	Univariate, Coefficient (CI)	p value	Multivariate, Coefficient (CI)	p value
Age at baseline	68.97 (63.24-74.86)	5.86 (4.85, 6.86)	<0.001		
Disease duration from diagnosis	1.12 (0.49-2.05)	0.07 (-0.05, 0.20)	0.240		
Gender, male (%)	165 (63.7)	0.05 (-0.20, 0.13)	0.692		
Motor severity outcomes					
H&Y	2.00 (1.00-2.00)	0.08 (-0.01, 0.16)	0.068	0.01 (-0.09,0.11)	0.835
MDS-UPDRS 3 Total	21.00 (15.00-29.00)	-0.73 (-2.37, 0.91)	0.382	-1.80 (-3.82,0.22)	0.080
Cognitive Outcomes					
MoCA	26.00 (23.00-28.00)	-0.60 (-0.04,0.00)	**0.021**	-0.38(-1.01,0.25)	0.236
Semantic fluency	21.00 (16.50-25.00)	-1.77 (-2.63, -0.92)	**<0.001**	**-1.10 (-2.16,0.04)**	**0.043**

4.313 Evaluation of NfL prediction of PD progression and mortality

I explored the ability of baseline NfL to predict motor, cognitive and functional progression with mixed effects linear models. A significant negative association with the intercept was noted between baseline NfL and patients overall total MDS-UPDRS 3 score (Coefficient -3.55, p=0.001). There was no association between the intercept for cognitive scores and NfL. Baseline serum NfL was associated with a more rapid motor progression (MDS-UPDRS 3: Coefficient 0.79, p=0.012; H&Y: Coefficient 0.06, p=0.001) (Table 4.3). Baseline serum NfL was not significantly associated with the changes in cognition scores (MoCA and SF).

Table 4.3 Relationship between baseline NfL level and change in motor, cognitive and functional scores using linear mixed effects models

Variable	Main effect, Coefficient – Intercept (CI), p value	Interaction with time - Slope Coefficient (CI), p value
H&Y	-0.11 (-0.23,0.01), 0.061	**0.06 (0.02,0.08), 0.001**
MDS-UPDRS 3 Total	**-3.55 (-5.68, -1.43), 0.001**	**0.79 (0.17, 1.43), 0.012**
MoCA	0.07 (-0.56, 0.69), 0.839	-0.17 (-0.34, 0.01), 0.062
Semantic Fluency	-0.61 (-1.68, 0.46), 0.263	-0.03 (-0.31, 0.24), 0.803

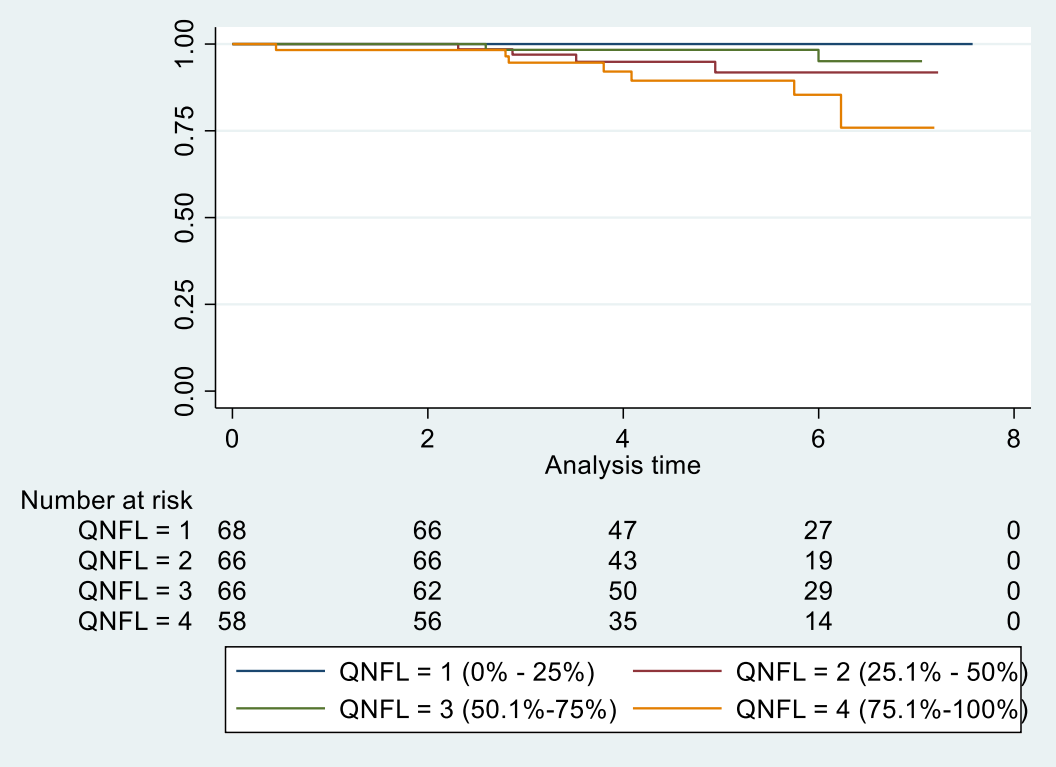
I then explored if baseline NfL could predict progression to postural instability, dementia and death using cox regression analysis (Table 4.4). Of the 258 patients studied, 93 developed postural instability over a mean follow-up interval of 3.27 years (SD 1.61). Thirty-five of the 258 patients (13.6%) developed dementia over an average interval of 3.70 years (SD 1.78) while 13 patients (5.0%) died during follow-up (mean 4.87 ±SD 1.52 years). A higher NfL concentration at baseline predicted a shorter progression to dementia, HR 2.50 (1.72-3.65), p<0.001). This remained significant following multivariate analysis 2.64 (1.58-4.41, p<0.001). Similarly, higher baseline NfL concentrations predicted a more rapid progression to postural instability, (Univariate HR 1.50, CI 1.24-1.81, p<0.001), Multivariate HR 1.32, CI 1.03-1.69, p=0.030).

Table 4.4 Relationship between baseline NfL levels and the development of dementia, postural instability and death using cox regression

Variables	Univariate HR (95% CI)	p value	Multivariate HR (95% CI)	p value
Postural instability	**1.50 (1.24-1.81)**	**<0.001**	**1.32 (1.03-1.69)**	**0.030**
Dementia	**2.50 (1.72-3.65)**	**<0.001**	**2.64 (1.58-4.41)**	**<0.001**
Death	**1.94 (1.36-2.76)**	**<0.001**	**1.89 (1.14-3.11)**	**0.013**

A higher NfL concentration at baseline predicted a shorter survival, HR 1.94 (1.36-2.76, p<0.001) (table 3.4). This remained statistically significant when corrected for age, and gender and baseline MDS-UPDRS 3 (HR 1.89, 1.14-3.11, p=0.013). The highest baseline NfL quartile conferred a two-fold higher risk of mortality in comparison to the lowest quartile (HR 2.04, 1.13- 3.69, p=0.018). (Figure 4.1)

Figure 4.2 Kaplan-Meier survival estimates by NfL quartiles in PD



4.314 Diabetes and NfL in PD

For this analysis I removed 9 cases who were re-diagnosed at follow-up, 1 extreme outlier with NfL of 2124.12 (who had co-existent T2DM), and 1 case where the patients diabetes status was not recorded. Of the 280 patients studied, 29 suffered from prevalent diabetes. PD patients with coexistent type 2 diabetes (PD-DM) were older (median 74.90 (IQR 68.57-80.18) versus 68.15 (IQR 63.18-74.40), p=0.0002), with higher BMIs (median 30.05 (IQR 27.12-34.49) vs 26.56 (IQR 24.40-29.58), p=0.0001). Serum NfL was higher in PD-DM patients (median 34.83 (IQR 27.97-49.60) versus 25.90 (IQR 19.51-34.63), p=0.0002). When adjusted for age and BMI, NfL levels were significantly associated with patients' diabetic status (0.58, 95% CI:0.24-0.91 p=0.001).

4.32 Group Data in PROSPECT MSA cohort

In total, 212 patients with MSA and 40 age-matched HC were included. Demographic and baseline clinical characteristics are presented in Table 4.5. There were no age or gender differences between MSA patients and HC, nor was there a significant difference in clinical characteristics between MSA-P (n=106) and MSA-C (n=106) cases (Table 4.5). At the time of data analysis, 147 MSA patients (69.3%) were still alive and 81 of them continued to be followed up clinically. MSA diagnosis was confirmed at autopsy in 18 cases. The median clinical follow-up of living patients after biofluid collection was 2 years (range 1-8, IQR 1-3 years), while median survival of deceased patients after biofluid collection was 2.5 years (range 2-11 years).

Table 4.5 Subject characteristic for MSA patients included in the biomarker study.

median (range, IQR)	MSA-C	MSA-P	p value	Controls
Total number	106	106		40
Gender, n female (%)	44(44%)	56 (56%)	0·09	20 (50%)
Age at onset, years	58 (51-63)	59 (53-64)	0·75	
Age at sample collection, years - median	64 (56-69)	64 (58-69)	0·69	64.5 (59-68)
Diagnostic certainty, n (%)			0·109	
Possible MSA	27 (25·5)	15 (14·2)		
Probable MSA	70 (66·0)	82 (77·3)		
Definite MSA	9 (8·5)	9 (8·5)		
Disease severity at baseline				
UMSARS total (part I and II)	45·4	46·6	0·622	
Biomarkers (number of samples per MSA-type group)				

Cross-sectional assessment (168 total number of cases), n (%)	75 (70·5)	92 (84·9)		
Longitudinal follow-up (44 total number of cases), n (%)	31 (29·5)	16 (15·1)		
Plasma and CSF matched samples (105 total number of cases), n (%)	50 (47·6)	55 (52·4)		

4.321 Evaluation of NfL as a diagnostic biomarker for MSA

Plasma samples were obtained from 212 MSA patients and CSF samples were obtained from 114 MSA patients. Paired plasma and CSF samples were obtained in 105 MSA cases and 36 HC. There was a significant correlation between NfL levels in matched plasma and CSF samples in MSA cases (n=105) (rho=0.41, p<0.001) and HC (rho=0.70 p<0.001).

Plasma NfL was positively associated with age in MSA patients (Coefficient 0.07 (CI 0.02-0.12), p=0.005). CSF NFL was not associated with age (Coefficient -0.00 (CI -0.00-0.00), p=0.640). Although CSF NfL was negatively associated with disease duration (Coefficient -0.00 (CI -0.00—0.00), p=0.003), plasma NFL was not.

NfL was higher in MSA patients compared to HC in CSF (4329 pg/mL, IQR 2577-5862 vs 560 pg/mL, IQR 420-855, p<0.001) and plasma (39.9 pg/mL, IQR 27-48 versus 9.1 pg/mL, IQR 8.7-9.8), (p<0.001). No significant differences in plasma or CSF NfL levels were noted between MSA-P and MSA-C subgroups. Both CSF and plasma NfL showed excellent AUCs (AUC=0·995; 95% CI 0.988 to 1·00 for CSF and AUC=0·966; 95% CI 0·938 to 0·944 for plasma) for distinguishing MSA from HC.

4.322 Evaluation of the relationship between NFL and clinical features

A statistically significant association between plasma NfL levels at baseline and disease severity represented by the UMSARS (Coefficient 0.23 (CI 0.13-0.33), p<0.001) was noted. This remained significant after adjustment for age, gender, and disease duration (Coefficient 0.21 (CI 0.12-0.32), p<0.001). CSF NfL was not associated with UMSARS total scores on univariate and multivariate regression analysis.

4.323 Evaluation of NfL prediction of MSA progression and mortality

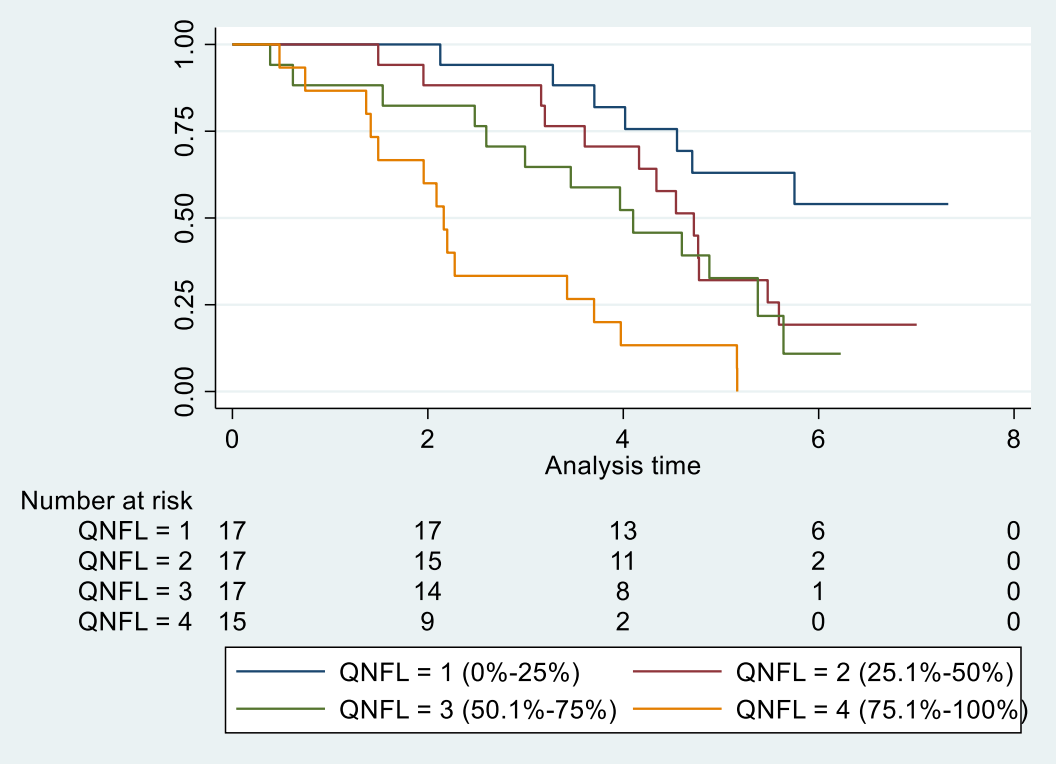
Of the 79 patients who were followed up longitudinally, 37 were assessed with an UMSARS after 6 months, 79 after 1 year, 44 after 2 years, and 3 after 3 years. Baseline plasma NfL was available in 67 of these cases and CSF NfL was available in 14 cases. Considering the small number of patients with baseline CSF NfL and longitudinal clinical assessments, I limited analysis to plasma NfL. I noted a significant association with the intercept but not the slope with baseline plasma (Intercept: Coefficient 0.33 (CI 0.03-0.63), p=0.029 Slope: 0.00 (CI-0.04-0.05), p=0.970). Baseline NfL did not significantly predict progression to disease milestones. (Table 4.6)

Higher plasma NfL levels at baseline were associated with a shorter overall survival when corrected for age, gender, and baseline UMSARS total (HR 1.02, CI 1.01-1.04, p=0.001). The highest baseline plasma NfL quartile conferred a 1.7-fold higher risk of mortality in comparison to the lowest quartile (HR 1.72, 1.35- 2.18, p<0.001). (Figure 4.3).

