

IMPROVING THE MANAGEMENT OF
PARKINSON'S DISEASE:
THE EXPERIENCE OF HOSPITALISATION
AND A NOVEL MRI-BASED DIAGNOSTIC
TOOL

By

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Abstract

Parkinson's disease (PD) motor and non-motor symptoms progress relentlessly leading to frequent hospitalisations and an increase in the economic and societal burden of the disease. A major challenge in assessing treatments which can potentially reduce neurodegeneration, is the lack of tools that can be used to identify individuals with early PD with high sensitivity and specificity.

There were several facets to this thesis, which were aimed at addressing these issues. Firstly a retrospective analysis of PD hospital admissions was conducted to provide background data on hospitalisation and clinical coding accuracy. A systematic review of literature for interventions to reduce hospital admissions was performed. Effect of treatments for PD motor symptoms on hospitalisation was also evaluated. Lastly, screening of potential lanthanide and ¹⁹ Fluorine based MRI probes for future use as diagnostic tools in PD was conducted.

Results of these studies highlight significant underreporting of PD hospitalisation which has a negative effect on PD resource allocation. A lack of robust evidence for measures which reduce PD admissions was demonstrated. Although the initial attempts to develop a novel MRI sensitive tool for use in PD were negative, the study refined a protocol that has potential for use in screening future probes.

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List of Abbreviations

AADC Aromatic amino acid decarboxylase

A β 1-42 Beta amyloid 1-42

bFGF Basic Fibrinogen growth factor

cAMP cyclic Adenosine monophosphate

CNS Central nervous system

COMTI Catechol-O-methyl transferase Inhibitor

CSF Cerebrospinal fluid

DAT dopamine transporter

DMEM Dulbecco's Modified Eagle Media

DOPAC 3, 4 dihydroxyphenylacetic acid

DTBZ [¹¹C] dihydrotetrabenazine

DTI Diffusion Tensor Imaging

FA Fractional Anisotropy

FAB (E-E) - 1- fluoro-2.5-bis (3-hydroxycarbonyl-4-hydroxy) styrylbenzene

FITC fluorescein Isothiocyanate

GBA Glucocerebrosidase

HEPES 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid)

HES Hospital episodes statistics

HVA Homovalinic acid

ICD International classification of diseases

ICP-MS inductively coupled plasma spectrometry

IQR- Interquartile range

L-DOPA dihydroxyphenylalanine

LED Laser emitting diode

LRRK2 Leucine-rich repeat Kinase 2

LUHMES Lund Human Mesencephalon Cells

MAOBI Monoamine Oxidase B Inhibitor

MD Mean diffusivity

MPTP 1-methy-4-phenyl-1, 2, 3, 6-tetrapyridine

MRI Magnetic resonance Imaging

MRS Magnetic resonance spectroscopy

MSA Multisystem atrophy (MSA),

MTR Magnetisation Transfer ratio

NEADL-Nottingham Extended Activities of Daily Living

NET Noradrenaline Transporter

NFL Neurofilament light chain protein

NGF Nerve growth factor

NHS National Health Service

NMR Nuclear Magnetic Resonance

NTUA Neurotransmitter Transporter Uptake Assay

PD Parkinson's disease

PDMED Parkinson's disease oral medication trial

PDQ39 Parkinson's Disease Questionnaire-39

PEG Polyethylene glycol

PET Positron Emission Tomography

PICS Prescription and Information Communications System

PINK1 PTEN- induced putative kinase

PSP Progressive supranuclear palsy

PSS Physiological Saline Solution

RBD Rapid eye movement (REM) behaviour sleep disorder

REM Rapid eye movement

ROI Region of interest

RPMI Roswell Park Memorial Institute

SERT Serotonin Transporter

SNCA Synuclein alpha

SPECT Single Positron Emissions Computed Tomography

SUS Secondary Uses Services

SWEDD Scans without evidence of dopaminergic deficit

SWI Susceptibility weighted imaging

TH Tyrosine hydroxylase

UCH-L1 Ubiquitin C—terminal hydrolase L1

UK United Kingdom

UPDRS Unified Parkinson's Disease Rating Scale

VMAT Vesicular monoamine transporter 2

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CHAPTER 1: INTRODUCTION

1.1. Epidemiology

Parkinson's disease (PD) is the second commonest progressive neurodegenerative disease, which predominantly affects individuals above the age of 60 years (Nussbaum and Ellis, 2003). In 2005, disease prevalence in Europe was estimated to range from 108 to 257 per 100 000 and an incidence rate of 11 to 19 per 10 000 was reported (Von Campenhausen et al., 2005). The wide variation in prevalence is probably due to different methodologies, age groups, and diagnostic criteria used in different studies (de Rijk et al., 1997).

Age is the most important risk factor for the disease. The effect of age on disease prevalence was demonstrated in a recent systematic review, which assessed the worldwide prevalence of PD (Pringsheim et al., 2014). Meta-analysis of data from included studies showed an increase in disease prevalence with age: from 41 per 100 000 in the 40-49 years age group, rising to 173 per 100 000 between 50-59 years, and 1087 per 100 000 in the 70-79 years group (Pringsheim et al., 2014). Therefore, the number of individuals affected by the disease is predicted to increase with improving quality of life and increasing life expectancy of populations in developed countries (de Lau and Breteler, 2006, Driver et al., 2009). An association between risk of the disease and gender has also been reported, with males having a 1.5-2 times higher incidence of the disease than females (Twelves et al., 2003).

The exact cause of PD is unknown but it is thought to involve an interaction between genetic and environmental factors (de Lau and Breteler, 2006). Mendelian genetic cases only contribute to a small proportion (10%) (de Lau and Breteler, 2006), suggesting that environmental factors play a larger part in disease causation. Controversy surrounds which environmental factors are relevant in PD. Some epidemiological studies reported an increased risk of the disease with exposure to pesticides, heavy metals such as manganese and iron, rural, and farm living, but others have produced conflicting results (de Lau and Breteler, 2006,

Wirdefeldt et al., 2011). Additional evidence for environmental factors playing a part in disease causation comes from animal studies which demonstrated nigrostriatal degeneration and increased alpha-synuclein expression following exposure to pesticides such as rotenone, paraquat, and organochlorines (Wirdefeldt et al., 2011).

A number of lifestyle habits maybe protective against the disease. For example, cigarette smoking is reported to halve the risk of PD, possibly via the stimulatory and potential neuro-protective effects of nicotine on dopaminergic neurons (Wirdefeldt et al., 2011). Vitamin E and caffeine consumption may also reduce the risk of the disease (Wirdefeldt et al., 2011, de Lau and Breteler, 2006). Lastly, an inverse, but weak association between exercise and risk of PD has also been reported (Wirdefeldt et al., 2011).

1.2 Pathophysiology

The pathological signature of the disease includes depletion of dopaminergic cells in the substantia nigra pars compacta and Lewy bodies in the cytoplasm of the remaining neurons (Greenfield and Bosanquet, 1953). The Lewy bodies are predominantly composed of alpha-synuclein (Spillantini et al., 1997). Underlying molecular mechanisms in the pathogenesis of both idiopathic and familial PD are complex. Mitochondrial dysfunction, abnormal protein accumulation, and oxidative stress are thought to be involved in processes leading to neuronal degeneration (Wood-Kaczmar et al., 2006, Lim and Zhang, 2013). Evidence for this comes from studies involving genes implicated in familial PD (Wood-Kaczmar et al., 2006). Both familial PD due to alpha-synuclein gene (*SNCA*) mutation and idiopathic PD are characterised by alpha-synuclein aggregation in the intra-cytoplasmic inclusions (Wood-Kaczmar et al., 2006, Devine et al., 2011), suggesting a role for alpha-synuclein accumulation in the pathogenesis of the condition. However, it remains unclear whether Lewy bodies are toxic to

cells or their deposition signifies a cellular response to another pathologic substrate or process (Devine et al., 2011, Wood-Kaczmar et al., 2006).

The ubiquitin-protease system and autophagy are the principle systems involved in the processes leading to degradation of misfolded proteins and damaged organelles (De Rosa et al., 2015). Target proteins bind to ubiquitin and are presented to proteasomes where degradation occurs (De Rosa et al., 2015). Dysfunction of these protein clearance systems may also play a role in PD pathogenesis. Inhibition of ubiquitin-protease system and proteasome function in rat ventral mesencephalon culture led to accumulation of alpha-synuclein and ubiquitin positive inclusions, and degeneration of dopaminergic neurons in a study by McNaught et al. (2002) (McNaught et al., 2002). Further evidence for the involvement of abnormal protein clearance in PD pathogenesis comes from the observation that mutation of genes involved in the ubiquitin-protease system also lead to familial PD (De Rosa et al., 2015). For example, parkin mutations lead to loss of activity of E3 ubiquitin ligase which is important in the ubiquitin proteasome system (Shimura et al., 2001), and the ubiquitin C-terminal hydrolase L1 (*UCH-L1*) mutation, has been described in familial PD (McNaught et al., 2002). Damaged organelles and aggregated proteins are also degraded by lysosomes via a process called autophagy (Hara et al., 2006) and mutation of the genes which encode for some lysosome proteins [e.g. glucocerebrosidase (*GBA*) and *ATP13A2*] are also associated with an increased risk of PD (De Rosa et al., 2015).