Table 4.6 Relationship between baseline plasma NfL levels and the development of disease milestones

Variables	HR (95% CI)	p Value
Wheelchair	0.98 (0.95-1.01)	0.412
Residential Home	3.54 (0.08-164.6)	0.518
PEG	0.93 (0.83-1.04)	0.210
Unintelligible speech	1.03 (1.00-1.07)	0.050
Death	**1.02 (1.01-1.04)**	**0.001**

Figure 4.3 Kaplan Meier survival estimates by NfL quartiles in MSA



4.33 Diabetes and NfL in atypical parkinsonian disorders

In chapter 3, I did not find the coexistence of T2DM in MSA and PSP to have any impact on the severity and progression of these disorders. Here, I explored if this was also the case for NfL levels in these subgroups and did not note any differences. (Table 4.6)

Table 4.7 Comparison of Nfl levels in atypical parkinsonian patients with and without type 2 diabetes

NfL, median (IQR)	NDM	DM	p value
PSP			
Serum NfL (DM group n=12, Non-DM group n=49)	31.31 (20.98-31.31)	34.41 (18.21-65.32)	0.7440
CSF NfL (DM group n=5, Non-DM group n=11)	3375.50 (2331.00-4327.00)	2734.00 (2399.00-2949.00)	0.5332
MSA			
Plasma Nfl (DM group n=7, Non-DM group n=42)	38.00 (25.75-52.50)	34.96 (29.47-68.45)	0.8542

CSF Nfl (DM group n=1, Non-DM group n=9)	4804.75 (3378.71-7962.74)	3058.27	0.3428

4.34 Value of NfL in a PD clinical trial cohort

I compared plasma NfL levels in 23 patients selected from the exenatide PD3 trial with HC, MSA and PSP patients recruited through the PROSPECT study. Characteristics of the cohorts are summarised in table 4.7. Although PSP patients were older than PD patients (68.65 vs 59.42, p=0.0018), no significant age difference was noted between PD patients and HC or MSA patients. PD patients also had a longer diagnostic duration compared to MSA (p=0.0040) and PSP patients (p<0.001). No gender differences were noted between groups. NfL levels were not significantly associated with age or diagnostic duration in the disease groups. Plasma NfL was however associated with age in the HC group (Coefficient 0.55 (CI 0.11,0.99), p=0.017). NfL was not significantly different in PD compared to HC (p=0.8124). NfL levels were similar in MSA and PSP patients (p=0.997). NfL levels were higher in MSA and PSP patients compared to HC and trial PD patients (p=0.001). Plasma NfL was not significantly associated with MDS-UPDRS 3 total scores assessed in the 'off' (Coefficient -8.77 (CI-26.05, 8.51), p=0.300) and 'on' (Coefficient -8.14 (CI-19.76, 3.47), p=0.300) states nor was it significantly associated with MoCA scores (Coefficient 3.01 (CI -0.17, 6.20), p=0.063) in PD trial patients when adjusted for age, gender and disease duration.

ROC analysis indicated that plasma NfL did not discriminate PD from HC well 0.69, 95%CI:0.54-0.84 in the exenatide PD3 group. NfL discriminated PD from all atypical patients with an AUC of 0.93, 95% CI: 0.84–0.98. Separately, plasma NfL distinguished PD from PSP patients with an AUC of 0.90, 95% CI: 0.78-0.98 and MSA patients with an AUC of 0.97, 95% CI: 0.85-1.00.

Table 4.8 Comparison of baseline demographics and NfL levels between PD trial patients and HC, PSP and MSA cases

Median (IQR)	Age (years)	Gender (male/female)	Diagnostic duration (years)	Plasma NfL (ng/L)
HC	63.50 (60.00-68.50)	18/6		12.90 (8.20-15.00)
PD	59.42 (54.41-67.71)	11/10	4.06 (2.02-6.46)	12.45 (9.72-14.07)
MSA	62.09 (59.51-67.38)	11/13	1.41 (0.81-3.57)	31.64 (21.00-37.92)
PSP	68.65 (64/40-73.60)	16/7	0.85 (0.50-1.70)	22.45 (15.84-40.31)

4.4 Discussion

In this study I explored the potential value of NfL as a diagnostic and prognostic biomarker in PD and MSA in two natural history studies and further clarified if it would be useful in providing biological evidence of a more severe disease subtype associated with T2DM. I then assessed if these biomarker properties were applicable in a PD clinical trial cohort. I found baseline NfL to be an acceptable marker for distinguishing PD from atypical disorders, though its ability to distinguish PD from controls varied between cohorts. The coexistence of T2DM in PD was related to higher NfL levels though this was not the case in atypical disorders. This was in keeping with clinical disease severity findings when comparing the two subgroups in the different disorders. I was also able to establish that serum NfL

could predict several aspects of PD clinical progression and mortality though my findings in MSA were less clear.

NfL levels rise with age in healthy controls (426). This presumably relates to increased axonal degeneration and decreased clearance that occurs with ageing (55, 428, 727). It is therefore widely accepted that if NfL is used as a diagnostic and/or prognostic tool then age adjusted/corrected measures will need to be used. Although I noted a significant association with age in the tracking and PROSPECT studies, I did not note this finding in the smaller replication study in disease groups despite replicating the finding in HC. Levels have not been consistently noted to rise with ageing in parkinsonian disorders (426). The neuropathological process in these conditions may cause plateau levels or mask age associations.

Serum NfL levels were elevated in PD compared to HC in the tracking PD study. This difference has not been universally noted in prior studies as was the case in my analysis of the exenatide PD3 trial where I did not note a difference (55, 435, 437, 728). Potential reasons for these variable findings in previous studies include underpowered sample sizes and variability in selection criteria for control groups (215, 724). Demographic differences to note between the studies include median diagnostic duration differences (tracking study 1.1 years versus exenatide trial 4.1 years) and age at recruitment with tracking cohort being approximately 10 years older. NfL levels rise as patients begin to appreciate motor symptoms and this is when patients typically start to seek a diagnosis (441, 729). Blood levels continue to rise gradually thereafter unlike healthy controls when adjusted for age and gender (730, 731). I did not directly compare NfL levels in the two PD cohorts as tests were performed on different sample types (serum versus plasma) and using different machines, kits, and matrix all of which could confound interpretation. On balance however, diagnostic duration does not fully explain differences noted between PD cohorts considering longitudinal studies suggest that levels in PD are consistently higher than HC once motor symptoms are manifest.

I did not find an association between NfL and baseline motor severity measures (MDS-UPDRS 3 and H&Y) though I noted a trend towards significance. The relationship between the MDS-UPDRS 3 (total and sub-scores) and NfL has varied between studies (435, 440, 441, 724). The association of H&Y status and NfL is more consistent in studies (435, 440, 441, 724) though this is more the case at H&Y stages >2.5 which reflect the occurrence of axial motor dysfunction. Reduced white matter integrity in the substantia nigra is a feature of PD at this H&Y stage as is higher NfL levels (732). The lack of significant association between H&Y scores and NfL levels at baseline in the tracking study is likely a reflection of the minimal representation of patients at this disease stage. Similarly, this may also in part explain the normal levels noted in the exenatide trial patients who only comprised of cases with a H&Y stage of ≤2. Taken together, motor disability stage/characteristics reflecting axonal damage is also an important consideration when determining the usefulness of NfL. This will particularly be the case for using NfL to track progression/measure treatment response in trials.

Baseline MoCA and semantic fluency scores were inversely associated with NfL levels. This finding is consistent with other studies exploring global cognitive function. The clearer association between semantic fluency and NfL noted is intuitive and consistent with a previous study that explored this subdomain of the MMSE (439). A deficit in this test reflects fronto-temporal dysfunction (733). Abnormalities in axonal tracts in these regions have been noted in the early stages of PD and seem to correlate with CSF NfL levels (724). This finding potentially highlights the value of more detailed neuropsychological testing, but this is of course more labour intensive than a simple blood test.

Baseline NfL levels predicted more rapid motor progression as well as the development of postural instability mirroring several other studies (440, 441, 448, 724, 734). Despite only noting a trend towards baseline NfL levels being associated with cognitive progression as determined by changes in the MOCA, I noted a significant predictive capacity for earlier development of dementia. These findings of NFL's ability to predict motor, and cognitive progression as well as death could potentially

be explained by it predicting a more malignant progression reflecting the magnitude of alpha synuclein deposition and anatomical dysfunction present (438).

Consistent with previous observations (735), the concentration of NfL in plasma correlated positively with those in CSF though this correlation was weak in the MSA cohort (rho=0.4). Several physiological confounding factors that influence the accuracy of blood NfL measurement such as lower concentrations compared to CSF, and degradation and clearance variability possibly contribute (736). An explanation specific to MSA is that axonal degeneration in the condition is predominantly a central process and NfL elevation in plasma is therefore mostly the result of spill-over through the blood–brain barrier. Impairment in the barrier is known to occur in MSA and therefore adds an element of variability to correlation (737).

CSF and plasma NfL distinguished MSA patients from HC subjects well. In addition to this, plasma levels distinguished PD patients from the exenatide trial from both MSA and PSP patients well. These findings are consistent with previous reports (441, 448, 454, 724, 725). This is partly explained by the significantly more widespread and severe axonal neurodegeneration that occurs in atypical disorders (738). I did not note a significant relationship between plasma NfL and disease duration. Plasma NfL was associated with clinical severity though this was not the case for CSF NfL. These findings mirror a separate study (739). This disconnect is possibly explained by the greater impact of falls and injuries on plasma levels as well as greater impairment of the blood-brain-barrier as the disease progresses (737, 740). I noted a positive interaction with the intercept but not the slope in progression modelling. Although I noted that NfL weakly predicted survival, it did not predict development of disease milestones. Other studies have either noted a weak association with higher plasma NfL and more rapid UMSARS progression or a non-significant relationship (456, 739, 741). The sample size of cases followed up in this study partly limits conclusions. An explanation for this weak relationship in contrast to PD however could also be that central axonal degeneration in MSA does not closely reflect the speed and magnitude of axonal loss in brain regions critical for loss of function or changes in

neurological signs which the UMSARS measures. Multidimensional assessment with quantitative imaging may help disentangle this. Ultimately however my findings do not suggest that NfL would significantly add to progression prediction over what is already established with the UMSARS.

My findings of higher levels of NfL in T2DM associated PD suggests more severe neuroaxonal damage occurs in these patients. Furthermore, the data indicate that the more severe phenotype in PD-DM noted to date by several studies is likely to be mediated by a range of factors that co-exist in these cases. T2DM and PD share several pathological processes encompassing neuroinflammation, lysosomal dysfunction, mitochondrial dysfunction and the development of central insulin resistance that leads to neurodegeneration (349). This process is in part mediated by its downstream impact on alpha synuclein aggregation (717). It is also possible that some of the observed associations are explained by diabetic neuropathy, as other peripheral neuropathies are known to increase blood NfL concentrations (742). Disentangling the mechanistic factors which contribute to this more rapidly progressive axonal damage is of critical importance in the development of disease modifying therapies for PD. Similar associations were not noted in atypical parkinsonian disorders. I did not find a convincing effect from T2DM on PSP and MSA patients in the PROSPECT cohorts I studied. This will however need to be explored further in larger natural history cohorts with better follow-up and *post mortem* diagnostic confirmation.

The strengths of this work are the large sample sizes and prolonged follow-up of the PD cohort. While I did not note significant differences between the smaller sample of the Tracking-PD study chosen and the broader study population, it is possible that the results might be confounded by unrecognised selection biases. The lack of neuropathological diagnostic confirmation in the PD cohort is also a limitation though the exclusion of patients with a diagnostic probability of <90% at the last available visit aimed to mitigate the potential inclusion of misdiagnosed patients. In the MSA cohort some of the cross-sectional and longitudinal correlations of NfL with existing outcome measures are weak, likely due to both biological and measurement variability. Furthermore, the CSF cohort was smaller

than the cohort of patients with available NfL concentrations in plasma thus limiting interpretation as to whether measurement in plasma is a sufficient alternative. Similarly, the small number of cases with available clinical follow-up makes interpretation of my findings of NFL's ability to predict progression limited. Diagnostic certainty in MSA increases with disease duration and considering only a limited number of cases have pathological confirmation some patients in early disease stages in the study may turn out to have other parkinsonian diseases or sporadic cerebellar ataxias.

Taken together, NfL measured in blood is a useful diagnostic, disease severity and prognostic biomarker for parkinsonian disorders. Caveats such as age, and disease stages at which measurement is performed such as duration from diagnosis and clinical severity characteristics do however need to be considered when determining when it maybe usefully applied in clinical trials. Ultimately, however it would be important to explore if NfL provides additional or alternative value to disease specific biomarkers for diagnostic distinction or simple clinical markers which can predict progression well.

Chapter five

Exploring analysis approaches for using the dopamine transporter striatal binding ratio in early to mid-stage Parkinson's disease modification trials

5.0 Summary of chapter

The dopamine transporter striatal binding ratio (DAT SBR) has been used as an outcome measure in Parkinson's disease (PD) modification trials. Both patient characteristics and current analysis approaches potentially complicate its interpretation. In this chapter, I explored whether DAT SBR reflects PD motor severity across striatal subregions and its relationships to disease duration and side of onset.

Associations between both mean and lateralised DAT SBR subregions (posterior and anterior putamen and caudate) and summed and lateralised motor characteristics were explored with regression analysis in the exenatide PD3 trial cohort. Analyses were repeated considering disease duration and limiting analysis to the less affected hemisphere.

Lateralized bradykinesia was most consistently associated with loss of DAT uptake in the contralateral anterior putamen. There was much higher variance in the posterior putamen and in all regions in those with longer duration disease (although bradykinesia remained robustly associated with anterior putaminal DAT uptake even in longer duration patients). Restricting analyses to the less affected side did not usefully reduce the variance compared to the overall cohort.

DAT SBR could be a useful biomarker in disease modifying trials, but a focus on striatal subregions and incorporating disease duration into analyses may improve its utility.

5.1 Background

Dopamine transporter (DaT) single-photon emission computed tomography (DaT-SPECT) assesses nigrostriatal dopaminergic denervation in PD (743). The specific binding ratio (SBR) is a quantitative index of DAT binding which can reflect symptom severity resulting from dopaminergic denervation (744-747). Striatal dopaminergic denervation typically begins in the posterior putamen in the premotor disease stage before gradually progressing in a non-linear manner to involve anterior striatal sub regions (748, 749). An analysis of the PPMI data showed a significant correlation between MDS-UPDRS part 3 scores and DAT SBR using either the mean bilateral putamen score, or the score of the putamen contralateral to the most clinically affected side at presentation. By year 4, the strength of the relationship between MDS-UPDRS part 3 score and DAT SBR is greatly diminished. Moreover, even in the early years, there are only weak correlations between the change in the MDS-UPDRS part 3 scores over time and the change in DAT SBR, considering either mean putamen, mean caudate, whole striatum or contralateral putamen. This theoretically undermines the potential of DAT SBR as an objective, sensitive measure of disease progression that might be used in trials of potential disease modifying agents.

Part of the explanation of the lack of this relationship is that MDS-UPDRS part 3 does not solely capture dopaminergic elements of PD, and this is particularly so with disease progression due to extra-striatal degeneration (e.g. the development of axial signs of PD). Indeed, there have been previous explorations of the relationships between DAT SBR and specific clinical features of PD indicating that bradykinesia is most consistently related to DAT SBR. Additionally, the relationship between DAT SBR and clinical severity may also be influenced by compensatory mechanisms (750), long duration effects of dopaminergic replacement therapies (751-755), and the extent of these confounders may vary with advancing disease.

DAT SBR values may also be limited by floor effects especially when considering the most affected striatum and/or if analyses include the posterior putamen which is often almost completely lost even

in early disease. There has therefore been interest in using the DAT SBR ipsilateral to clinical onset (i.e. the least affected side) which may be less vulnerable to floor effects. An alternative approach is to restrict analyses to striatal subregions such as the anterior putamen or caudate, as these will be less likely to have reached floor effects.

Despite these recognised difficulties with DAT SBR as a quantitative outcome measure, it remains of major potential importance in the conduct of trials exploring disease modifying agents in PD (4). While there is a surge in interest in recruiting patients at the earliest stages of PD, or even prior to the onset of motor manifestations, there will inevitably be a need to also assess the potential of candidate disease modifying interventions in the 10 million individuals who have already developed motor manifestations of PD but wish to avoid developing the falls, dementia and swallowing issues associated with advanced PD.

For these reasons, I sought to further explore how DAT SBR relates to clinical severity among patients with established PD, by performing analyses restricted to striatal subregions on both the most and least affected sides and considering the relationship between clinical sub-item severity (lateralised bradykinesia, rigidity, tremor, axial features) and these subregions, the ultimate goal being to inform whether/what should be the best approach for analysis of DAT SBR as a disease modifying interventional trial outcome measure in patients with established PD.

In this study I aimed to explore the feasibility for using the SBR as a biomarker in a clinical trial exploring disease modification in PD. A priori, I expected;

1) In established PD, mean anterior putamen/caudate DAT SBR would be more strongly associated, with MDS-UPDRS 3 score overall, than mean posterior putamen DAT SBR.

2) The mean of bilateral whole striatal DAT SBR would be more strongly associated with axial features than lateralised striatal DAT SBR, but this relationship would lessen in advancing disease.

3) Lateralised anterior putamen and caudate DAT SBR would be more strongly associated with contralateral bradykinesia and rigidity than whole lateralised striatal DAT SBR in advancing disease.

AIMS:

To evaluate whether severity of denervation on DaT-SPECT is related to motor signs in a well characterised PD trial cohort.

To compare global denervation with regional denervation taking account of lateralisation of features and duration of disease.

To explore these aims and hypotheses, I have analysed the imaging data systematically considering mean values of both hemispheres against summed MDS-UPDRS part 3 scores. I then analysed lateralised imaging scores against lateralised clinical scores, considering bradykinesia, tremor, and rigidity. I further explored whether there were differences according to the disease duration or when analysis is restricted to the least affected side.

5.2 Methods

5.21 Participants

Participants for this study were from a subgroup of recruits from the Exenatide PD3 trial who consented to undergo a DAT-SPECT scan at trial baseline, prior to exposure to any investigational medications. Detailed trial recruitment criteria are summarised in chapter 2. Briefly, patients were aged between 25 and 80, had a clinical diagnosis of PD guided by Queen Square brain bank criteria, a Hoehn and Yahr stage ≤2.5 in the ON medication state, and had used dopaminergic treatment for at least 4 weeks. Participants with a suspicion of other causes for parkinsonism, with significant cognitive impairment and/or concurrent severe depression were excluded.

5.22 Clinical assessments

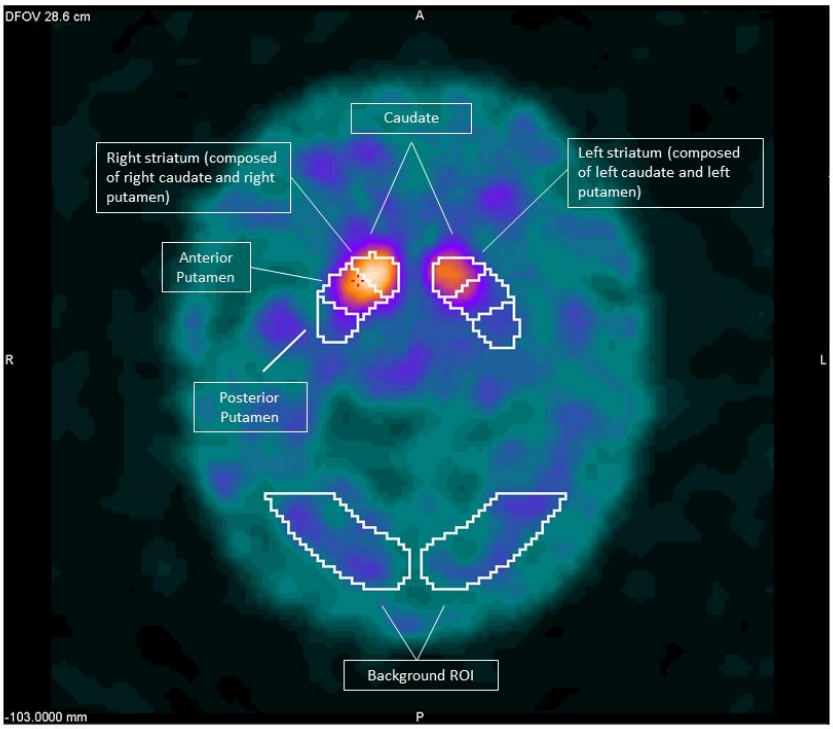
Demographic data included age, gender, and disease duration since diagnosis (DD). Motor characteristics were explored in the practically defined 'OFF' medication state using the MDS-UPDRS 3. The 'OFF' state was predefined as withholding all short acting conventional PD medications for at least 8 hours and all long-acting conventional PD medications for at least 36 hours. Overall motor status was defined by the MDS-UPDRS part 3 total score. Axial motor features were assessed using a composite Posture, Postural Instability & Gait (PIGD) score (Items 3.9+3.10+3.11+3.12+3.13). Lateralised motor features were defined using right and left hemi body scores for tremor (Items 3.15, 3.16, 3.17), rigidity (Item 3.3), and bradykinesia (Items 3.4, 3.5, 3.6, 3.7, 3.8).

5.23 DAT imaging analysis

DaT imaging was performed three hours after the intravenous injection of 185 MBq Iodine-123 Ioflupane using one of two GE Discovery 670 SPECT/CT scanners. No adjustments were made given the very similar performance of the two scanners. Data was acquired for 40 minutes and reconstructed using OSEM iterative reconstruction with two iterations and 10 subsets with an image voxel size of 3mm x 3mm x 3mm. Following tomographic reconstruction, data analysis was performed by GE Datquant software (DaTQUANT SA). This is a fully automated quantification method which has been previously well described (756). Briefly, Datquant contains an image template based in MNI space which was defined on co-registered T1 MR and DAT-SPECT from the PPMI study. Within this template striatal volumes of interest (VOI) covering the caudate and anterior and posterior putamen were defined. The division of anterior and posterior putamen was made arbitrarily during the software development. When using the software, reconstructed DAT-SPECT data from the subject was automatically spatially registered to this template with the registered data visually assessed/adjusted by the operator (JD) to ensure that the striatal contours fit the targets. (Figure 5.1) Using an occipital lobe region to represent non-specific uptake in the brain, SBR values for the right and left overall

striatum as well as anterior and posterior putamen and caudate were generated. SBR values were calculated by taking the count concentration in the region of interest and subtracting the count-concentration in the non-specific uptake volume before dividing this by the non-specific count concentration (757). A mean score for the whole striatum and each subregion was determined by averaging right and left scores for further analysis.

Figure 5.1 DaTQUANT SA VOIs superimposed on a patient's automatically registered DAT-SPECT registered image



5.24 Subgroups Explored

Disease duration: Participants were divided into disease duration subgroups of ≤4 years and > 4years for sub-analysis. No consensus on what a suitable disease duration cutoff would be currently exists for this approach. This cut-off was chosen based on a natural history study following newly diagnosed

de novo patients demonstrating weaker DAT-SPECT correlation with clinical markers at the 4-year interval scan in contrast to the first and second years (623).

Less affected side: We also explored analysis confined to the less affected side only. Cases with no DAT SBR asymmetry were excluded from this analysis.

5.25 Statistical analysis

Given non-normally distributed data, medians and interquartile ranges were reported for continuous variables while frequencies, and percentages were reported for categorical variables. A Mann-Whitney U test was used for group comparison (disease duration ≤4 years vs >4years). Chi-squared tests were used for comparing categorical data. Multivariate linear regression analysis was performed to investigate the relationship between mean and lateralized clinical scores (dependant variables) and different subregional (anterior putamen, posterior putamen and caudate) hemispheric mean and lateralized SBR (independent variables). Age and gender were included in multivariate regression analysis due to their previously reported influence on clinical and DAT SBR outcomes (758, 759). The relationship of MDS-UPDRS 3 total scores and PIGD scores were assessed against mean bilateral DAT SBR. The relationship between lateralized clinical features (tremor, bradykinesia, rigidity) was assessed against contralateral DAT SBR. Given the previous literature allowed clear hypotheses to be generated, we accepted a $p<0.05$ indicated statistical significance. All analyses were performed using Stata V.17.0.

5.3 Results

Seventy-seven patients with DAT-SPECT assessments were included. All patients had abnormal imaging consistent with a parkinsonian disorder on visual inspection. Demographics and DAT SBR values are summarised in table 5.1. No significant age or gender differences were noted comparing subgroups with a disease duration ≤4 years and > 4years. While there were significant differences in

the total MDS-UPDRS 3 off, PIGD and bradykinesia scores as well as DAT SBR in all striatal regions, there were no significant differences in rigidity, or tremor scores between subgroups according to disease duration (Table 5.1).

Table 5.1 Cohort Characteristic

	Trial Cohort with DAT (n=77)	Disease duration <4 yrs (n=36)	Disease duration >4 yrs (n=41)	P value for cohort comparison
Age	59.95 (54.41-66.47)	62.26 (56.37-67.71)	59.30 (53.54-64.46)	0.2571
Disease duration	4.12 (2.31-6.00)	2.16 (1.44-2.77)	5.64 (4.79-7.63)	
Gender (M/F)	56/21	25/11	31/10	0.3673
MDS-UPDRS 3 Off	34.0 (29.0-40.0)	31.5 (25.5-36.5)	37 (31-44)	**0.0125**
PIGD	3.0 (2.0-4.0)	2.0 (2.0-3.0)	4.0 (3.0-6.0)	**0.0021**
Tremor	3.0 (1.0-4.0)	3.0 (1.0-4.0)	3.0 (1.0-4.0)	0.6804
Rigidity	2.0 (1.0-4.0)	2.0 (1.0-3.5)	2.5 (1.0-4.0)	0.2344
Bradykinesia	6.0 (5.0-8.0)	5.5 (4.0-7.0)	7.0 (5.0-8.0)	**0.0043**
Mean SBR				
Whole Striatum	0.92 (0.74-1.13)	1.03 (0.86-1.36)	0.85 (0.68-1.36)	**0.0005**

Posterior putamen	0.39 (0.30-0.51)	0.48 (0.37-0.63)	0.33 (0.26-0.42)	**0.0001**
Anterior putamen	0.86 (0.68-1.01)	0.92 (0.82-1.27)	0.75 (0.59-0.88)	**0.0002**
Caudate	1.43 (1.12-1.71)	1.55 (1.28-1.93)	1.30 (1.07-1.62)	**0.0060**
Least Affected SBR				
Whole Striatum	1.01 (0.81-1.28)	1.17 (0.94-1.49)	0.94 (0.74-1.07)	**0.0013**
Posterior putamen	0.40 (0.26-0.51)	0.49 (0.41-0.59)	0.33 (0.23-0.42)	**0.0001**
Anterior putamen	0.92 (0.75-1.15)	1.10 (0.91-1.38)	0.83 (0.68-0.97)	**0.0001**
Caudate	1.55 (1.23-1.90)	1.75 (1.36-2.11)	1.46 (1.18-1.76)	**0.0137**

Table 5.2 summarises the association between mean bilateral whole striatum and mean bilateral subregion SBR with the MDS-UPDRS 3 total score and the PIGD sub-score. There was a significant relationship between overall striatal DAT SBR and MDS UPDRS 3. This relationship was strongest in the anterior putamen. There was a strong relationship between both whole bilateral striatum and regional DAT SBR for PIGD scores. No significant relationship was noted in the different disease duration subgroups.