The role of mitochondrial dysfunction in PD was demonstrated in pathological studies showing reduced mitochondrial complex 1 levels in PD brains (Keeney et al., 2006). Additional support comes from the observation that exposure to the toxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrapyridine (MPTP), a mitochondria complex 1 inhibitor results in a phenotype similar to that seen in sporadic PD (Keeney et al., 2006). In addition, rotenone, another

mitochondrial complex 1 inhibitor, induced dopaminergic neuron degeneration in animal studies (Sherer TB, 2003). Furthermore, mitochondrial abnormalities have been demonstrated in cells derived from individuals with Leucine-rich Repeat Kinase 2 (*LRRK2*) G2019S mutation, an autosomal dominantly inherited mutation (Wang et al., 2012a, Cooper et al., 2012). Mitochondrial dysfunction is a common pathway that potentially leads to neuronal death in early onset autosomal recessive PD secondary to Parkin, PTEN- induced putative kinase (*PINK1*) and *DJ-1* mutations (Thomas et al., 2011). How this results in neurodegeneration remains uncertain, but is thought to interfere with mitochondrial function either by accumulation of an unknown pathogenic substrate or ubiquitin dependent signalling (Dawson et al., 2010). PINK1, a mitochondrial protein kinase plays an important part in mitochondrial homeostasis (Trempe and Fon, 2013). DJ-1 protein acts as molecular chaperone in mitochondrial oxidative pathways (Dawson et al., 2010). Thomas et al 2011 showed that DJ-1 loss of function led to mitochondrial fragmentation suggesting a possible role of oxidative stress in dopaminergic degeneration (Thomas et al., 2011). These studies demonstrate the complexity of PD pathogenesis. Several mechanisms are implicated but the exact pathophysiology for the disease remains unknown.

1.3. Clinical features of PD

Motor features

It is now well recognised that PD has a prodromal phase, and that motor symptoms only appear when there is at least 80% reduction in striatal dopamine levels (Bernheimer et al., 1973). The cardinal motor symptoms of the disease are: tremor, rigidity, bradykinesia, and postural instability. The first three are often present when a clinical diagnosis is made but

postural instability is frequently seen in more advanced stages of the disease. Motor symptoms in PD typically have a unilateral onset. With time, symptoms progress to involve the contralateral side. A 4-7 Hz rest tremor is characteristic of the condition, (Pfeiffer, 2015) but a postural re-emergent tremor may also be present (Pfeiffer, 2015). The tremor often starts in one upper limb but other parts including the lower limbs, lips or chin tremor can be involved. Pathophysiology of rest tremor in PD is uncertain. The observation that the rest tremor does not always respond to dopaminergic treatments, suggests that pathways other than the nigrostriatal dopaminergic neurons may be involved (Pfeiffer, 2015). Some authors postulate that basal ganglia dysfunction acts as a trigger and cerebellar networks drive the rest tremor, but it remains uncertain if these pathways interact at the level of the thalamus or the cortex (Hallett, 2012).

Bradykinesia is defined as slowness of movements and is secondary to dopaminergic cell loss and in the nigrostriatal pathway (Pfeiffer, 2015). In addition to abnormal speed of movement, the definition of bradykinesia also encompasses hesitation, loss of rhythm of movements, absence and reduction in amplitude of movements: akinesia and hypokinesia respectively (Heldman et al., 2011, Pfeiffer, 2015). When using clinical diagnostic criteria, bradykinesia has to be present before a parkinsonian syndrome can be considered (Gibb and Lees, 1988). Patients often complain of generalised slowness of movement, difficulty with movement initiation, and problems with performing fine motor tasks. On clinical examination, affected individuals may exhibit the following features: freezing, reduced arm swing, expressionless faces, and difficulty in initiating movements (Berardelli et al., 2001). Other signs include micrographia, reduced blinking rate, hypophonic speech, hesitation and arrests on foot or finger tapping.

Rigidity in PD, is characterised by uniform resistance to passive movement also termed ‘lead-pipe rigidity’, but cogwheel rigidity may also be detected when tremor is superimposed on rigidity (Broussolle et al., 2007). In the early stages of the disease, rigidity may be difficult to detect (Pfeiffer, 2015). In such cases, the Froment’s manoeuvre can be used to augment the clinical finding: facilitation manoeuvres such as repetitive movements of the contralateral limb or arithmetic tests are used to reinforce the rigidity (Broussolle et al., 2007). The pathophysiology of rigidity in PD is uncertain but may involve reticulospinal tracts. Changes in the activity of spinal inhibitory neurons lead to tonic facilitation of the alpha motor neurons, hence the rigidity in PD (Santens et al., 2003).

Postural instability is more common in the later stages of the disease and is a major contributor to the risk of falls in PD (Kim et al., 2013). Basal ganglia dysfunction is thought to play a role in the development of postural instability in PD (Kim et al., 2013). Other motor features, such as dyskinesias and ‘on off’ fluctuations, are a consequence of long-term dopaminergic treatment.

Non-motor features

The neurodegenerative process is not limited to the dopaminergic system, but involves other neuronal networks which results in the non-motor symptom complex (table 1) (Chaudhuri et al., 2006). For example, cholinergic denervation has been linked to Rapid eye movement (REM) behaviour sleep disorder (RBD): a sleep disorder marked by loss of muscle atonia during REM sleep (Kotagal et al., 2012). Cholinergic dysfunction has also been demonstrated in PD dementia (Bohnen et al., 2003). Anxiety and mood disorders in PD were shown to correlate with a reduction in dopamine and noradrenaline innervation in the locus coeruleus and limbic regions in PD (Remy et al., 2005).

Table 1: Spectrum of Non-motor symptoms in Parkinson's' disease

<p><i>Neuropsychiatric symptoms</i> Depression, apathy, anxiety Anhedonia Attention deficit Hallucinations, illusion, delusions Dementia Obsessional behaviour (usually drug induced), repetitive behaviour Confusion Delirium (could be drug induced) Panic attacks</p> <p><i>Sleep disorders</i> Restless legs and periodic limb movements Rapid eye movement (REM) sleep behaviour disorder and REM loss of atonia Non-REM-sleep related movement disorders Excessive daytime somnolence Vivid dreaming Insomnia Sleep disordered breathing</p> <p><i>Autonomic symptoms</i> Bladder disturbances Urgency Nocturia Frequency Sweating Orthostatic hypotension Falls related to orthostatic hypotension Coat-hanger pain Sexual dysfunction Hypersexuality (likely to be drug induced) Erectile impotence Dry eyes (xerostomia)</p>	<p><i>Gastrointestinal symptoms (overlaps with autonomic symptoms)</i> Dribbling of saliva Ageusia Dysphagia and choking Reflux, vomiting Nausea Constipation Unsatisfactory voiding of bowel Faecal incontinence</p> <p><i>Sensory symptoms</i> Pain Paraesthesia Olfactory disturbance Other symptoms Fatigue Diplopia Blurred vision Seborrhoea Weight loss Weight gain (possibly drug induced)</p>
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Other PD non-motor symptoms include: olfactory dysfunction, sleep disturbances, gastrointestinal disturbances, autonomic dysfunction, fatigue, pain, and hallucinations (Chaudhuri et al., 2006).

Clinical phenotypes

The clinical phenotype in PD is heterogeneous. This may be due to multiple underlying pathophysiological mechanisms as well as a result of influence from various genetic factors (Lewis et al., 2005). Grouping patients into different subtypes may have prognostic implications and also influence treatment choices (van Rooden et al., 2010). Two main disease subtypes have been described and these are based on predominant motor phenotype. Firstly, tremor dominant PD is characterised by slow disease progression (Thenganatt and Jankovic, 2014). The second subtype is the akinetic rigid syndrome or postural instability and gait disorder (PIGD) which is associated with a poor prognosis, more rapid disease course, and higher non-motor symptom burden including depression, apathy, cognitive decline, hyposmia, and RBD (Thenganatt and Jankovic, 2014, Wu et al., 2016). Finally, a mixed or indeterminate group which overlaps between the two main subtypes has also been described (Marras, 2015). Other authors have used statistical methods such as cluster analysis to classify patients into different subgroups. Lewis et al. (2005) described four disease subtypes using cluster analysis of clinical data derived from 120 PD patients with early disease (Lewis et al., 2005). A ‘young onset’ subgroup was identified and this was characterised by a benign disease course with slow progression, mild motor symptoms, no cognitive impairment, but more treatment related motor complications when compared to other clusters (Lewis et al., 2005). The ‘tremor dominant’ subgroup showed a slow disease progression, and no cognitive impairment or depression (Lewis et al., 2005). A ‘non-tremor dominant’ cluster was also described. This group had a more rapid disease course, higher depression scores and poor

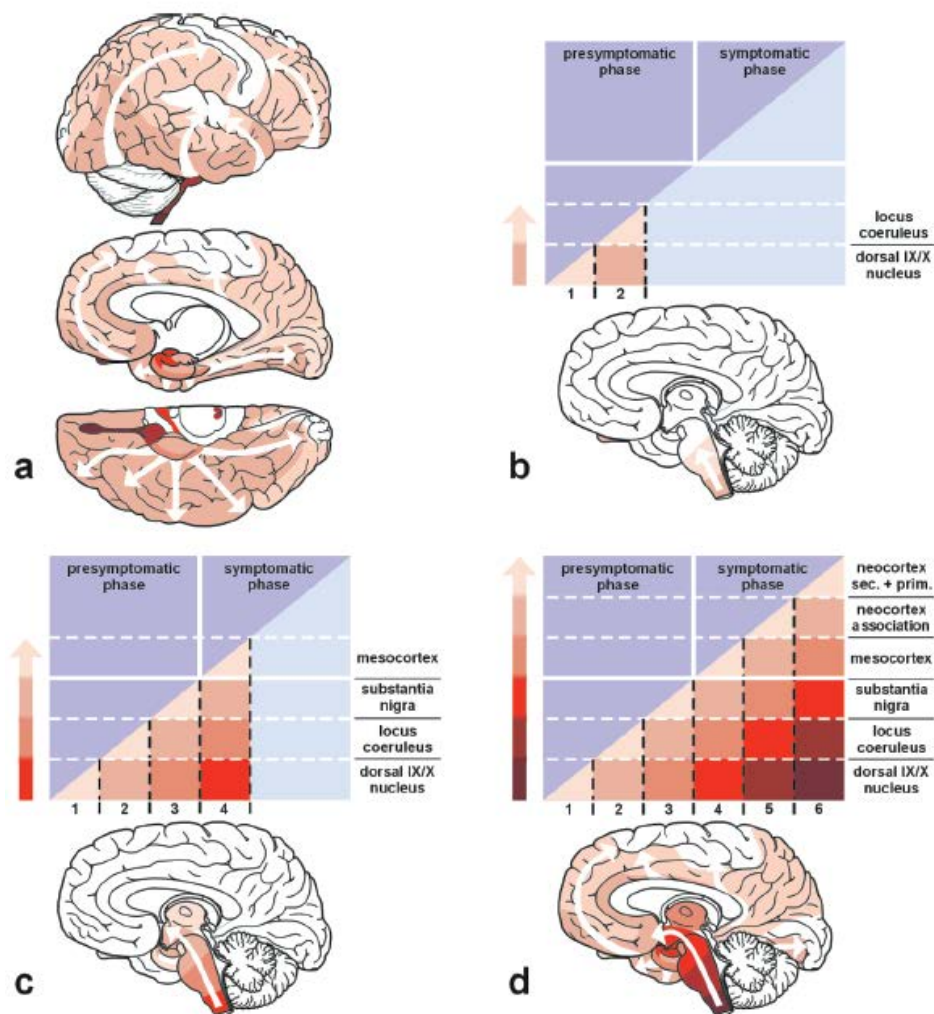
cognition (Lewis et al., 2005). Lastly, ‘rapid disease progression’ subgroup with minimal cognitive impairment and less levodopa response when compared to other subgroups (Lewis et al., 2005). This group may represent patients with atypical PD, although study authors thought this was unlikely since standard clinical criteria were used to make a diagnosis. Similar clusters were also identified in recent studies (Liu et al., 2011, Ma et al., 2015). A systematic review of data driven methods to identify PD subtypes showed inconsistencies in the clusters described across the included studies (van Rooden et al., 2010). The differences may be a result of variability in the parameters included in the cluster analysis and also inclusion of patients with different disease duration (van Rooden et al., 2010).

Braak hypothesis and evolution of symptoms

Some non-motor symptoms have been shown to predate the onset of the motor syndrome by many years (premotor phase), but others including dementia tend to occur in the later stages of the disease (Chaudhuri et al., 2006). The evolution of both motor and non-motor symptoms from the premotor phase to advanced stages of the disease was delineated in the Braak hypothesis discussed below (figure 1). Braak et al. (2002), used a six stage system to describe the progression of Lewy body pathology. This hypothesis provides an explanation why some symptoms occur before the onset of motor symptoms, and others in more advanced stages of the disease (Braak et al., 2002). In Stage 1, Lewy body pathology is seen in the olfactory bulb, the myenteric plexus of the gut, and the dorsal motor nucleus of the vagus nerve (Braak et al., 2002). This may explain the occurrence of olfactory dysfunction (Stiasny-Kolster et al., 2005) and gastrointestinal symptoms in the premotor phase of the disease. From the vagus nerve, the pathology ascends into the brain stem to involve various nuclei including the locus coeruleus, caudal raphe nuclei, and the reticular formation in Braak stage 2 (Braak et al., 2002). RBD is thought to occur during this stage (Stiasny-Kolster et al., 2005). The classical

motor symptoms of the disease appear in Stage 3 when there is involvement of the substantia nigra (Braak et al., 2002). As the disease continues to progress, the neuropathological changes worsen in previously affected regions and the pathology also ascends to involve the forebrain and cerebral cortex in Stages 5 and 6 (Braak et al., 2002), leading to cognitive impairment

Figure 1: Braak hypothesis, spread of Lewy body pathology in Parkinson's disease



Braak et al 2002, upward spread of Lewy body pathology in PD (b) Stage 1 and 2: Lewy body deposition in olfactory bulb and involvement of brainstem nuclei. (c) Stage 3: substantia nigra, basal forebrain (d) stage 5 and 6

Figure obtained from Braak H, Del Tredici K, Bratzke H, Hamm-Clement J, Sandmann-Keil D, Rub U (2002) Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). *Journal of Neurology* **249**, 1-1, with permission from Springer.

And dementia. Although this staging system is widely accepted, it has a number of limitations. Post-mortem studies have shown that the disease pathology does not strictly follow the caudo-rostral ascension described by Braak and colleagues. For example, Dickson et al.(2008) showed that asymptomatic individuals who were found to have incidental Lewy body deposition at post-mortem, had a similar pattern of alpha-synuclein deposition in multiple brain regions to that found in the group with clinical PD, but the alpha-synuclein body load was lower in the earlier group (Dickson et al., 2008). Furthermore, tyrosine hydroxylase immunostaining was reduced in cardiac sympathetic terminals of both individuals with PD and those with incidental Lewy bodies (Dickson et al., 2008). Since individuals with incidental Lewy body deposition are thought to have preclinical PD (DelleDonne et al., 2008), Dickson et al. (2008) findings suggest widespread Lewy body deposition in the preclinical stage of the disease with the load increasing as the disease progresses (Dickson et al., 2008). This is in contrast to Braak et al. (2002) who proposed that the pathology spreads upwards from the lower brainstem nuclei (Braak et al., 2002). Another post-mortem study showed that 55% of patients with extensive alpha-synuclein pathology corresponding to stage 5-6 of Braak staging, did not have extrapyramidal signs or dementia (Parkkinen et al., 2008). In addition a subgroup had pathology in the substantia nigra, basal forebrain, and cortex, but brain stem nuclei including locus coeruleus and the dorsal motor nuclei of the vagus were spared (Parkkinen et al., 2008). These findings suggest that the Braak staging system may not be useful in predicting progression of neurodegeneration in PD or Lewy body dementia (Parkkinen et al., 2008). It is also important to highlight that neurodegeneration and Lewy body deposition in PD is not only localised to the central nervous system. For example, Lewy body deposition has been demonstrated in the enteric (Hilton et al., 2014, Beach et al., 2010) and sympathetic nervous system (Beach et al., 2010),

which suggest that both peripheral and central nervous system neurodegeneration occurs in PD. Lewy body deposition in the peripheral nervous system may explain the occurrence of non-motor symptoms such as postural hypotension and gastrointestinal symptoms.

1.4. Differential diagnosis

In clinical practice, a diagnosis of PD is made when motor symptoms emerge and is based on the United Kingdom (UK) Queens Square Brain Bank Criteria: table 2 (Gibb and Lees, 1988). The presence of bradykinesia is an essential requirement. Either rest tremor, rigidity and or postural instability have to be present in a parkinsonian syndrome (Gibb and Lees, 1988). The following atypical features must be excluded during patient assessment: previous history of head trauma, stroke, encephalitis, neuroleptic exposure, absence of levodopa response, supranuclear gaze palsy, and early autonomic involvement (Gibb and Lees, 1988). Unilateral onset of symptoms, progressive rest tremor, levodopa responsiveness, dyskinesias, and hyposmia are all supportive of a diagnosis of idiopathic PD (Gibb and Lees, 1988). More recently, the Movement Disorders Society (MDS) task force, published the MDS-Clinical Diagnostic Criteria for PD: MDS-PD criteria (Postuma et al., 2015). These new criteria include some of the core features of the UK PD Brain Bank Criteria but also incorporated non-motor symptoms, since these are a vital component of the disease. Both clinical criteria use a staged approach in making a PD diagnosis, and require the presence of bradykinesia and either rest tremor or rigidity for Parkinsonism to be considered (Postuma et al., 2015, Gibb and Lees, 1988). But, there are a number of differences between the two criteria. Firstly, the MDS-PD Criteria do not include postural instability in defining Parkinsonism, since the presence of postural

Table 2: The UK Parkinson's Disease Society Brain Bank criteria

<p><i>Step 1 Diagnosis of Parkinsonian syndrome</i> * Bradykinesia (slowness of initiation of voluntary movement with progressive reduction * in speed and amplitude of repetitive actions) And at least one of the following: muscular rigidity 4-6 Hz rest tremor postural instability not caused by primary visual, vestibular, cerebellar, or proprioceptive dysfunction.</p> <p><i>Step 2 Exclusion criteria for Parkinson's disease</i> * History of repeated strokes with stepwise progression of parkinsonian features * History of repeated head injury * History of definite encephalitis * Oculogyric crises * Neuroleptic treatment at onset of symptoms * More than one affected relative * Sustained remission * Strictly unilateral features after 3 years * Supranuclear gaze palsy * Cerebellar signs * Early severe autonomic involvement * Early severe dementia with disturbances of memory, language, and praxis * Babinski sign * Presence of cerebral tumour or communicating hydrocephalus on CT scan * Negative response to large doses of levodopa (if malabsorption excluded) * MPTP exposure</p> <p><i>Step 3 Supportive prospective positive criteria for Parkinson's disease</i> (Three or more required for diagnosis of definite Parkinson's disease) * Unilateral onset * Rest tremor present * Progressive disorder * Persistent asymmetry affecting side of onset most * Excellent response (70-100%) to levodopa * Severe levodopa-induced chorea * Levodopa response for 5 years or more * Clinical course of 10 years or more</p>

Table 2 obtained from Gibb W, Lees A (1988) The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. *Journal of Neurology, Neurosurgery, and Psychiatry* **51**, 745-752, with permission from BMJ publishing group.

instability in the early stages of the disease indicates an alternative parkinsonian syndrome (Postuma et al., 2015). Because of the problems with sensitivity and specificity of available diagnostic criteria, the MDS Criteria divided the diagnosis into clinically established PD and clinically probable PD which allows use of this criteria for research purposes and also in making a diagnosis of PD in routine clinical practice (Postuma et al., 2015).