Table 5.2 Relationship between non lateralised motor scores and mean bilateral DAT SBR

Coefficient (SE), p value	Whole cohort MDS-UPDRS 3 Total	MDS-UPDRS 3 Total DD≤4 years	MDS-UPDRS 3 Total DD> 4 years	Whole cohort PIGD	PIGD DD≤4 years	PIGD DD> 4 years
Overall Striatum	**-8.55 (3.51), 0.0172**	-3.17 (5.98), 0.6002	-5.61 (5.50), 0.3398	**-2.57 (0.76), 0.0013**	0.31 (0.92), 0.7362	-2.29 (1.33), 0.0947
Posterior putamen	**-12.20 (5.72), 0.0362**	-3.99 (8.51), 0.6425	-11.67 (9.83), 0.2426	**-3.07 (1.28), 0.0189**	0.40 (1.31), 0.7602	-3.45 (2.29), 0.1406
Anterior putamen	**-10.46 (3.63), 0.0051**	-5.85 (6.26), 0.3573	-7.73 (6.18), 0.2193	**-2.86 (0.79), 0.0006**	-0.01 (0.97), 0.9913	-2.66 (1.42), 0.0696
Caudate	-4.47 (2.40), 0.0660	-0.29 (3.95), 0.9427	-2.35 (3.83), 0.5427	**-1.58 (0.52), 0.0034**	0.39 (0.60), 0.5234	-1.32 (0.88), 0.1425

Table 5.3 summarises the associations between lateralized whole striatum and lateralised striatal subregion DAT SBR and different lateralised motor characteristics. Bradykinesia scores were significantly associated with DAT SBR in all subregions, whereas tremor and rigidity scores were associated with more anterior striatal structures. Bradykinesia remained strongly associated with anterior striatal DAT SBR values even with advancing disease whereas tremor and rigidity were no longer associated.

When analysis was restricted to the less affected side similar relationships were observed, with the anterior putamen being more consistently associated with bradykinesia than the posterior putamen again likely due to the high variance seen in the posterior putamen DAT SBR results. However, the smaller sample size led to fewer significant associations when only least affected sides were included, particularly in the cohort split according to disease duration.

Table 5.3 Association between specific motor scores and lateralized SBR

	Including most and least affected sides			Including least affected side only		
	Combined Trial Cohorts	Disease duration ≤4 years	Disease duration >4 years	Combined Trial Cohorts	Disease duration ≤4 years	Disease duration >4 years
	Coefficient (SE), p value					
Contralateral tremor						
Whole Striatum	**-1.41 (0.54), 0.0096**	**-2.95 (0.89), 0.0015**	-0.71 (0.85), 0.4064	-0.68 (0.62), 0.2745	-0.66 (1.14), 0.5644	0.37 (1.01), 0.7177
Post Putamen	**-1.95 (0.79), 0.0149**	**-3.11 (1.17), 0.0097**	-1.13 (1.32), 0.3958	-0.19 (0.94), 0.8381	-0.61 (1.59), 0.7031	1.79 (1.55), 0.2539

Ant Putamen	**-1.65 (0.55), 0.0032**	**-3.26 (0.89), 0.0005**	-0.96 (0.89), 0.2814	-1.08 (0.66), 0.1072	-0.61 (1.19), 0.6141	-1.16 (1.12), 0.3092
Caudate	**-0.74 (0.37), 0.0487**	**-1.58 (0.63), 0.0146**	-0.30 (0.56), 0.5879	-0.30 (0.43), 0.4871	-0.27 (0.82), 0.7458	-0.05 (0.63), 0.9334
Contralateral bradykinesia						
Whole Striatum	**-2.84 (0.63), <0.0001**	-1.32 (1.08), 0.2269	**-3.07 (0.96), 0.0020**	**-2.21 (0.80), 0.0071**	0.15 (1.44), 0.9154	-2.40 (1.24), 0.0602
Post Putamen	**-2.57 (0.97), 0.0090**	-1.54 (1.39), 0.2687	-2.10 (1.58), 0.1860	**-2.98 (1.45), 0.0437**	-0.44 (1.89), 0.8168	-0.96 (2.67), 0.7224
Ant Putamen	**-3.15 (0.64), <0.0001**	**-1.82 (1.09), 0.0989**	**-3.40 (1.00), 0.0011**	**-2.68 (0.85), 0.0024**	-0.47 (1.58), 0.7679	**-3.00 (1.33), 0.0305**
Caudate	**-1.75 (0.44), 0.0001**	-0.42 (0.75), 0.5800	**-1.95 (0.63), 0.0027**	**-1.48 (0.56), 0.0108**	1.07 (0.97), 0.2776	**-2.06 (0.80), 0.0140**
Contralateral rigidity						
Whole Striatum	**-0.91 (0.36), 0.0123**	**-1.71 (0.55), 0.0029**	-0.47 (0.60), 0.4326	-0.52 (0.46), 0.2673	-1.25 (0.82), 0.396	-0.01 (0.77), 0.9947
Post Putamen	-0.88 (0.53), 0.1003	-0.97 (0.74), 0.1958	-0.59 (0.94), 0.5334	-0.69 (0.75), 0.3630	-0.04 (1.05), 0.9660	0.11 (1.49), 0.9436
Ant Putamen	**-1.02 (0.37), 0.0063**	**-1.70 (0.56), 0.0036**	-0.74 (0.63), 0.2401	-0.56 (0.48), 0.2430	-1.00 (0.84), 0.2391	-0.16 (0.82), 0.8472
Caudate	**-0.55 (0.25), 0.0259**	**-1.21 (0.38), 0.0020**	-0.17 (0.40), 0.6685	-0.17 (0.32), 0.5829	-0.53 (0.56), 0.3489	0.24 (0.50), 0.6285

5.4 Discussion

In this study, I aimed to evaluate the relationships between the severity of dopaminergic denervation in the whole striatum and its subregions, and the severity of lateralised clinical motor features, to help

inform on how DAT SBR might be optimally analysed as an outcome measure in disease modifying trials. In this cross-sectional dataset, I found that comparing early versus more advanced disease, MDS UPDRS part 3 scores increase, and DAT SBR scores fall when considering the mean score across the whole striatum or in all striatal subregions. PIGD items have a bilateral contribution hence the strong association with mean DAT SBR, however when I restricted focus to the DAT SBR in lateralised anterior putamen and lateralised scores of clinical subitems, I found the most statistically significant relationships were between lateralised anterior putamen DAT SBR scores and the contralateral bradykinesia score. This is maintained even beyond 4 years disease duration.

My finding that tremor and rigidity scores do not change significantly between earlier disease patients and more established patients, is of interest. This is likely explicable in view of higher variability of these clinical items between patients, even in early disease. Lack of consistent progression in these items may mean that clinical progression may be somewhat diluted when considering overall MDS UPDRS part 3 scores rather than exploring specific subitems. Tremor has differentially lateralized rest, postural and kinetic aspects and the underlying dopaminergic and non-dopaminergic basis of these tremor elements vary (760, 761) and can change over the disease course (760, 762), perhaps also under the influence of long duration effects of dopaminergic replacement therapies.

Nevertheless, MDS UPDRS part 3 scores in the off-medication state do change over time, and in this cohort, a significant contribution to this appears to be the increase in the PIGD subitems in this scale. The change in PIGD subitems is associated with changes in all DAT SBR subregions but particularly the anterior putamen. While PIGD scores may be a useful clinical measurement of change related to striatal denervation in early disease, with more advanced disease, the relationship is again likely diluted due to additional contributions from extra-striatal denervation.

The most consistent relationship between lateralized DAT SBR and lateralized motor characteristics was noted in the anterior putamen even across the patient subgroups studied despite the inevitably smaller sample sizes. This may partly reflect that denervation in the posterior putamen has reached a

floor effect very early in the course of disease progression, while denervation in the caudate may be less closely related to these motor characteristics. Restricting analysis to the less affected side did not profoundly influence these findings. In addition, there are also challenges with the registration of the posterior putamen region to the template given that it has low or no signal thus further impacting on reliability of measurement in this region. Denervation in the caudate may be less closely related to the motor characteristics we explored (lower association coefficients in our study and previous studies demonstrating stronger associations with cognitive performance (763, 764). Loss of DAT SBR in the caudate tends to occur later as suggested by its significant association with bradykinesia only being noted in later disease.

DAT SBR reflects loss of functioning dopaminergic terminals in the striatum. Ratios therefore correlate best with clinical deficits that are related to the dopamine transporter system (747). My findings are in line with this and broadly mirror previous studies (747, 765-768) with contralateral bradykinesia and to a lesser extent with rigidity. If considering an intervention that is targeted to rescue degenerating dopaminergic neurons, DAT SBR may therefore be a more sensitive measure of change than routine clinical evaluations of bradykinesia. This does not however capture non-dopaminergic degeneration, and it may be less likely that any disease modifying intervention would be restricted to dopaminergic terminals alone, but DAT SBR may nevertheless potentially still be a more useful, objective, and sensitive measure of target engagement and potential efficacy even among individuals with > 4 years of disease.

Disease stage is a fundamental issue in the design of clinical trials in PD with increasing attention towards intervening early or even in premotor PD (4). Nevertheless, any early signal of success will likely require replication/confirmation among people with established motor PD which will need a sensitive measure to detect efficacy. The incorporation of DAT-SPECT SBR as a potential outcome measure in trials of disease modifying interventions will therefore likely remain of great interest (747). Correlation between DAT SBR and motor deficits becomes weaker with advancing disease duration

from diagnosis (623). This is in part related to floor effects with one pathological study suggesting a virtual absence of fibres in the dorsal striatum 4 years after diagnosis (769). These findings are broadly in line with the disease duration differences I noted, though the larger variances in the longer duration group may also be partly explained by smaller sample sizes (770). The reduced strength of association noted does not however entirely exclude the use of DAT SBR subregions in patients with later disease duration, particularly given my findings regarding contralateral bradykinesia and anterior putaminal DATSCAN uptake.

Lateralized DAT-SBR usefully predicts the severity of some motor characteristics in this cohort of PD patients. If applied as an outcome measure in a disease modifying trial, there are a number of potential methods of analysing DATSCAN data. Changes in each lateralized anterior putamen DAT SBR could be analysed according to active treatment/placebo i.e. two data points per participant in an early phase trial as an early sensitive signal, whereas in a later phase 3 trial, the same lateralised anterior putamen DAT SBRs might contribute to a composite trial endpoint that also encompasses clinical or patient reported measures. The analysis of the least affected side was designed to disentangle floor effects. While consistent with the findings using both DAT SBRs, isolating analysis to the lateralized region which is least affected, did not provide additional value in this cohort and reducing the amount of data by 50% might result in a negative impact on power for any given trial sample size calculation.

The relatively small sample size and further subgrouping according to disease duration may have impacted on my ability to detect significant associations. The cohort had very few participants with young onset PD to address the impact of heterogeneity arising from this subgroup. My overarching goal was to explore relationships by different DAT SBR analysis approaches to inform best approaches for demonstrating disease modification. The analysis used cross sectional data and relied on accurate disease duration data which might be subject to recall bias or lack of recognition of motor PD in its early stages. Furthermore while I adjusted for age and gender in association analysis, several other biological and technical factors can influence the relationship between DAT SBR and clinical severity

and have not been considered in my modelling (771). In addition, we analysed association between off state motor scores and DAT SBR in the ON state. Impact on striatal DAT levels from dopaminergic medication is however difficult to adjust for considering the varying impact of levodopa and dopamine agonists, short and long-acting agents as well as differential impacts based on disease duration (772, 773). While a disease modifying intervention may have different or overlapping effects on both motor and non-motor features of PD, future analysis might consider limiting DAT SBR measurement to the anterior putamen for demonstrating disease modifying effects of interventions in patients with clinically established PD. Whether or not DAT SBR proves to be a useful outcome measure in trials of candidate disease modifying interventions will depend on the identification of a successful treatment. Once proven, it may become a useful means of shortening the length of follow up needed to confirm/refute effect

Chapter six

Combining biomarkers for diagnosing, reflecting disease severity and predicting progression of Parkinson's disease

6.0 Summary of chapter

Patients with PD have variable disease presentations and progression. More accurate measurement of disease severity and prediction of clinical variability progression could improve clinical trial design. Although some variance can be predicted by age at onset and phenotype, this can potentially be improved by biomarkers.

The objective of this chapter was to determine if blood (NfL and QUICK1) and imaging (DAT SBR) biomarkers and genetic status (GBA and APOE) are useful in addition to clinical measures for diagnosing, predicting clinical characteristics and prognostic modelling in PD.

The study was divided in to cross-sectional and progression sections. In the cross-sectional section I evaluated the relationship between the biomarkers explored in previous chapters (QUICK1, plasma NfL and DAT SBR) and their ability to improve diagnosis and predict motor and non-motor severity in PD individually and in combination. In the longitudinal section, I also explored serum NfL and baseline clinical measures as well as patients' genetic (GBA and APOE) status in predicting motor and cognitive progression in a large clinical dataset. I classified patients as having a favourable or an unfavourable outcome based on a previously validated model and explored whether blood biomarkers could distinguish prognostic phenotypes and improve on validated predictive clinical variables.

The QUICK1 index, NfL and DAT SBR did not correlate with one another. NfL was useful at distinguishing PD from atypical disorders. The addition of the QUICK1 index did not improve this. Combining plasma NfL and DAT SBR potentially improved prediction of motor severity. Baseline NfL was associated with the progression of motor and functional impairment and with increased mortality. Baseline NfL levels predicted unfavourable progression to a similar extent as previously validated clinical predictors. The combination of clinical, NfL and genetic data produced a stronger predication of unfavourable outcomes as compared to age and gender alone.

Clinical trials of disease-modifying therapies might usefully stratify patients using clinical, genetic and NfL status at the time of recruitment.