Another important distinction is that the occurrence of dementia in the early stages of the disease is not considered as an exclusion criteria, therefore the MDS-PD Criteria allows dementia with Lewy bodies to be classified as a subtype of PD (Postuma et al., 2015). The MDS-PD Criteria also include investigations such as cardiac metaiodobenzylguanidine (MIBG) scintigraphy and olfactory tests in its supportive criteria (Postuma et al., 2015).

Although the UK Brain Bank Criteria have been widely adopted, it is associated with a high misdiagnosis rate. Conditions such as essential tremor, Alzheimer's disease, along with other parkinsonian syndromes including Multiple System Atrophy (MSA), vascular Parkinsonism, and Progressive Supranuclear Palsy (PSP) are often misdiagnosed as idiopathic PD and vice versa (Hughes et al., 1992, Meara J, 1999). An early clinico-pathological study of 100 cases with a clinical diagnosis of PD made by neurologists and geriatricians reported a diagnostic error rate of 24%: 76 cases had pathological findings characteristic of PD and the 24% cases misdiagnosed as PD during life had MSA, PSP, and Alzheimer type pathology (Hughes et al., 1992)). In another study from North Wales, only 53% of patients treated with PD medications in the community fulfilled the UK PD Brain Bank Criteria for PD (Meara J, 1999). In a more recent clinico-pathological study, only 10% of cases diagnosed as PD using the clinical diagnostic criteria were redefined to other diagnoses at post-mortem, showing an improved diagnostic accuracy compared to previous studies (Hughes et al., 2001). In its early stages, PD is often difficult to diagnose. A study by Adler et al. (2014) used neuropathological

confirmation as the gold standard for PD diagnosis and showed low diagnostic accuracy of a clinical diagnosis of probable or possible PD especially in early disease: 26% and 88% respectively (Adler et al., 2014). In this study responsiveness to dopaminergic medication, longer disease duration and hyposmia increased the positive predictive value for probable PD (Adler et al., 2014).

The gold standard or definitive diagnosis of idiopathic PD can only be confirmed at post-mortem when characteristic features of nigrostriatal dopaminergic cell loss and intracytoplasmic inclusions or Lewy bodies are detected (Greenfield and Bosanquet, 1953). At present, there is no validated tool that can accurately diagnose PD and differentiate it from atypical Parkinsonism pre-mortem. Implications of incorrectly labelling patients with PD as atypical Parkinsonism or vice versa are multiple. Firstly, atypical parkinsonian syndromes such as MSA and PSP have a worse prognosis when compared to PD and response to the available dopaminergic treatments is often poor (Christine and Aminoff, 2004). Patients with non-degenerative Parkinsonism may be exposed to PD medications and their associated side effects, which may impair quality of life. Further, when assessing the effect of potential neuroprotective treatments, it is essential that the correct diagnosis is assigned since including patients with other diagnoses who might not respond to the treatment as well, might interfere with interpretation of results from such trials (Chahine and Stern, 2011).

1.5 Investigations

PD diagnosis is clinical and investigations are useful in excluding conditions that may mimic PD. In the UK, two imaging modalities are used in clinical practice: Magnetic Resonance Imaging (MRI) is used to exclude structural abnormalities; and functional imaging which aids in visualising dopaminergic cell loss in the nigrostriatal pathway using radionuclide imaging

techniques [Positron emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT)] (Brooks, 2010).

Magnetic resonance imaging

MRI has the advantage of being widely available, non-invasive and does not use radioisotopes (Peran et al., 2010). The utility of MRI in PD in clinical practice is limited to exclusion of secondary causes of Parkinsonism such as strokes, hydrocephalus and other structural abnormalities (Brooks, 2010, Brooks and Pavese, 2011). In the majority of patients with idiopathic PD, MRI brain is normal (Brooks, 2010). There may be a potential for use in distinguishing PD from MSA and PSP (Bhattacharya et al., 2002), but studies have shown conflicting results. Specific MRI features such as putaminal atrophy, middle cerebellar peduncle (MCP) signal abnormality and atrophy of the brainstem were found in patients with MSA-P (MSA- Parkinsonism) and not in any of the PD patients, so the authors proposed a potential role of MRI brain in aiding the diagnosis of MSA (Bhattacharya et al., 2002). Significantly more patients with MSA-P had putaminal hypodensity when compared to PD patients (Bhattacharya et al., 2002). In contrast, another study reported that putaminal hypodensity was not specific for either PD or MSA (Lee et al., 2004). Hyperintensity of MCP has also been shown to have high sensitivity and specificity: 85.2% and 100% respectively, in differentiating patients with MSA-C from PD, PSP, and controls. The ‘hot cross bun’ sign is another characteristic radiological feature which supports a clinical diagnosis of MSA (Shrivastava, 2007), and can be used to differentiate MSA cases from PD, but the sign has low specificity, as it also been demonstrated in patients with spinocerebellar ataxias (Lee et al., 2009). Measuring MCP width has also been proposed as a way of differentiating PD from MSA, with MSA patients showing significantly smaller MCP width compared to idiopathic PD patients (Nicoletti G, 2006). Longoni et al. (2011), used MRI as a diagnostic tool and

authors were able to separate PSP patients from PD and healthy controls. The authors measured the ratio of the pons to midbrain area (pons/midbrain: P/M), the MCP to superior cerebellar peduncle (SCP) width ratio (MCP/SCP) and calculated the $[P/M * MCP/SCP]$, a parameter called MR Parkinsonism index (Longoni et al., 2011). When compared to PD patients and controls, individuals with PSP had higher P/M ratios and MR Parkinsonism index, suggesting that midbrain atrophy can be used to separate these groups of patients (Longoni et al., 2011). The MR Parkinsonism index was shown to have high sensitivity and specificity in differentiating PD from PSP-Richardson syndrome patients: 100% and 92% respectively (Longoni et al., 2011). Similarly the P/M ratio also had high sensitivity (90%) and specificity (96%) in differentiating the two groups, but the same parameters did not achieve similar sensitivity in separating PSP-Parkinsonism from PD patients (Longoni et al., 2011). Other studies have also reported low sensitivity when MRI is used to differentiate between PD from atypical Parkinsonism (Lee et al., 2004, Meijer et al., 2012).

Radionuclide imaging

PET and SPECT imaging provide a direct way of estimating dopaminergic cell loss, a key pathological process in PD (figure 2). This is done by using radiotracers which bind to targets on the presynaptic or post synaptic dopaminergic terminals (Cummings et al., 2011). Examples of these radiotracers are shown in figure 2. SPECT imaging is more widely available, but PET provides better sensitivity and image resolution (Zhu et al., 2014). A significant drawback of using PET is radiotracers have shorter half-lives compared to SPECT ligands and therefore require onsite production of ligands which makes PET more expensive (Thobois et al., 2001).

Figure 2: PET and SPECT radio ligands and binding sites on the dopaminergic terminal

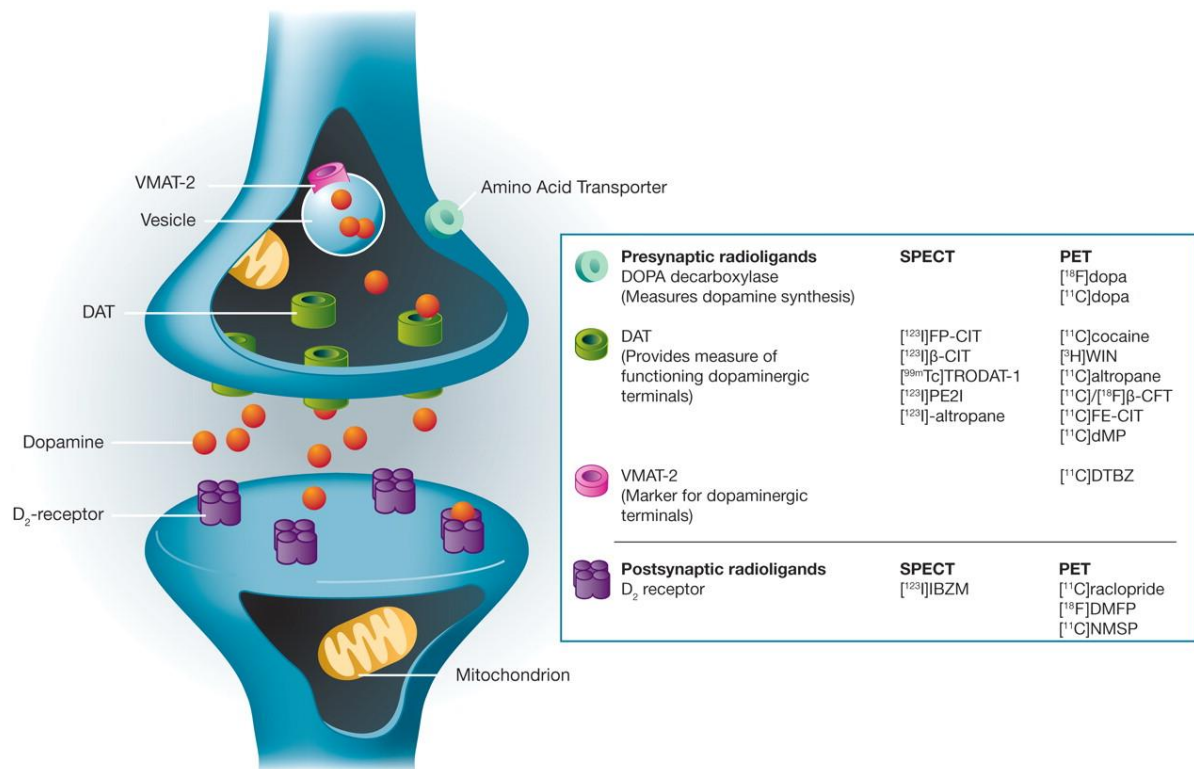


Figure obtained from Cummings JL, Henchcliffe C, Schaier S, Simuni T, Waxman A, Kemp P (2011). The role of dopaminergic imaging in patients with symptoms of dopaminergic system neurodegeneration. *Brain* **134**, 3146-3166, with permission from Oxford University press.