6.1 Introduction

Parkinson's disease (PD) is a heterogenous disorder with varying clinical characteristics, severity presentations and progression (1, 774). Reasons for this are complex and likely to relate to an interplay of the degree of cell to cell spread of pathogenic proteins, downstream cellular mechanisms predominantly affected at different disease stages being assessed (e.g., inflammation, mitochondrial dysfunction, insulin resistance), underlying cellular susceptibility and compensatory mechanisms (388). This at least in part is influenced by genetic variation (261, 775, 776).

Different biomarkers are useful in assessing these separate aspects. In previous chapters I explored biomarkers which reflect the occurrence of insulin resistance, axonal injury, and dopaminergic denervation in PD. These biomarkers were differentially impacted upon by age and disease duration and variably related to motor and non-motor symptom severity. I also demonstrated that neurofilament light chain (NfL), a marker of axonal injury was able to predict unfavourable disease progression. Considering the complexity of disease pathogenesis, it is likely that utilizing several biomarkers in combination will be important for distinguishing PD from atypical parkinsonian disorders (in the absence of a well validated disease specific biomarker that could achieve this), reflecting disease severity and predicting progression. Prior to this, it will also be critical to determine the relationship of these biomarkers with one another. Previous studies have demonstrated a positive correlation between axonal injury as determined by serum and CSF NfL and dopaminergic denervation (443, 777). Preclinical studies suggest impaired glucose metabolism and the presence of insulin resistance in the brain can negatively impact on dopamine metabolism (778-780). Furthermore, there is also evidence that T2DM can result in more severe dopaminergic denervation (781) and axonal degeneration based on my findings in Chapter 4.

As genetic variation is likely to influence this, it will also need to be considered when examining this relationship. The strongest candidates in PD are the E4 allele of apolipoprotein E (*APOE*) and glucocerebrosidase (*GBA*) mutations. *APOE-E4* affects progression to cognitive decline in PD (261, 267) as do *GBA* mutations although the risk of development of dementia in *GBA* mutation carriers varies based on the type of mutation while their impact on motor progression (261, 782) and survival (783, 784) is less clear.

Unbalanced randomisation in clinical trials can have a significant effect on the power of the study to detect the impact of an intervention (785). Using biomarkers that can better distinguish PD from atypical disorders, reflect disease severity and predict prognosis is likely to be key in improving this and these concepts are likely to be best achieved with a combination of markers. In this chapter, I explore this in biomarkers reflecting different disease aspects (peripheral insulin resistance (QUICK1), dopaminergic denervation (DAT-SBR), axonal injury (NfL)) and prognostic markers (clinical and genetic status) in the 2 previously described PD cohorts. A priori - I expected;

1) Markers of peripheral IR, dopaminergic denervation, and axonal injury may correlate with one another.

2) The addition of peripheral IR markers to NfL would not improve diagnostic distinction of PD from HC and atypical parkinsonian disorders considering a lack of difference previously noted in addition to NfL's excellent ability to distinguish the disorders.

3) Combining NfL, DAT-SBR and peripheral IR markers may improve disease severity prediction modelling.

4) Genetic status would predict subsequent motor and cognitive progression and survival like NfL considering previous study findings.

5) NfL levels would not vary with genetic status if clinical severity was not altered by the same genetic status.

6) Combining NfL levels with clinical predictors of progression and genetic status may improve progression modelling

Aims:

To evaluate if PD biomarkers which reflect different disease aspects correlate with one another.

To determine if combining biomarkers can improve the diagnosis of PD, better reflect disease severity, and predict progression.

6.2 Methods

6.2.1 Participants:

Details of clinical cohorts where participants were recruited from are outlined in chapter 2. I divided this chapter into a ***cross-sectional study section*** where I explored the relationship between biomarkers, and their combination in predicting disease severity and disease diagnosis and a ***progression study section*** where I explored the value of combining biomarkers to predict worse progression. Data utilized for the ***cross-sectional study*** was derived from cases recruited into the exenatide PD3 trial, exenatide MSA trial and the prospect study that were explored in Chapters 3, 4 and 5. Data utilized for the ***progression study*** was derived from cases recruited into tracking PD study as this study had high quality longitudinal data and patients' genetic status was available.

The characteristics of patients who had peripheral insulin resistance tested and its association with clinical severity in PD as well as its ability to distinguish PD from HC and atypical parkinsonian disorders are summarised in Chapter 3. The characteristics of patients who had NfL tested and its association with clinical severity and progression in PD as well as its ability to distinguish PD from HC and atypical parkinsonian disorders are summarised in Chapter 4. The characteristics of patients who underwent

DAT-SPECT imaging and the association of DAT-SBR with clinical severity are summarised in Chapter 5. Considering my findings in the previous chapter I have limited analysis to the QUICK1 index and anterior putamen SBR. As the biomarkers being considered are 'not lateralised', I selected hemispheric averaged anterior putamen SBR taking the impact of disease duration into account.

6.2.2 Clinical Assessments:

Patient age, gender and disease duration were recorded for PD, MSA and PSP patients for this study. Age, and gender were noted for HC. In the ***cross-sectional study*** motor symptom severity was defined using the MDS-UPDRS part 3 OFF state scores and non-motor symptom severity was defined using the total score NMSS. In the ***progression study,*** motor symptoms with the MDS-UPDRS part 3 and H&Y scale and cognitive symptoms were assessed with the MoCA scale and by assessing semantic fluency.

6.2.3 Favourable vs. Unfavourable prognosis subgroups:

Patients were classified as having Favourable or Unfavourable outcomes based on a previously validated model of progression (786). A binary outcome measure was created for unfavourable progression PD (U-PD) when patients had postural instability (defined by a H&Y scale score of 3 or higher) or dementia (defined by adapted Movement disorders society criteria for PD dementia (MOCA<21 and impairment in at least two domains, cognitive deficits impacting on daily living -MDS UPDRS 1.1≥2 and no severe depression- MDS UPDRS 1.3<4) (267) at the last available assessment, or if they had died during follow-up. Although the premise for grouping was identical to the previously validated model, our definition of dementia varied (level 1 criteria from the Movement Disorder Society Task Force and operationalized using MMSE and either clock drawing or phonemic fluency tests was used in the model development study) (786). All other patients were classified as having favourable progression PD (F-PD). Patients already demonstrating U-PD characteristics at baseline were excluded from the progression to U-PD analyses but were retained in the baseline analysis and the mixed effects regression analysis. The three baseline variables (age at baseline, MDS-UPDRS axial

score, and animal SF) that were previously identified to predict the development of U-PD (786) were then explored individually and in combination with NfL and patients genetic status to compare clinical, genetic and biomarker data in predicting progression.

6.2.4 Sample Collection and measurement:

Sample collection approaches are outlined in chapter 2. QUICK1, NfL and DAT-SBR measurement techniques are summarised in Chapters 3, 4 and 5 respectively.

6.2.5 Genetic status classification:

Molecular genetic analysis techniques for determining patients *APOE* and *GBA* status are outlined in previous publications (261, 787). As *APOE* ε4 status is known to be a determinant of cognitive progression, thus patients were classified into groups of either being ε4 carriers (homozygous and heterozygous) and non-carriers (261). Mutations identified and classification approaches for determining *GBA* prognostic status in the Tracking Parkinson's study have previously been detailed (787). Patients in this study were classified into groups where a *GBA* variant was identified as either being pathogenic in Gaucher disease (GD) and associated with PD in the heterozygous state (GD causing) (L444P (5 cases), p.R463C (1 case), p.R395C (1 case), p.G377S (1 case), p.N370S (1 case) and p.D409H/L444P/A456P/V460V (1case)), or non-synonymous genetic variants that are associated with PD (non-GD causing) (E326K (10 cases), T369M (7 cases), and p.D140H/p.E326K (1 case). Two cases with variants of unknown significance were excluded from the group analysis (p.M123T, p.R262H).

6.2.6 Statistical Analysis:

Descriptive statistics including mean, SD, frequencies, and percentages were used to describe demographic and clinical characteristics by groups. Differences were compared using Kruskal-Wallis

tests for continuous data and chi^2 tests for categorical data. A natural logarithm (Ln) transformation was performed to reduce right skewness for NfL levels as indicated by inspection of residuals.

In the ***cross-sectional study*** the correlation between QUICK1, DAT-SBR, and NfL was examined using spearman correlation. The interaction between biomarkers and MDS-UPDRS3 'off' and NMSS total scores was explored with multivariate linear regression with MDS-UPDRS3 'off' and NMSS total scores as the dependent variable and the individual and combination of biomarkers as the independent variable(s). Considering the nested nature of models studied, the adjusted R-squared metric was examined to determine the appropriateness of each model.

In the ***longitudinal study***, the interaction between *GBA* and *APOE* status with NfL was explored with univariate and multivariate linear regression with NfL as the outcome measure and the respective positive gene status being compared with those who were negative. Associations between genetic status and change in motor, cognitive and quality of life outcomes over time (disease duration from diagnosis as the time axis) were then investigated by linear mixed effects analysis, adjusted for age at diagnosis and gender. The mixed models had both a random intercept and a random slope. Cox proportional hazards regression was then used to investigate whether genetic status individually and when combined with baseline NfL levels predicted, postural instability, dementia, and mortality after adjustment for age, gender, and baseline MDS-UPDRS 3. Logistic regression was repeated using previously validated baseline predictive clinical variables (MDS-UPDRS axial score and SF) individually and in combination with NfL levels, and the patients' *GBA* and *APOE* status to explore the ability to distinguish predetermined outcome groups (U-PD vs F-PD). The area under the curve (AUC) for each combination of variables was statistically compared against age and gender alone, and then together with different biomarker combinations using Delong's test. All statistical analysis and figures were generated using Stata version 17.0.

6.3 Results

6.3.1 Cross sectional study

6.3.1.1 Correlation between biomarkers

The relationship between biomarkers using spearman correlation. Correlation between QUICK1 and DAT-SBR was performed in 73 cases while correlation between NfL and DAT-SBR was performed in 17 cases and QUICK1 and NfL in 23 cases. No significant correlation was noted between the three different biomarkers. (Table 6.1)

Table 6.1 Correlation between respective biomarkers

Spearman rho (p value)	QUICK1	NfL
DAT-SBR	0.06 (0.6218)	0.26 (0.3045)
NfL	0.22 (0.3236)	

6.3.1.2 Combining biomarkers to distinguish PD from HC and atypical parkinsonian disorders

Table 6.2 summarises AUC comparisons of the ability of different biomarkers and combinations of them for distinguishing HC and different disease groups. NfL distinguished PD from atypical disorders well but this was not the case for PD versus HC in line with my findings in Chapter 4. The QUICK1 index was a poor biomarker for distinguishing PD from HC and atypical disorders. Adding the QUICK1 index to NfL did not provide additional benefit distinguishing the groups. I was not able to establish this in the PSP group as NfL and QUICK1 was not tested in the same cases.

Table 6.2 AUC comparisons of different biomarkers and combinations for distinguishing PD from other groups

AUC (CI)	PD vs HC	PD vs MSA	PD vs PSP
1. Age+gender alone	0.65 (0.51-0.79)	0.65 (0.51-0.79)	0.60 (0.45-0.74)
2. +NfL	0.65 (0.51-0.79)	0.99 (0.98-1.00)	0.98 (0.96-1.00)
3. +QUICK1	0.63 (0.48-0.78)	0.65 (0.50-0.79)	0.63 (0.49-0.77)
4. +NfL+QUICK1	0.63 (0.50-0.77)	1.00 (1.00-1.00)	N/A
AUC comparison Chi (p-value)			
4 vs 1	0.49 (0.4843)	24.18 (<0.001)	N/A
4 vs 2	0.18 (0.6716)	0.85 (0.3568)	N/A
4 vs 3	0.00 (0.9711)	24.07 (<0.001)	N/A

* Items 2, 3, 4 include age and gender in modelling

6.3.1.3 Combining biomarkers for predicting disease severity

I explored the performance of models for predicting motor and non-motor symptom severity with the different biomarkers being treated as predictors in addition to age and gender. (Table 6.3) I noted that the addition of NfL to age and gender resulted in the regression model performing more poorly in predicting motor severity. The addition of the QUICK1 index only resulted in a small improvement in predicting motor severity. The addition of DAT SBR improved motor and non-motor severity prediction in addition to age, gender, and disease duration. Adding NfL to DAT SBR substantially improves motor severity prediction however combining the QUICK1 index to this resulted in a small improvement to the model. Adding NfL to the model improved NMSS prediction. Combining all 3 biomarkers best predicted NMSS severity.