Schematic diagram showing location of the dopamine transporter (DAT), vesicular monoamine transporter (VMAT), amino acid transporter, and D₂ receptors on the dopaminergic terminal and examples of SPECT and PET radioligands which bind to each of these receptors. DAT (green) is present on the presynaptic membrane. Dopamine (orange), a DAT substrate and is transported into the presynaptic terminal via DAT, and is subsequently pumped into intracellular vesicles via VMAT (pink). D₂ receptors (purple) are present on the postsynaptic membrane.

Targets for radioligands in PD

Aromatic Acid Decarboxylase (AADC) activity

[¹⁸F]-DOPA, is a fluorinated levodopa analogue used in PET imaging for measuring dopamine synthesis, uptake into storage vesicles, and metabolism (Forsback et al., 2004). The probe is actively taken up by striatal dopaminergic neurons and decarboxylated to fluoro-dopamine by aromatic acid decarboxylase (AADC). Fluoro-dopamine is then transported into

storage vesicles (Ravina et al., 2005). [¹⁸F]-DOPA uptake has been shown to correlate with nigral cell counts (Snow et al., 1993). However, cell loss results in a compensatory AADC up regulation, therefore [¹⁸F]-DOPA uptake tends to overestimate dopaminergic innervation in the striatum (Bruck et al., 2009, Lee et al., 2000). In Parkinson's disease, conversion of DOPA to dopamine by AADC is thought to be the rate limiting step, therefore [¹⁸F]-DOPA is likely to reflect AADC activity (Ravina et al., 2005, Lee et al., 2000).

[¹⁸F]-DOPA PET shows reduced striatal uptake in PD patients compared to controls: figure 3 (Rinne et al., 2001). The reduced striatal uptake in PD is not homogeneous, with marked reduction in uptake in the putamen compared to the caudate: figure 3 (Bruck et al., 2009, Rinne et al., 2001, Lee et al., 2000). Tracer uptake in the putamen and caudate was shown to correlate with disease stage and severity of rigidity and bradykinesia but not tremor (Otsuka, 1996). In some studies, putamen uptake only correlated with motor and total Unified Parkinson's Disease Rating Scale (UPDRS) scores (Morrish, 1996).

Vesicular monoamine transporter (VMAT) ligands

[¹¹C] dihydrotetrabenazine (DTBZ) and (¹⁸F)-9-fluoropropyl- +-dihydrotetrabenazine PET ligands bind to VMAT2, which actively pumps dopamine into storage vesicles (Lin et al., 2014, Wilson and Kish, 1996). Up to 95% of VMAT binding in the striatum is associated with dopaminergic terminals (Frey et al., 1996)(Frey et al., 1996). (¹¹C) DTBZ is therefore considered to be a sensitive marker of presynaptic dopaminergic terminals with studies demonstrating reduced contralateral striatal binding in PD patients compared to controls: figure 3 (Frey et al., 1996, Bohnen et al., 2006, Martin et al., 2008). The pattern of uptake is similar to that reported with the dopamine transporter (DAT) showing more marked reduction in putamen than caudate uptake in PD compared to control (Bohnen et al., 2006, Frey et al., 1996). Reduction in (¹¹C) DTBZ striatal binding correlates with bradykinesia and disease

duration (Martin et al., 2008, Bohnen et al., 2006). A recent study used PET ligand (¹⁸F)-9-fluoropropyl- + -dihydrotetrabenazine to differentiate PD patients from healthy control showed sensitivity and specificity of 100% in differentiating PD patients from healthy controls (Lin et al., 2014). In another study ¹⁸F-9-fluoropropyl- + -dihydrotetrabenazine binding correlated with disease duration and UPDRS scores (Hsiao et al., 2014).

VMAT ligands are less likely to be influenced by compensatory mechanisms that occur secondary to dopaminergic terminal loss such as the up-regulation of AADC activity and reduced DAT expression (Vander Borght et al., 1995). In addition, VMAT binding is thought to be less susceptible to regulation by treatments that interfere with synaptic dopaminergic levels and post synaptic receptor function (Vander Borght et al., 1995, Wilson and Kish, 1996). This suggests that VMAT binding can be used as an accurate marker of dopaminergic neuronal integrity and also as potential tool for assessing response to treatment (Vander Borght et al., 1995). However, VMAT tracer binding in the brain is not specific to dopaminergic neurons and has been demonstrated in other regions rich in serotonergic and noradrenergic neurons such as locus coeruleus, raphe nucleus, and the hypothalamus (Wilson and Kish, 1996).

Post synaptic receptor ligands

PET ligand (¹¹C) raclopride and SPECT [¹²³I] iodobenzamine (1BZM) have been used to label post synaptic dopamine receptors (Brooks and Pavese, 2011). Striatal ¹¹C raclopride uptake is increased in PD compared to control, reflecting an up-regulation of dopamine D2 post synaptic receptors which occurs with dopaminergic neuronal loss (Rinne, 1995).

Dopamine transporter ligands

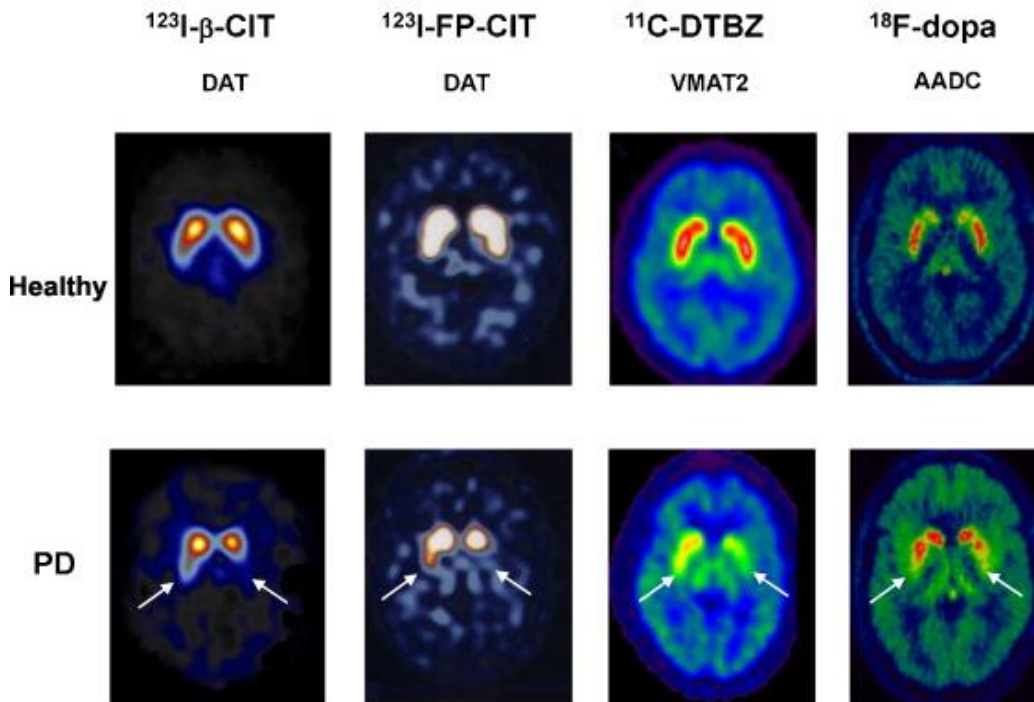
Most of the ligands used in PET and SPECT imaging are cocaine analogues with high affinity for DAT (Thobois et al., 2001). Examples of ligands used for SPECT imaging include Iodine-¹²³I-3-carbomethoxy-3-(4-iodophenyl)tropane (¹²³I B-CIT), ¹²³I FP-CIT, and [^{99m}Tc] TRODAT-1 (Cummings et al., 2011). PET DAT ligands include, [¹¹C] Cocaine, [³H] WIN, [¹¹C] altopane, [¹¹C]/ [¹⁸F] B-CFT and [¹¹C] FE-CIT (Cummings et al., 2011).

Nigrostriatal neuronal degeneration has been shown to be paralleled with a reduction in dopamine transporter density (Fischman, 1998). SPECT [¹²³I] B-CIT binding is reduced in both the PD striatum contralateral and ipsilateral to the affected side compared to controls (Vermeulen et al., 1995). [¹²³I] FP-CIT striatal uptake has also been assessed in early PD, late PD, and healthy controls. Results were in concordance with [¹²³I] B-CIT findings showing reduced tracer uptake in ipsilateral and contralateral caudate and putamen of PD patients compared to controls (Vermeulen et al., 1995). A more marked reduction in uptake in the contralateral striatum particularly the posterior putamen, was seen in PD patients than controls (Vermeulen et al., 1995, Booij et al., 1997). Similarly, striatal hypofunction has been demonstrated in PD using other DAT ligands: [¹²³I]PE21 (Prunier et al., 2003) [¹²³I]altropane (Fischman, 1998), [^{99m}Tc]TRODAT-1 (Huang et al., 2001), [¹¹C]B-CFT (Laihinen et al., 1995), and [¹⁸F]CFT (Rinne et al., 2001). The SPECT ligands [¹²³I] B-CIT and [¹²³I] altopane showed comparable sensitivity to [¹⁸F]-DOPA PET in distinguishing early PD from healthy controls (Ishikawa et al., 1996, Fischman, 1998). DAT radiotracer uptake has also been shown to correlate with disease stage (Booij et al., 1997, Laihinen et al., 1995, Prunier et al., 2003), bradykinesia, and rigidity scores (Benamer et al., 2003).