Table 6.3 Summary of performance of models predicting motor and non-motor symptom severity with different variables included

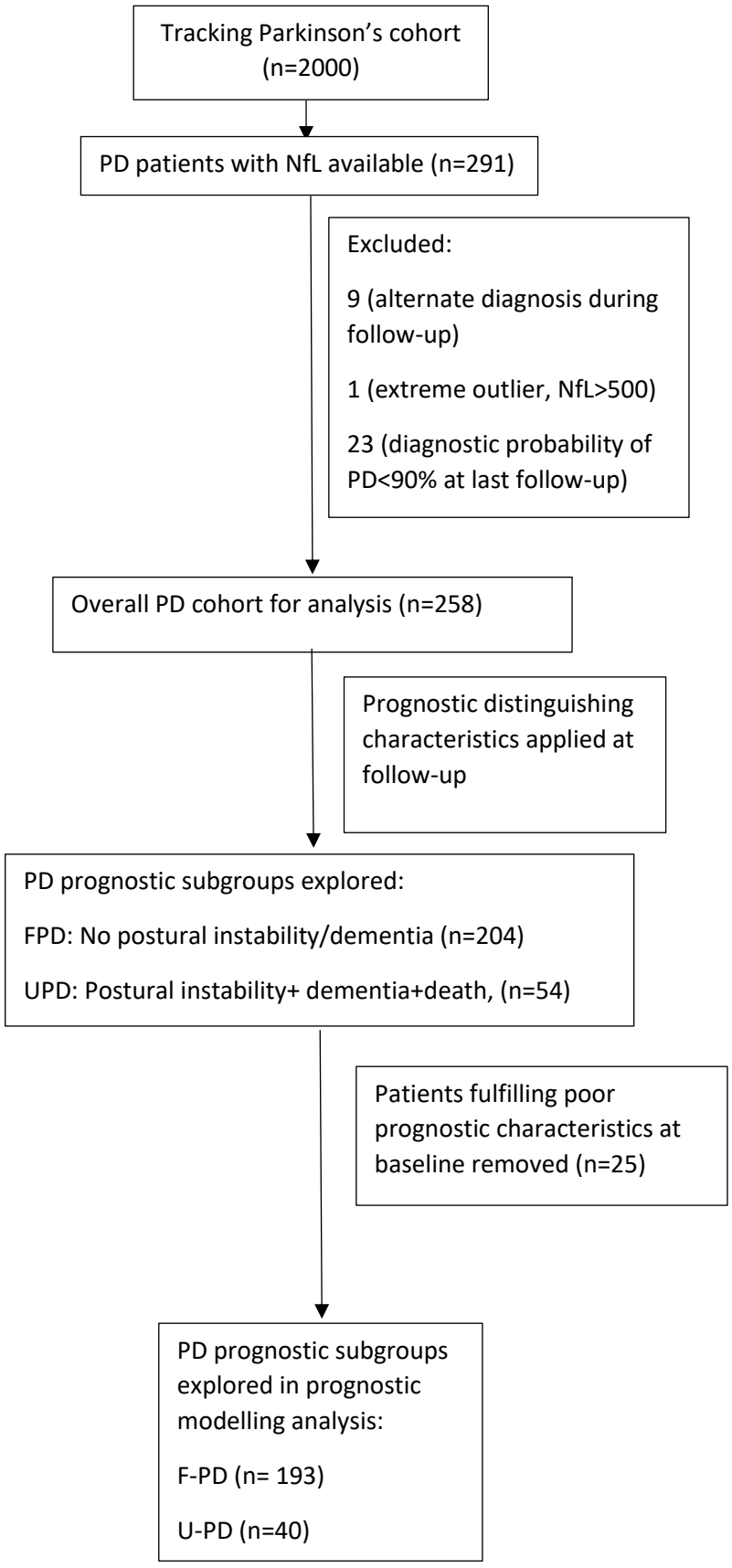
Adjusted R-squared	MDS-UPDRS 3 'off'	NMSS
1. Age+gender	0.0523	0.0726
2. Age+gender+ disease duration	0.1408	0.0372
3. NfL (n=23)	-0.1297	0.1665
4. QUICK1 (n=187)	0.0703	0.0345
5.DAT SBR (n=77)	0.1888	0.0828
6. NfL+QUICK1 (n=17)	-0.1925	0.1611
7. NfL+ DAT SBR (n=17)	0.8112	0.2329
8. QUICK1+ DAT SBR (n=77)	0.1961	0.0700
9. NfL+ QUICK1+ DAT SBR (n=17)	0.8511	0.3169

* Items 3, 4, 6 include age and gender in modelling, Items 5, 7, 8, 9 include age, gender, and disease duration

6.3.2 Progression study

Of the 2000 patients enrolled into the Tracking Parkinson's study, 258 were included in the analysis based on previously outlined selection criteria (chapter 4) and summarised in Figure 6.1. Of these cases, 252 were assessed at 18 months while 217, 128 and 60 were assessed at 36, 54 and 72 months, respectively.

Figure 6.1 Summary of study design



6.3.2.1 Relationship between genetic status, serum NfL levels and PD clinical variables at baseline

The demographic (age, gender, disease duration from diagnosis) and baseline clinical characteristics (MDS-UPDRS 3, H&Y and MoCA) of the cohort based on genetics status is outlined in table 6.4. No differences in serum NfL levels were noted between patients who were positive for either GBA genes or APOE ε4 status compared to those who were not. No significant association between NfL levels and predefined GBA or APOE status was noted when compared to those who were negative for mutations. (6.5)

Table 6.4 Comparison of baseline characteristics of patients with and without genetic abnormalities

Mean (SD)	*GBA* negative PD (n=213)	Non-GD variant PD (n=17)	GD variant PD (n=10)	Non ε4 allele PD (n=165)	Heterozygous ε4 PD (n=63)	Homozygous ε4 PD (n=8)
Age	68.6 (8.8)	67.6 (8.3)	62.0 (11.2)	69.7 (8.7)	65.4 (8.2) **	67.5 (4.9)
Gender, male (%)	132 (62.0)	15 (88.2)	6 (60.0)	112 (67.9)	36 (57.1)	5 (62.5)
Disease duration	1.3 (0.9)	1.1 (0.7)	1.1 (1.1)	1.3 (0.9)	1.3 (0.9)	1.0 (0.7)
H&Y	1.8 (0.6)	1.8 (0.6)	1.7 (0.7)	1.8 (0.6)	1.7 (0.6)	1.4 (0.6)
MDS-UPDRS 3	23.2 (11.5)	20.1 (12.6)	19.7 (16.8)	23.0 (11.8)	23.1 (10.5)	15.9 (10.4)
MoCA	25.1 (3.6)	25.7 (2.3)	24.9 (2.8)	25.2 (3.3)	24.9 (3.4)	24.0 (2.9)

SF	20.9 (6.3)	22.8 (6.6)	26.2* (4.2)	21.4 (6.3)	21.2 (6.4)	18.5 (10.4)
NfL	30.5 (17.3)	32.2 (17.4)	25.8 (17.0)	30.6 (17.2)	27.4 (13.2)	36.7 (22.9)

* GD variant PD vs *GBA* negative PD p<0.05, ** Heterozygous ε4 PD vs Non ε4 allele PD p<0.01

Table 6.5 Univariate and multi variate analysis of relationship between NFL and genetic status

Genetic status	Total (%)	Univariate, Coefficient (CI)	p value	Multivariate, Coefficient (CI)	p value
GBA positive (non-GD variant)	18/240 (7.5)	0.14 (-0.37, 0.65)	0.590	0.30 (-0.12, 0.72)	0.155
GBA positive (GD variant)	10/240 (4.2)	-0.45 (-0.37, 0.65)	0.590	0.02 (-0.51, 0.55)	0.945
ApoE ε4 heterozygous	63/236 (26.7)	-0.19 (-0.48, 0.09)	0.186	0.09 (-0.14, 0.33)	0.433
ApoE ε4 homozygous	8/236 (3.4)	0.34 (-0.37, 1.04)	0.350	0.52 (-0.04,1.08)	0.07

Univariate and multivariable (age at baseline, gender, and disease duration) linear regression analysis on baseline NfL and genetic status, NfL was treated as the outcome measure and patients who were positive for a genetic abnormality were compared to those who were not.

6.3.2.2 Evaluation of biomarker prediction of PD progression and mortality

I explored the ability of baseline genetic status to predict motor, cognitive and functional progression with mixed effects linear models. Baseline *GBA* status did not predict progression of any of the measures while *APOE* status predicted a more rapid cognitive decline (MoCA Coefficient -0.43, $p<0.001$) (Table 6.6). Previously noted NfL findings (Chapter 4) are also included in table 6.6.

Table 6.6 Relationship between baseline NfL level, *GBA* and *APOE* status and change in motor and cognitive scores using linear mixed effects models

Variable	**Main effect, Coefficient – Intercept (CI), p value**			**Interaction with time - Slope Coefficient (CI), p value**		
	NfL	*GBA*	*APOE*	NfL	*GBA*	*APOE*
H&Y	-0.11 (-0.23,0.01), 0.061	0.02 (-0.18,0.21, 0.880	-0.12 (-0.28,0.04), 0.151	0.06 (0.02,0.08), **0.001**	0.00 (-0.06,0.06), 0.967	0.02 (-0.02,0.07), 0.335
MDS-UPDRS 3 Total	-3.55 (-5.68, -1.43), **0.001**	-0.25 (-1.03, 0.92), 0.218	-2.48 (-5.46, 0.51), 0.104	0.79 (0.17, 1.43), **0.012**	-0.05 (-1.03, 0.92), 0.912	0.69 (-0.17, 1.56), 0.116
MoCA	0.07 (-0.56, 0.69), 0.839	-0.14 (-1.11, 0.83), 0.775	-0.47 (-1.27, 0.34), 0.258	-0.17 (-0.34, 0.01), 0.062	0.14 (-1.32, 0.41), 0.312	-0.43 (-0.66, -0.19), <**0.001**
Semantic Fluency	-0.61 (-1.68, 0.46), 0.263	**2.26 (0.54, 3.98), 0.010**	-0.90 (-2.41, 0.61), 0.243	-0.03 (-0.31, 0.24), 0.803	-0.37 (-0.81, 0.07), 0.100	-0.34 (-0.71, 0.04), 0.077

I then explored if baseline genetic status could predict progression to postural instability, dementia and death using cox regression analysis. (Table 6.7) Of the 258 patients studied, 93 developed postural instability over a mean follow-up interval of 3.27 years (SD 1.61). Thirty-five of the 258 patients (13.6%) developed dementia over an average interval of 3.70 years (SD 1.78) while 13 patients (5.0%) died during follow-up (mean 4.87 ±SD 1.52 years).

Patients' *GBA* status did not predict progression to dementia though their *APOE* ε4 status did (Univariate HR2.08, CI 1.16-3.73, p=0.014, Multivariate HR 3.12, CI 1.63-6.00, p=0.001). *GBA* and *APOE* ε4 status did not predict progression to postural instability. Although *GBA* status predicted survival when corrected for baseline age, gender and MDS-UPDRS 3 (HR 2.66, 1.04-6.79, p=0.041), *APOE* ε4 status did not. (Table 6.7) Previously noted NfL findings (Chapter 4) are also included in table 6.7.

In modelling combining all biomarkers with baseline age, gender and MDS-UPDRS 3, only *APOE* ε4 status (HR 2.75, 1.44-5.24, p=0.002) and Nfl (HR 2.09, 1.16-3.76, p=0.014) continued to significantly predict progression to dementia. Only NfL levels predicted progression to postural instability (HR 1.44, 1.04-2.01, p=0.029) in the model with all variables combined. NfL levels predicted survival (HR 2.18, 1.17-4.05, p=0.014). A trend towards *GBA* status predicting survival (HR 2.33, 0.92-5.95, p=0.076) was noted.

Table 6.7 Relationship between baseline NfL levels, *GBA* and *APOE* status alone and the development of dementia, postural instability and death using cox regression

		HR (95% CI)			
Variables	**Baseline Status**	**Univariate**	**p Value**	**Multivariate**	**p Value**
Postural instability	NfL	1.50 (1.24-1.81)	**<0.001**	1.32 (1.03-1.69)	**0.030**
	GBA	0.76 (-.42-1.39)	0.378	1.03 (0.54-1.98)	0.927
	APOE	0.83 (0.52-1.33)	0.443	0.98 (0.61-1.57)	0.920
Dementia	NfL	2.50 (1.72-3.65)	**<0.001**	2.64 (1.58-4.41)	**<0.001**
	GBA	0.54 (0.16-1.88)	0.337	0.60 (0.15-2.38)	0.471
	APOE	2.08 (1.16-3.73)	**0.014**	3.12 (1.63-6.00)	**0.001**
Death	NfL	1.94 (1.36-2.76)	**<0.001**	1.89 (1.14-3.11)	**0.013**
	GBA	1.66 (0.71-3.86)	0.241	2.66 (1.04-6.79)	**0.041**
	APOE	0.43 (0.10-1.79)	0.246	0.79 (0.19-3.25)	0.744

Univariate and multivariable (age at baseline, gender and MDS-UPDRS 3 score at baseline) cox regression analysis on baseline NfL, *GBA* and *APOE* status with progression to dementia and postural instability at the last available visit and death treated as outcome measures. The *GBA* group includes GD causing & non-GD causing mutation carriers; the *APOE* group includes *APOE* ε4 heterozygous and homozygous carriers.

6.3.2.3 Evaluation of biomarker use in progression modelling

I applied distinction criteria (summarised in Figure 1) for determining a poor prognosis at the last available follow-up to separate patients into 2 groups (U-PD & F-PD). PD patients with an unfavourable prognosis (U-PD) had higher serum NfL levels at baseline than those with a favourable prognosis, F-PD (41.9 (SD 21.7) versus 29.6 (SD 36.6), $p<0.001$). Baseline NfL levels were able to distinguish these phenotypes with an AUC of 0.79, 95% CI 0.72–0.85. (Figure 6.2A)

Baseline variables (MDS-UPDRS axial score, SF and NfL) explored in logistic regression individually and in combination with age at the baseline assessment and gender as covariates are summarised in supplementary table 6.8.