The ability of SPECT and PET tracers to discriminate early PD from healthy controls supports its use as a diagnostic tool in early PD. [¹²³I] FP-CIT (DaTSCAN) is now widely available for

use in clinical practice and is recommended for use in cases of diagnostic uncertainty (Berardelli et al., 2013).

Figure 3: Images of healthy control and PD striatum using SPECT (DAT and VMAT) and [18F]-DOPA PET ligands.



Images above show the reduction in ligand uptake in the posterior putamen in PD
 Figure obtained from Brooks DJ, Pavese N (2011) Imaging biomarkers in Parkinson's Disease. *Progress in Neurobiology* **95**, 614-628, with permission from Elsevier

Transcranial sonography (TCS)

In some countries, transcranial ultrasonography (TCS) is used in clinical practice to provide supportive information for PD diagnosis. TCS typically shows substantia nigra hyperechogenicity in PD subjects which is not found in healthy individuals (Li et al., 2016). Iron accumulation is thought to contribute to substantia nigra hyperechogenicity in PD (Berg et al., 2002). A systematic review and meta-analysis which included 31 studies from 13 different countries assessed the diagnostic accuracy of TCS in PD (Li et al., 2016). Data for

1 926 individuals with PD and 2 460 controls was included. Pooled data showed that TCS had a high sensitivity and specificity of 83% and 87% respectively when used in making a PD diagnosis (Li et al., 2016).

In addition to its role in discriminating PD from healthy controls, TCS has also been shown to be useful in differentiating PD from atypical Parkinsonism. Normal substantia nigra echogenicity is suggestive of MSA-P and hyperechogenicity of the lentiform nucleus combined with dilatation of the third ventricle is indicative of PSP (Walter et al., 2007). However, TCS has its limitations. Up to 60% of individuals in the Asian population may have an insufficient temporal window and it is not always possible to perform TCS in these individuals (Li et al., 2016). Sensitivity and specificity figures reported across different studies have not been consistent (Li et al., 2016). Lastly substantia nigra hyperechogenicity may not be specific to PD as it was also reported in 70% of patients with amyotrophic lateral sclerosis (Fathinia et al., 2013) and 9% of healthy controls (Berg et al., 2002).

1.6. Biomarkers

According to the Biomarkers Definitions Working Group, a biomarker is a ‘characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacological response to a therapeutic intervention’ (Biomarkers Definitions Working Group, 2001). Several clinical, biological and imaging tools have been studied in PD and some of the imaging tools are now in use in clinical practice. These tools have shown promising results as potential biomarkers, but most are associated with major drawbacks. Figure 4 summaries the role of biomarkers in PD. In PD biomarkers maybe used in pre-symptomatic diagnosis, which provides a window for use of neuroprotective treatments. A tool which provides high diagnostic sensitivity and specificity is required. This will aid in identifying patients with early stage disease before significant neurodegeneration

occurs, and such patients may benefit from disease modifying treatments or cure when these treatments become available. Tools for assessing disease severity and progression are also required in PD. At present, the UPDRS is the standard tool for assessing disease severity, progression, and response to treatment in clinical practice and trials (Movement Disorder Society Task Force on Rating Scales for Parkinson's Disease, 2003). Its use has been limited by poor inter-rater reliability as reported in some studies (Movement Disorder Society Task Force on Rating Scales for Parkinson's Disease, 2003). UPDRS scores also improve with symptomatic treatment for PD making it less ideal to assess disease progression in clinical trials of disease modifying treatments (Morgan et al., 2010). More recently, the Movement Disorders Society has modified the UPDRS to deal with these shortcomings and to include non-motor symptoms assessment (Goetz et al., 2008). A recent systematic review showed the lack of evidence for a robust biomarker for assessing disease progression in PD and also highlighted the significant methodological flaws in the 183 included studies. (McGhee et al., 2013) Major limitations were the small sample sizes, insufficient data reporting, limited statistical analyses, and the cross-sectional nature of a large proportion (85%) of the studies. (McGhee et al., 2013) In view of these shortcomings, the review authors made several recommendations for future studies. Careful selection of a specific pathophysiological aspect of the disease which can be measured reliably across studies is pivotal in biomarker development. (McGhee et al., 2013) In addition, further progress can be made improving the quality of the studies. Ideally biomarker studies should include of large sample sizes and be conducted in a longitudinal, and prospective fashion with sufficient follow up periods to allow measurement of clinical and biomarker outcomes at several time points. (McGhee et al., 2013)

Figure 4: The role of biomarkers in premotor and symptomatic phases of Parkinson's disease

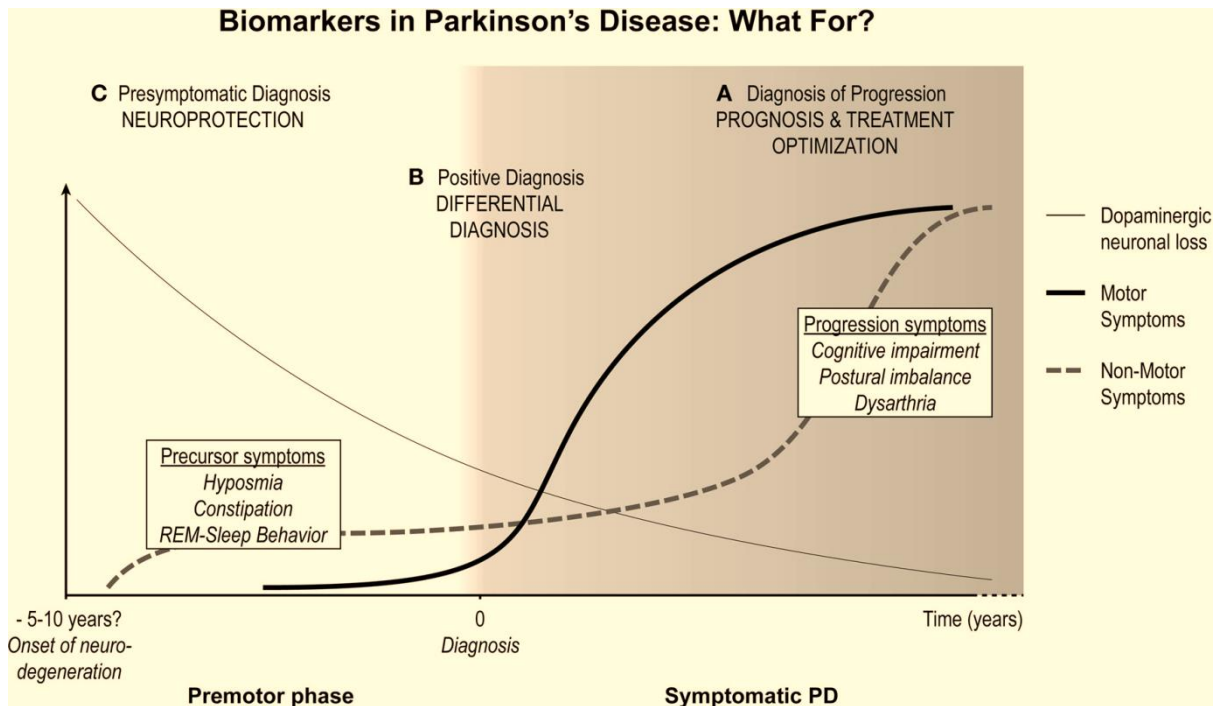


Figure 4 obtained from Lebouvier T, Tasselli M, Paillusson S, Pouclet H, Neunlist M, Derkinderen P (2010) Biopsable neural tissues: toward new biomarkers for Parkinson's disease? *Frontiers in Psychiatry* 1. Open access article

1.6.1. Clinical markers

As alluded to in the previous section, the presence of motor features is critical in making a clinical diagnosis of PD but these symptoms occur when a significant proportion of dopaminergic neurons have been lost (Bernheimer et al., 1973). Olfactory dysfunction, RBD, constipation, and depression occur in the premotor phase and therefore have a potential role in premotor diagnosis: figure 4 (Morgan et al., 2010, Lebouvier et al., 2010). Olfactory deficits are prevalent in people with PD and a recent meta-analysis showed that this non-motor symptom occurs in approximately 76% of PD patients, (Chen et al., 2015) and predates the motor syndrome by at least 4 years (Ross et al., 2008). Thus, screening for hyposmia may

be useful for premotor diagnosis of PD. Further, other Parkinsonian syndromes (MSA, PSP, and CBD) are often associated with little or no olfactory deficit, allowing this clinical marker to be used to differentiate PD from other parkinsonian syndromes (Hawkes, 2003), although this may be limited by low specificity and sensitivity (Hawkes, 2003). In addition, hyposmia cannot be used to reliably distinguish PD from other neurodegenerative conditions such as Alzheimer's disease and DLB which may also be associated with marked olfactory deficits (Hawkes, 2003, Chahine and Stern, 2011). Another limitation is that olfactory dysfunction does not correlate with disease activity, hence is not a useful marker for disease progression (Chahine and Stern, 2011). The short latency period between hyposmia detection and PD diagnosis, (Ross et al., 2008) may also limit the window for potential use of neuroprotective treatments.

RBD also predates the development of motor features in the synucleopathies by over 10 years (Iranzo et al., 2006). The long latency before onset of motor symptoms makes it ideal in the preclinical diagnosis of PD. The 10 year risk of developing a neurodegenerative disorder in individuals with RBD was reported to be approximately 41%, in a study by Postuma and colleagues (Postuma et al., 2009a). A more recent study showed that an even higher proportion of patients (82%) with RBD developed a synucleopathy (Iranzo et al., 2013).

Constipation also predates the motor syndrome in PD (Abbott et al., 2003). A major disadvantage of employing this as a clinical marker is that, the prevalence of constipation is much higher than that of PD in the general population, which reduces its specificity as a preclinical marker (Postuma et al., 2010).

Other non-motor symptoms such as depression and anxiety are also potential preclinical markers, but utility as preclinical markers is limited by low specificity (Postuma et al., 2010).

Another study showed that other preclinical markers of PD such as olfactory dysfunction, abnormal colour vision, and autonomic dysfunction were more common in patients with RBD compared to controls (Postuma et al., 2009b). Therefore, a combination of these clinical markers may increase the utility of RBD in preclinical diagnosis.

1.6.2. Biochemical markers

Biological markers in both saliva, blood and CSF have shown potential utility in diagnosis and disease progression (Chahine and Stern, 2011, Morgan et al., 2010). Use of these markers is advantageous because samples are easy to obtain and costs are low. CSF markers including alpha-synuclein, and beta amyloid 1-42 (A β 1-42), neurofilament light chain protein (NFL), DJ-1, and tau protein have been evaluated, but results have not been consistent across studies (Bäckström et al., 2015, Herbert et al., 2015, Waragai et al., 2006). A recent prospective study including 128 patients with Parkinsonism and 30 healthy controls evaluated the utility of several CSF markers including NFL, alpha-synuclein, A β 1-42, and heart fatty acid binding protein as diagnostic markers in PD (Bäckström et al., 2015). Alpha-synuclein levels were similar in both PD and healthy controls, (Bäckström et al., 2015) which contrasts with findings from previous studies which demonstrated lower alpha-synuclein levels in PD patients compared to controls (van Dijk et al., 2014, Mollenhauer et al., 2011). Bäckström and colleagues also reported lower A β 1-42 levels and elevated NFL levels in the PD group compared to controls, (Bäckström et al., 2015) suggesting a role of NFL and A β 1-42 in PD diagnosis. In the same study, the authors were able to separate PD patients from those with PSP using CSF NFL and A β 1-42 levels: PSP patients had higher CSF NFL levels and low CSF A β 1 protein compared to PD and at 1 year follow up (Bäckström et al., 2015). The

NFL/A β 41 ratio had high specificity (93%) and sensitivity (89%) for differentiating PSP from PD (Bäckström et al., 2015). However, MSA patients could not be separated from PD based on these markers, (Bäckström et al., 2015) which conflicts with the findings of a previous report: Hebert et al. (2015) reported higher CSF NFL levels in MSA than PD and suggested that this marker could be used with a high degree of accuracy to discriminate PD from MSA (Herbert et al., 2015). In terms of predicting patients who may develop PD dementia, high CSF NFL, low A β 1-42, and high heart fatty binding protein may be useful (Bäckström et al., 2015). Similarly, Terrelonge and colleagues reported an association between low baseline CSF A β 1-42 levels and development of cognitive impairment at 2 years in patients with early drug naïve PD (Terrelonge et al., 2015). The role of plasma and CSF DJ-I levels in PD diagnosis has also been assessed and was shown to be significantly higher in PD patients compared to non-PD controls (Waragai et al., 2006, Waragai et al., 2007). Conversely, another study found no difference in DJ-1 levels in the serum of PD patients and age-matched healthy controls (Maita et al., 2008). It has also been shown that CSF DJ-1 has no correlation with disease stage (Waragai et al., 2006).

Correlation between CSF markers and dopaminergic deficit on Positron Emission Tomography (PET) imaging was evaluated in a small study which included eight patients with PD secondary to *LRRK2* mutation and 18 symptomatic carriers (Shi et al., 2012). They found no association between CSF DJ-1 or alpha-synuclein with imaging findings and no correlation between the CSF marker levels and disease progression in asymptomatic *LRRK2* carriers (Shi et al., 2012). Others studies have compared salivary DJ-1 levels in PD patients and healthy controls and found no significant difference between the two groups (Kang et al., 2014). Salivary DJ-1 levels were significantly higher in patients with advanced disease (H&Y stage 4) compared to earlier stages of the disease and healthy controls (Kang et al., 2014).

The same study also demonstrated a weak correlation between DJ-1 levels and putamen (99m)Tc-TRODAT-1 uptake suggesting a potential role of this marker in assessing disease progression (Kang et al., 2014). Other authors have shown an increased risk of cognitive decline with low plasma epidermal growth factor levels (Chen-Plotkin et al., 2011) suggesting a role predicting cognitive decline in PD.

So far, studies which assessed utility of biological fluids as diagnostic or prognostic markers have produced conflicting results. This may be due to the use of different assays or methods for assessing the markers. Standardisation of these assays may provide more reliable or uniform results. Another limitation is the small sample sizes used in the studies. Larger studies employing standardised techniques are required to confirm or refute usefulness of these markers in PD.

1.6.3. Imaging markers

There has been major progress in imaging biomarker research in PD in the last 20 years. MRI, PET, and SPECT imaging techniques have shown promising results as potential biomarkers in PD but these tools also have their disadvantages.

1.6.3.1. Magnetic resonance Imaging in Parkinson's disease

Although the role of MRI as a clinical diagnostic tool at present is only limited to excluding structural abnormalities, various MRI techniques have been developed, and shown to have potential use as biomarkers in PD.

MRI and iron imaging

Iron accumulation in the substantia nigra is one mechanism thought to underlie the pathological process in PD, by facilitating free radical formation and lipid peroxidation

(Griffiths et al., 1999, Dexter et al., 1987). Pathological studies have shown increased iron content in the substantia nigra of PD brains compared to non-PD controls (Dexter et al., 1987, Griffiths et al., 1999). MRI relaxometry including T2 and T2* weighted images have been used to measure iron content in the brain, and on these sequences iron containing structures appear hypointense (Lehericy et al., 2012). An increase in T2 relaxation rate (R2) and shortening of T2 relation time correspond with increased iron concentration (Wallis et al., 2008). Studies have shown significantly higher R2 in the substantia nigra of PD patients compared to healthy volunteers, which correlated with increased iron content in PD (Graham et al., 2000, Ulla et al., 2013). Iron accumulation measured by these MRI techniques also correlated with disease severity, suggesting a potential role of MRI as a prognostic marker for motor progression (Wallis et al., 2008, Ulla et al., 2013). Nevertheless, other studies did not find a similar correlation between disease severity and iron accumulation (Graham et al., 2000). Susceptibility weighted imaging (SWI) is another technique for estimating iron deposition using phase imaging and is dependent on the magnetic susceptibility of tissues relative to their surroundings (Haacke et al., 2004, Zhang et al., 2010). SWI has been used to discriminate PD from controls and SWI parameter showed correlation with UPDRS scores (Zhang et al., 2010). Another small study used SWI to differentiate PD from MSA patients on the basis of higher iron deposition in the putamina of MSA-P patients (Wang et al., 2012b).

Magnetisation Transfer

Magnetisation Transfer (MT) improves the differentiation between grey and white matter by measuring energy transfer between protons in dense tissues such as myelin and free water after MT pulse (Lehericy et al., 2012). MT ratio (MTR) corresponds to the degree of axonal and myelin loss which gives a reflection of the underlying pathology (Lehericy et al., 2012). MTR can be abnormal when there are no detectable changes on conventional MRI sequences

(Tambasco et al., 2011). MTR has been used in differentiating parkinsonian syndromes but other studies showed inconsistent results (Eckert et al., 2004, Tambasco et al., 2003, Hanyu et al., 2001). When used to assess brain regions that are affected by the disease in patients with different disease severity, Tambasco et al. (2011) reported reduced MTR in the following brain regions of all PD patients compared controls: substantia nigra, putamen, parietal and periventricular white matter (Tambasco et al., 2011). When PD patients with more advanced disease were compared to those with mild disease and controls other brain regions including the frontal white matter, lateral thalamus, caudate, and pons were also affected (Tambasco et al., 2011). These findings suggest that this technique may be used to track progression of neurodegeneration, but this requires further verification in larger studies.

Magnetic Resonance Diffusion Tensor Imaging (DTI)

Magnetic Resonance DTI is an MRI technique which measures two parameters: mean diffusivity (MD), which increases with alterations of microstructures and increase in fluid accumulation in the extracellular space, and anisotropy which, measures the ease of diffusion of water molecules along white matter tracts (Lehericy et al., 2012). Disruption of neural tracts due to any pathology, restricts movement of water molecules, which is reflected by a reduction in fractional anisotropy: FA (Brooks, 2010). In MPTP animal model of PD, DTI showed increased mean diffusivity (MD) and reduced fractional anisotropy (FA) in the substantia nigra compared to control (Boska et al., 2007). A significant correlation between dopaminergic cell loss in the substantia nigra and transverse diffusivity was also found (Boska et al., 2007). DTI has also been used in human studies to differentiate patients with PD from healthy controls and demonstrated significant reduction in substantia nigral FA of PD patients (Vaillancourt et al., 2009, Chan et al., 2007, Peran et al., 2010). Vaillancourt et al. (2009) reported sensitivity and specificity of 100% in differentiating PD from healthy controls when

using 3.0T MRI DTI sequences (Vaillancourt et al., 2009). The study demonstrated reduced FA in the middle and caudal part of the substantia nigra with the greatest significance in FA of caudal of the substantia nigra (Vaillancourt et al., 2009). This finding corresponds to the pattern of regional neurodegeneration in the substantia nigra which has been demonstrated in pathological studies (Fearnley and Lees, 1991). In contrast to this, Acquino et al. (2013) reported no difference in substantia nigra FA and MD between controls and PD patients (Acquino et al., 2013). Also, a recent systematic review and meta-analysis of studies on FA and MD in PD showed an increase in MD in substantia nigra and no significant reduction in FA (Schwarz et al., 2013).