Table 6.8 Regression coefficients of the final combination models explored

		Coefficient	Standard error	p value
Model 1				
	Intercept	-5.40	2.03	
	Age	0.06	0.03	0.054
	Gender	-0.90	0.45	0.045
	NfL	0.81	0.23	0.001

Model 2				
	Intercept	-6.65	2.12	
	Patient age	0.09	0.03	0.001
	Gender	-0.63	0.45	0.164
	UPDRS axial	0.33	0.09	<0.001
	Semantic fluency	-0.10	-0.04	-0.007
Model 3				
	Intercept	-3.95	2.27	
	Age	0.04	0.03	0.194
	Gender	-0.89	0.49	0.066
	UPDRS axial	0.37	0.10	<0.001
	Semantic fluency	-0.08	0.04	0.046
	NfL concentration	0.82	0.26	0.001
Model 4				
	Intercept	-6.20	2.71	
	Age	0.07	0.04	0.044

	Gender	-0.85	0.54	0.114
	UPDRS Axial	0.34	0.11	0.001
	Sematic Fluency	-0.09	0.04	0.041
	NfL concentration	0.64	0.27	0.020
	ApoE status	0.68	0.38	0.071
	GBA status	0.45	0.53	0.403

The AUC for models incorporating variables individually were SF (0.78, 95% CI 0.71-0.85), MDS-UPDRS axial (0.79, 95% CI 0.71-0.86) and combined genetic status (0.76, 95& CI 0.68-0.84). An AUC of 0.82 (95% CI 0.74-0.88) was noted in the model combining SF and MDS-UPDRS axial scores. The AUC for this model did not significantly differ from the model with NfL alone (0.79 vs 0.82, p=0.3073) or combined genetic markers (0.76 vs 0.82, p=0.1098). (Figure 6.2A) The addition of NfL to clinical markers did not result in a significant improvement in comparison to clinical markers alone (AUC 0.82 vs 0.85, p=0.1691). The combination of NfL with both clinical markers did however result in a higher AUC for distinguishing PD progression phenotypes in comparison to NFL alone (0.79 versus 0.85, p=0.0163). (Table 6.9) The addition of patient's combined genetic status and baseline NfL levels to clinical variables in the model resulted in an AUC of 0.84. (Figure 6.2B) This combination resulted in a significantly higher AUC for distinguishing progression phenotypes in comparison to age and gender (0.74 vs 0.84, p=0.0121). (Table 6.10) The model combining all markers (MDS-UPDRS axial, SF, NfL, *APOE* and *GBA* status resulted in a similar AUC to the model incorporating both clinical variables and NfL with either genetic status (AUC 0.84 versus 0.85). (Table 6.9)

Table 6.9 Summary of ROC analysis for models using different baseline predictive variables and comparison of models

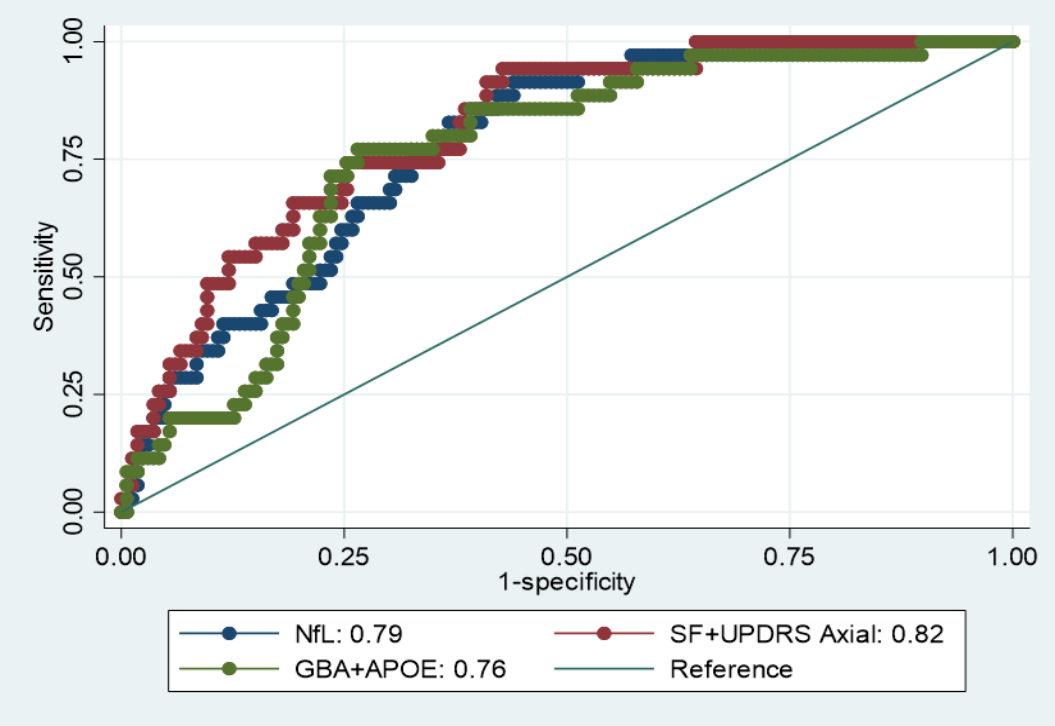
	AUC	CI
Age+gender	0.74	0.67-0.82
1. NfL	0.79	0.72-0.85
2. UPDRS Axial	0.79	0.71-0.86
3. SF	0.78	0.71-0.85
4. *GBA* status	0.75	0.68-0.83
5. *APOE* status	0.75	0.67-0.83
6. UPDRS Axial/NfL	0.82	0.76-0.89
7. SF/NFL	0.81	0.75-0.88
8.*GBA*/NFL	0.79	0.72-0.86
9.*ApoE*/NfL	0.80	0.73-0.86
10. *ApoE/GBA*	0.76	0.68-0.84
11. SF/UPDRS Axial	0.82	0.74-0.88
12. SF/UPDRS Axial/NfL	0.85	0.79-0.91
13. SF/UPDRS Axial/*GBA*	0.83	0.76-0.89
14. SF/UPDRS Axial/*ApoE*	0.82	0.75-0.89
15. SF/UPDRS Axial/NfL/*GBA*	0.85	0.78-0.91

16. SF/UPDRS Axial/NfL/*ApoE*	0.85	0.78-0.91
17. SF/UPDRS Axial/NfL/*ApoE/GBA*	0.84	0.78-0.91
AUC comparison	Chi	p-value
1 vs 11	1.04	0.3073
1 vs 12	5.77	0.0163*
1 vs 15	4.68	0.0305*
1 vs 16	5.49	0.0192*
1 vs 17	3.98	0.0461*
10 vs 11	2.56	0.1098
11 vs 15	1.83	0.1761
11 vs 16	2.53	0.1118
11 vs 17	2.07	0.1505

All models incorporate age and gender as covariates.

Figure 6.2 Receiver operator characteristic curves of (A) individual biomarker components and (B) all biomarker components combined for predicting unfavourable progression

(A)



(B)

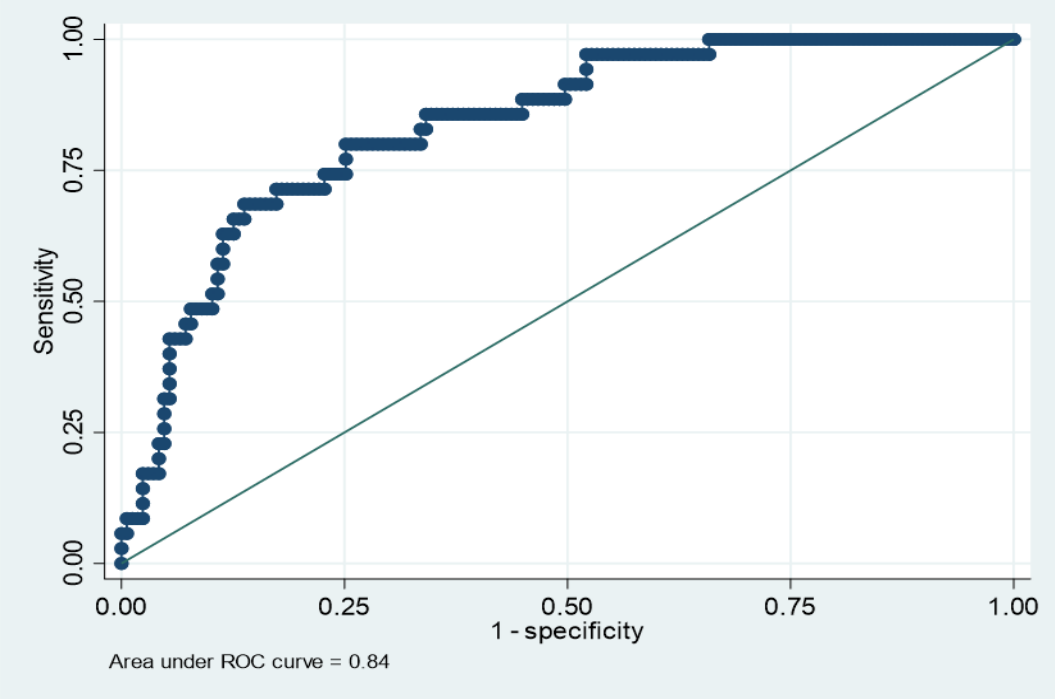


Table 6.10 Summary of ROC analysis for models combining baseline predictive variables and comparison of models against model with age and gender

	AUC (CI)	p value
Age + Gender	0.74 (0.67-0.82)	
Genetic status	0.76 (0.68-0.84)	0.4712
Genetic status + NfL	0.80 (0.74-0.87)	**0.0364**
Genetic status + NfL+ Clinical variables	0.84 (0.78-0.91)	**0.0103**

All models incorporate age and gender as covariates. AUC of each model is compared to Age+Gender

6.4 Discussion

In this study, I explored the interaction of biomarkers that I studied in previous chapters and the potential for combining them to improve diagnosing and predicting disease severity in a clinical trial cohort of mild to moderate PD. I then went on to explore the use of serum NfL and candidate genetic variables as potential prognostic biomarkers in a large and well-studied cohort of recently diagnosed PD patients with prolonged follow-up and high clinical diagnostic certainty. While I did not find a significant correlation between the QUICK1 index, NfL and DAT SBR, I noted an improvement in the ability to predict motor symptom severity in a model which combined plasma NfL and DAT SBR. I also established that serum NfL in combination with genetic variables (*APoE* and *GBA* status) and previously validated clinical measures could provide a better prediction of several aspects of PD progression in prognostic modelling, then clinical measures alone.

Greater dopaminergic dysfunction and axonal injury has been noted in the setting of dysregulated glucose metabolism in pre-clinical models and human studies (363, 443, 777-781). This is particularly

the case when the threshold for prediabetes and T2DM has been reached. I did not note a significant correlation between the QUICK1 index, mean anterior putamen DAT SBR and NfL levels. This may in part relate to differences in my biomarker selection approach which was justified based on findings in previous chapters (e.g., using QUICK1 index over HbA1c or mean anterior putamen rather than lateralized caudate approach). Also, smaller sample sizes particularly for NfL in addition to the low representation of patients with IR based on the predefined threshold in chapter 3 could potentially explain my discrepant findings.

My analysis of the combined value of NfL and QUICK1 in diagnosing PD reinforces my findings in chapter 4 that plasma NfL distinguishes PD from atypical disorders well and the addition of the QUICK1 index does not improve on this or help distinguish PD cases from HC. The QUICK1 index similarly does not improve modelling for predicting disease severity and therefore would not be additionally useful when combined with the other markers for reflecting the disease. The improvement on the model by combining NfL and DAT SBR over each individual marker for predicting motor severity is of interest and may suggest that these markers which reflect different aspects of disease pathology could be useful as a combination in trials though this will need to be explored with larger sample sizes and then studied longitudinally to explore if they track disease better in combination.

Despite a previous study suggesting higher blood NfL levels in patients with more pathogenic variants of *GBA* (55), I did not replicate this finding. Furthermore, I did not note significant differences in NfL levels when comparing patients with a heterozygous or homozygous *APOE* ε4 status to those who did not. These genetic markers are of interest considering their variable association with more severe cognitive and motor progression (261, 782). I have however confirmed the predictive capacity of *APOE* ε4 status on cognitive progression and development of dementia (261, 267), while the lack of impact of *GBA* variants on motor and cognitive progression in my study compared with previous publications (782, 788) is likely explained by the relatively short duration of follow up and by the small number of patients in this cohort.

PD progression and prognosis can be highly variable. Several phenotypes have previously been explored with the goal of predicting future outcomes (777). To date, studies focusing on the potential role of NfL in predicting more severe progression phenotypes have suggested that patients with a more prominent postural instability phenotype have more substantial increases in NfL levels over time (448). My goal was to explore if NfL levels and /or genetic variables could play a role in a model which predicts PD progression in a more encompassing and practical manner that could potentially be utilised in disease modifying clinical trials. I found that baseline NfL levels could replace or complement a number of simple clinical markers previously identified to predict PD progression in a well validated model (786), and while I did not find that NfL alone provided significant additional value to the clinical variables previously identified, the predictive model was strongest when NfL was combined with clinical variables and patient's genetic status. This finding highlights the potential use of combining biomarkers with clinical scales and could support its future use in randomising patients between active treatment and placebo arms in clinical trials.

A major limitation of the cross-sectional study is the small sample size of some biomarkers and potential selection bias which may compound applicability of the findings to the entire PD trial cohort. The strengths of the longitudinal study are its large sample size and prolonged follow-up of up to 72 months although this was only available in 23.2% of cases. I was limited by a lack of assessment in the 'OFF' medication state for the longitudinal study which restricted my ability to interpret NfL associations with motor progression of the dopa responsive elements of the disease and therefore limits my ability to estimate its value in clinical trial modelling where MDS-UPDRS OFF state changes may be the primary outcome. While I found no significant differences between the smaller sample of the Tracking-PD study and the broader study population, it is possible that my results might be confounded by unrecognised selection biases. I also lacked neuropathological diagnostic confirmation in my cohorts, although the exclusion of patients with a diagnostic probability of <90% in the tracking study at the last available visit and stringent selection criteria for the exenatide PD3 trial aimed to mitigate the potential inclusion of misdiagnosed patients.

I was able to demonstrate that the combination of plasma NfL and DAT SBR could potentially better predict motor symptom severity while serum NfL with baseline clinical outcomes and patients' genetic status could be useful for prediction of PD progression. In the appropriate setting, the former combination could potentially be used to track disease progression better though this will require further exploration while the latter combination could potentially be used to enrich a clinical trial cohort for individuals likely to have more rapid disease progression, which might then shorten the follow up time required to detect a disease modifying signal, or alternatively to help ensure that randomised groups are more likely to be balanced in terms of progression rates, thus facilitating detection of agents with true disease modifying properties.

Chapter seven

Discussion

7.0 Summary of main findings

Modifying the relentless deteriorating course of parkinsonian disorders remains a critical yet currently elusive goal. There is a growing interest in recruiting patients into trials even prior to symptom onset, given that intuitively earlier intervention may provide a better chance of preventing irreversible cell death. Alongside trials in prodromal cohorts, there will remain a need to identify whether any disease modifying intervention has an impact on the 6-10 million people already struggling with symptoms, and in need of prevention of further decline.

Despite decades of trials evaluating promising candidates, no treatments have yet been proven to achieve this definitively. While this may be due to lack of trial evaluation of truly effective agents, other potentially contributing factors include imprecise patient selection, inadequacies in trial design, failure to confirm target engagement, and the absence of objective measures of disease progression. One way of improving likelihood of success is by identifying better biomarkers.

Suboptimal patient selection in disease modifying trials may be related to poor diagnostic accuracy. Pathological modification (phosphorylation and conformational transformation) of the physiological protein, α-synuclein or tau (depending on the underlying parkinsonian disease) to misfolded oligomeric and fibrillary forms is the most consistent pathological feature of these disorders. The accumulation and interplay of these abnormal protein forms with the organelles/cellular pathways involved in their clearance as well as normal cellular maintenance and survival results in neuronal dysfunction and ultimately axonal injury and neuronal death.

The α-synuclein seed amplification assay (α-syn SAA) has high sensitivity and specificity for PD diagnostic accuracy and is now proposed as a core aspect of a potential staging system for PD (789, 790). This is potentially a pivotal step in clarifying eligibility criteria for inclusion in trials and distinguishing PD patients from those with atypical forms of parkinsonism. Distinguishing PD from MSA with the assay requires further clarification as does a similar tau assay for diagnosing tauopathies. The

α-syn SAA is at the present time largely a binary measure simply indicating the presence/absence of the pathophysiological process of alpha synuclein aggregation and cannot yet be used to track disease severity which instead relies on clinical measurements.

As such there is still a need for additional biomarkers that might enrich treatment arms for PD subgroups most likely to respond and allow early exploratory analyses according to engagement of the therapeutic with its putative target. Current trials typically rely on clinical end points with scales and questionnaires which are subject to inter-rater variability, limited by compliance and recall bias while potentially being confounded by symptomatic drug effects. Biomarkers that are robustly demonstrated to track disease progression and treatment effects could potentially shorten periods of assessment and reduce the number of patients required for preliminary demonstration of efficacy. Ideally, short-term changes in the biomarker should anticipate long-term clinical outcomes. Furthermore, by confirming target engagement by the dose(s) of the agent under study, biomarkers can be used to improve the distinction between an intervention's disease-modifying effects from purely symptomatic improvements. Prior to establishing this, it is important to determine if biomarkers uniquely reflect disease status in the parkinsonian disease studied.

The overarching goal of the work performed in this thesis was to inform on the best potential combination of biomarkers that could improve demonstration of disease modification in the exenatide PD3 and MSA trials. A major challenge for trials will be in the choice of selection of suitable combinations of fluid and imaging biomarkers that complement each other. This will need to be strongly guided by the biological action of the agent being tested and the stage of the disease of their participants being treated, though those biomarkers that appear to align with disease progression most closely should be prioritised. The three biomarkers selected for this work reflected different disease aspects with a predominant focus on determining if they would be useful for predicting and tracking progression. This included insulin resistance which was of interest as this pathway is a key target of GLP-1 receptor agonists which are being studied in the trials, and dopaminergic denervation

and axonal injury which are general downstream features of the neurodegenerative process. Demonstrating improvements in each of these aspects in the trials and potentially utilizing them in combination with genetic status for balancing trial arms could potentially provide important support for demonstrating disease modification in addition to addition to potential changes that maybe noted with clinical markers. Although the diagnostic value of some of these biomarkers were explored in previous chapters, this was more to understand if they were able to specifically reflect the underlying disease process differently in the individual disorders rather than to establish them for future diagnostic use considering this role will likely be well served by more specific disease markers such as the seed amplification assay in the future. In addition to this, I explored if known risk factors of disease severity and progression which may represent a disease subtype in PD when present such as T2DM has similar implications for atypical parkinsonian disorders and if biomarkers reflecting the neurodegenerative process could provide additional evidence for sub-characterizing this cohort. This could potentially be important for informing on how trial recruitment could be enriched to better demonstrate effects depending on which disease is being recruited.

Each chapter in this thesis therefore aimed to evaluate the performance of these biomarkers in using baseline data from the clinical trials as well as natural history studies and in some instances exploring if findings in natural history studies would be applicable to the trial cohorts. In addition to this the value of combining biomarkers for diagnostic and prognostic purposes over utilizing individual biomarkers was also explored.

In the first study, I aimed to evaluate if peripheral insulin resistance was more prevalent in PD, MSA, and PSP than HC and explored its relationship with patient demographics and clinical severity. I also aimed to explore if co-morbid T2DM influenced MSA and PSP severity and progression as it does in PD. I noted a similar proportion of patients had peripheral IR in all groups and that peripheral IR was not associated with disease severity. The presence of T2DM also did not impact on disease severity and progression in MSA and PSP patients. I concluded that peripheral IR makers were unlikely to be

of value in reflecting the disease severity of parkinsonian diseases and therefore not likely to be able to track progression well in trials. T2DM clearly influences disease severity and progression in PD based on several previous studies though this was not the case for MSA and PSP based on my findings.

Following this I explored the value of NfL in two natural history studies and aimed to see if findings would be replicable in the exenatide PD3 trial. NfL's usefulness for distinguishing PD from HC was mixed and dependent on the cohorts in which it was assessed. NfL was however an excellent biomarker for diagnosing MSA and distinguishing PD from MSA and PSP. NfL weakly reflected disease severity in PD and MSA. Baseline NfL predicted different aspects of PD progression and weakly predicted MSA progression. I concluded that NfL measured in blood is useful for distinguishing PD from atypical parkinsonian disorders and can predict progression in PD and MSA.

NfL levels were higher in PD patients with coexistent T2DM when compared to those without T2DM supporting clinical differences I noted. This provided support that more severe axonal degeneration was occurring in this subtype of patients. This was not the case in PSP and MSA, consistent with the clinical findings. This was important in providing additional evidence to support the conclusion that T2DM and potentially insulin resistance status would need to be considered differently depending on the type of parkinsonian disorder being studied if used as a recruitment criterion or for balancing trial arms.

The dopamine transporter striatal binding ratio (DAT SBR) has been used as an outcome measure in Parkinson's disease modification trials. Both patient characteristics and analysis approaches potentially complicate its interpretation. I explored whether DAT SBR reflected PD motor severity across striatal subregions and its relationships to disease duration, and side of onset. I noted that lateralized scores were most consistently associated with loss of DAT uptake in the contralateral anterior putamen. There was much higher variance in the posterior putamen and in all regions in those with longer duration disease (although bradykinesia remained robustly associated with

anterior putaminal DAT uptake even in longer duration patients). Restricting analyses to the less affected side did not usefully reduce the variance compared to the overall cohort. I therefore concluded that DAT SBR could be a useful biomarker in disease modifying trials even at this disease stage, but a focus on striatal subregions and incorporating disease duration into analyses may improve its utility.

The objective of chapter 6 was to explore the relationship between the three biomarkers that were previously studied and to determine if blood (NfL and QUICK1) and imaging (DAT SBR) biomarkers and genetic status (*GBA* and *APoE*) were useful in addition to clinical measures for diagnosing, predicting clinical characteristics and prognostic modelling in PD. I found that the QUICK1 index, NfL and DAT SBR did not correlate. NfL's excellent ability for distinguishing PD from atypical disorders was not improved by the addition of the QUICK1 index. Combining plasma NfL and DAT SBR potentially improved prediction of motor severity. NfL predicted PD progression and mortality well and the combination of simple, easily obtainable clinical variables with NfL and genetic data produced a stronger predication of unfavourable outcomes as compared to age and gender alone.

7.1 Limitations of study

Despite promising findings suggesting potential validity for the biomarkers explored, several limitations have impacted on interpretation and potential future application of findings. Firstly, several analysis datasets comprised small sample sizes from a single centre with further subgrouping based on demographics such as disease duration. This potentially resulted in underpowering of the groups for demonstrating the effects noted. In addition to this, there may be unrecognised selection bias by exploring findings in smaller groups taken from larger cohorts despite best efforts to minimise this.

Similarly, the limited follow-up timeframe in a relatively small sample of atypical parkinsonian patients aiming to establish the impact of T2DM on severity and progression limits the ability to interpret the

negative findings. Only a minority of cases in the atypical parkinsonian cohorts had brain bank diagnostic confirmation though efforts were made to select cases with maximum follow-up when analysing NfL for one of the sub-studies which potentially improved diagnostic certainty. This is critical as diagnostic inaccuracies can be high in these groups. The lack of neuropathological diagnostic confirmation in the tracking PD cohort is also a limitation though the exclusion of patients with a diagnostic probability of <90% at the last available visit aimed to mitigate the potential inclusion of misdiagnosed patients. This limitation also applies to the clinical trial recruitment in both PD and MSA though best attempts were made to adhere to sanctioned diagnostic criteria at recruitment.

Considering insulin resistance can be influenced by ethnicity (718) and other demographic factors such as obesity, the overwhelming recruitment of Caucasians into all groups, in addition to the PD trial cohort only comprising of a relatively small proportion of cases who were in the obese range, suggests that these aspects potentially limit applicability of findings in a more general population sense. The analysis however incorporated the entire exenatide PD3 and MSA trial cohorts and therefore is likely to be well powered to form conclusions on whether peripheral IR markers are reliable for use in trial analysis. The work however lacks the exploration of central IR markers such as IRS-1p 312 and IRS-1p TYR and their potential relationship with peripheral IR markers which is a critical limitation for determining how to use these markers in secondary trial analysis.

I noted contrasting findings when I separately explored the value of blood NfL in the tracking PD study and the exenatide PD3 trial. Naturally some of this will be accounted for by demographic differences, studying relationships between different variables (MDS-UPDRS 3 ON and OFF scores or combination of both) and NfL and the lack of power of the small sample size chosen from the exenatide trial. I did not directly compare NfL levels in the two cohorts as tests were performed on different sample types (serum versus plasma) and using different machines, kits, and matrix all of which could confound interpretation. This highlights one of the biggest limitations to using biomarkers in trials though with

advancing techniques and standardization of technical approaches between laboratories this is likely to become less of a concern.

Most of my analysis focused on exploring the relationship between biomarkers and motor severity scores. The relationship between biomarkers and non-motor symptoms were only minimally explored and when performed only demonstrated weak relationships indicating that they are likely to poorly reflect overall non motor severity. Considering the significant prevalence of these symptoms in parkinsonian disorders and their substantial impact on quality of life, the lack of relationship noted with chosen biomarkers is a critical limitation of their potential value. Although I adjusted for age, gender and disease duration in association analysis, these variables and other biological and technical factors can differentially influence the relationship between biomarkers and clinical severity and have not been considered in my modelling.

The major limitation in biomarker discovery is undoubtedly difficulty with validation. Association between a change in a biological assay alongside a clinical state need not equal causation. For example, biological changes may represent healthy compensatory responses to a pathological process. Furthermore, even biomarkers that do reflect active processes of neurodegeneration may not have linear relationships over the course of disease particularly if production ultimately declines because of widespread tissue death. My work largely focused on validation using cross sectional data. This limits interpretation of determining their value in trials as the possibility for changes in the relationship between the biomarkers and clinical severity over time have not been determined. Demonstrating this over follow-up will therefore be essential in addition to showing that the biomarker is amenable to change based on a potential drug response. This will however depend on the identification of a successful treatment. Once proven, it may become a useful means of shortening the length of follow up needed to confirm or refute effects.

7.2 Future work

Specific findings from this work will need to be re-explored in larger sample sizes incorporating data from several cohorts or sites, in addition to mitigating discrepant results between studies attributable to preanalytical and analytical confounders from variations in techniques employed and type of blood samples used. An example of this would be to reconsider the value of plasma NfL in all PD samples from all 6 exenatide PD3 sites (n=194) and performing levels on the entire tracking PD cohort (n=2000) in the same analysis. This would more definitively assess its potential value. Furthermore, this combined analysis would be sufficiently powered to assess potential cutoff levels that could be employed at baseline for excluding atypical cases and predicting progression which are NfL's strengths. This can then be explored in modelling to determine if potential treatment effects from exenatide in the trial could be enhanced by adjusting for baseline NfL level in addition to potentially exploring the number needed to treat to demonstrate effects. This information could also support future disease modification trial design. A recent NfL study supporting data driven cut-offs by employing this approach across neurodegenerative diseases including PD and atypical parkinsonian disorders in two large cohorts demonstrate the potential value of this approach (791).

The potential for combining NfL and DAT SBR as an improved outcome measure should also be considered further in a larger sample size. This could be achieved by firstly assessing the ability of this combination to predict motor and non-motor severity in all cases in the exenatide PD3 trial who have had DAT scans and then to re-explore this relationship in a separate cohort with longitudinal assessments of both markers. If the findings hold in these exploratory studies this combination could be tested in at baseline and at the 96-week follow-up of the PD3 trial as a potential combined outcome measure.

Central IR markers such as IRS-1p 312 and IRS-1p TYR have been shown to correlate with disease severity and progression. In addition to this, these markers tracked the MDS-UPDRS 3 off score which

was the primary outcome in the exenatide PD2 trial. These biomarkers will therefore need to be tested in the same cohorts where peripheral IR markers were assessed in a future study. Their potential correlation with peripheral IR markers will also need to be explored. If these central markers are demonstrated to be significantly elevated in either PD or MSA and correlate well with peripheral markers, then the current conclusion reached in this work of a lack of value of peripheral markers would need to be reconsidered as peripheral IR markers are likely to be cheaper and simpler to obtain from most healthcare laboratories.

The lack of neuropathological validation is a critical limitation particularly in the atypical cohorts. A future study exploring my negative findings of the effect of T2DM on MSA and PSP severity and progression in a brain bank cohort will be valuable. This will ensure diagnostic certainty and more prolonged follow-up. Despite the lack of objective measurement of disease progression with rating scales and issues arising from retrospective data ascertainment this study will complement my findings in the PROSPECT study which has strengths in this regard.

7.3 Concluding remarks

Clinical trials of disease-modifying therapies might usefully stratify patients using biomarkers at the time of recruitment. The identification of a better framework for the certainty of a diagnosis based on positivity of a biomarker such as the seed amplification assay or cutoff levels of NfL is a major step forward. The further development of reliable biomarkers of neurodegeneration in parkinsonian disorders could further facilitate prognostication and identification of disease subtypes as demonstrated in this work. This could potentially improve the conduct of clinical trials and identification of agents that may slow down or stop these processes. The precise role for biomarkers will depend on the mechanism of action of the agent in question, and the decision made regarding the stage of the illness at which the intervention is being applied. Rapid progress with validation is of

outmost importance and potentially achieved by routinely collecting biomarker samples as part of trials, differently incorporating them into inclusion criteria depending on the mechanism of action of the tested drug and the stage of disease being studied. Where appropriate, the same panel of biomarkers which are predictive of more rapid progression and can confirm target engagement of the intervention should be incorporated. This work has provided some preliminary proof of concept evidence of the value of these recommendations and future studies will aim to build on this.

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