DTI has been used to differentiate PD from atypical Parkinsonism (MSA and PSA) with 90% sensitivity and 100% specificity when combining different measures of the technique (Prodoehl et al., 2013).

DTI is a promising diagnostic technique but it has produced inconsistent results across different studies. This may be a result of different image analysis techniques and MRI field strength used in these studies (Chan et al., 2007, Vaillancourt et al., 2009, Schwarz et al., 2013).

The role of MR DTI in preclinical diagnosis has also been explored. Scheffler et al. (2011) showed reduced FA in the midbrain and rostral pons and increased MD in pontine reticular formation of patients with idiopathic RBD compared to control (Scherfler et al., 2011). These findings are consistent with the location of brainstem nuclei that may be involved in RBD pathogenesis (Scherfler et al., 2011) and RBD is known to precede neurodegenerative disorders including PD. Longitudinal studies are now required to provide information on whether MRI techniques such as DTI are useful tools in the preclinical diagnosis of PD.

Magnetic resonance spectroscopy

Magnetic resonance spectroscopy (MRS) provides spectral information on different metabolites including creatine, N-acetyl aspartate (NAA), choline (Cho), myoinositol, and lactate (Lehericy et al., 2012). Levels of metabolites in tissues give an indication of the ongoing molecular or metabolic processes in the particular brain region where they are measured (Lehericy et al., 2012). The disadvantage of using such a technique in PD is that the substantia nigra is small, which causes difficulties in detecting metabolic changes, and iron accumulation in the structure interferes with the quality of the data produced on MRS (Groger et al., 2011). Use of high field strength is likely to resolve this problem (Zhou et al., 2014).

Groger et al. (2011) used 3T MRS to compare substantia nigra metabolic changes between PD patients and controls (Groger et al., 2011). The authors reported decreased NAA/Cr ratio in the rostral and increased NAA/Cr ratio in caudal substantia nigra in PD patients when compared to healthy subjects (Groger et al., 2011). Similarly, a recent study which used 3T MRS measured the ratio of sum of the NAA and *n*-acetyl aspartate glutamate concentration and Cr (tNAA/Cr) in the substantia nigra of PD patients and compared it to age-matched controls and reported lower tNAA/Cr in PD patients at baseline (Seraji-Bozorgzad et al., 2015). There was a 4.4% decline in the ratio at three months follow up in the PD group but this was not seen in the control group, implying a potential role of MRS in assessing progression (Seraji-Bozorgzad et al., 2015). Study authors also reported an asymmetry in the tNAA/Cr in the substantia nigra of PD patients when the symptomatic and asymptomatic sides were compared (Seraji-Bozorgzad et al., 2015). Several other studies have also measured NAA/Cr ratios in other brain regions including the motor cortex, (Lucetti et al., 2001), temporo-parietal cortex, (Hu et al., 1999) and posterior cingulate cortex (Camicioli et al.,

2004) and reported lower values in PD patients compared to control groups. The low NAA levels suggest neuronal loss and also mitochondrial dysfunction, a potential pathophysiological mechanism in PD (Ciurleo et al., 2014). In contrast, Federico et al. (1997) did not find any difference in metabolite ratios between PD patients and controls when MRS of the lentiform nucleus was performed (Federico et al., 1997). However, they did report significant differences between PSP and MSA patients compared to control, (Federico et al., 1997) implying a potential use of MRS in differentiating between PD and atypical Parkinsonism (Federico et al., 1997).

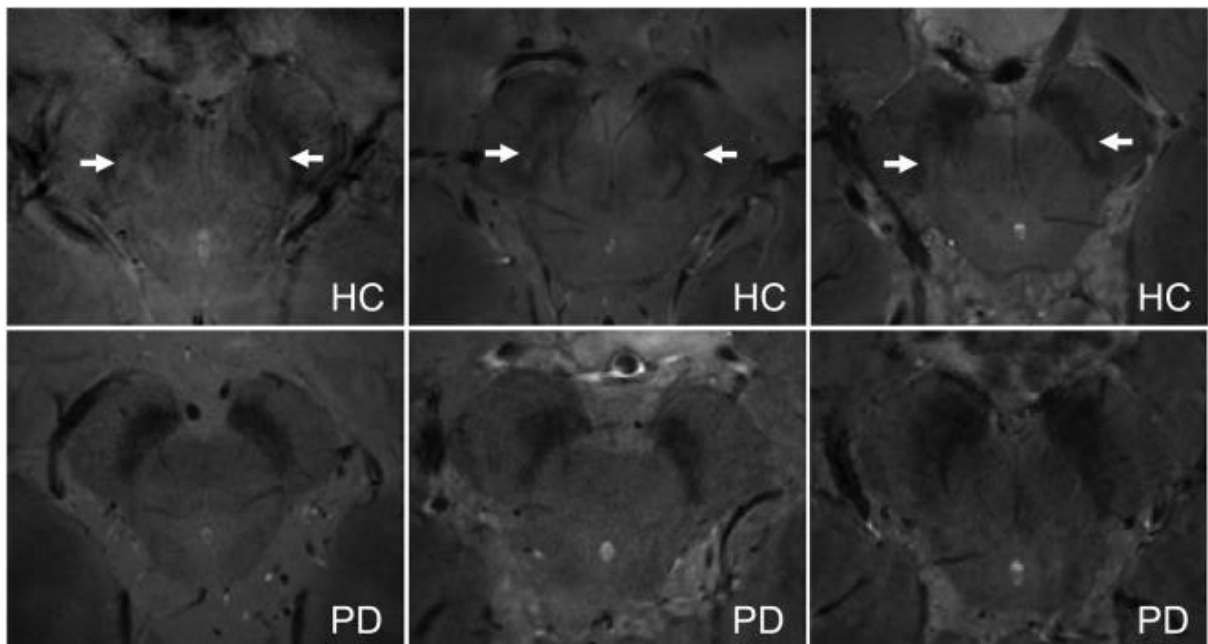
MRS has also been used to assess the effect of dopaminergic treatments on different metabolites and reported conflicting results. For example, Chaudhuri et al. (1996) reported reduced NAA/Cr and NAA/Cho ratio in the putamen of dyskinetic and levodopa treated PD patients compared to controls (Chaudhuri et al., 1996). In contrast Ellis et al. (1997) showed reduced NAA/Cho in the putamen of untreated PD patients compared to controls but no difference in the ratio when levodopa treated PD patients were compared with controls.(Ellis et al., 1997) There was no significant difference in NAA/(Cr+ProCr) when PD and control groups were compared (Ellis et al., 1997). Ciurleo et al. (2015) measured NAA/Cr and NAA/cho and Cho/Cr ratio in the motor cortex of PD patients and controls and reported lower NAA/Cr and NAA/Cho ratios in PD patients at baseline. After 10 months of ropinirole treatment, PD patients showed a significant increase in NAA/Cr and NAA/Cho ratio which correlated with UPDRS motors scores (Ciurleo et al., 2015).

These findings suggest that MRS may have a potential role in diagnosis, differentiating PD from its atypical forms, and in assessing effects of treatment (Ciurleo et al., 2014).

High field strength MRI

Dopaminergic neuron degeneration is difficult to image using MRI techniques currently available in routine clinical practice using field strengths up to 3T. Recent small studies have shown that MRI sensitivity as a diagnostic tool in PD can be increased if ultra-high field strengths are used. Using 7T MRI, Blazejewska and colleagues were able to delineate nigrosome 1, a substructure of the substantia nigra pars compacta containing the highest density of dopaminergic neurons (Blazejewska et al., 2013). Five nigrosomes make up the pars compacta which stain poorly for the protein calbindin D_{28k}, and Nigrosome 1, which is lens shaped, is the largest of these sub-regions (Damier et al., 1999). Blazejewska et al. (2013) compared midbrains of 10 PD patients and eight healthy controls using 7T MRI (T2*, and MT-T weighted images). They were able to differentiate the two groups based on the presence of T2* weighted and MT-T1 hyperintense structure (nigrosome 1) in the dorsal part of the substantia nigra pars compacta in healthy controls (figure 5). This area of hyperintensity was not seen in PD brains because of increased iron accumulation and loss of dopaminergic neurons in the nigrosome 1 (Blazejewska et al., 2013). This was consistent with MRI and histology finding which confirmed low neuromelanin and tyrosine hydroxylase levels in the nigrosome 1 of the PD brain (Blazejewska et al., 2013).

Figure 5: 7T MRI (T2* weighted images) of Parkinson's disease patients versus controls.



PD: Parkinson's disease, HC: healthy control.

Figure 5 obtained from Blazejewska AI, Schwarz ST, Pitiot A, Stephenson MC, Lowe J, Bajaj N, Bowtell RW, Auer DP, Gowland PA (2013) Visualization of nigrosome 1 and its loss in PD: Patho-anatomical correlation and in vivo 7 T MRI. *Neurology* 81, 534-540. With permission from Wolters Kluwer Health.

Similarly, an area of hyperintensity in the dorsomedial substantia nigra pars compacta was seen in controls subjects but not in PD patients in another study, which used 7T MRI brain (Kwon et al., 2012). The authors performed a retrospective analysis of MRI data and showed that nigrosome 1 could be clearly visualised using 3T MRI and they were able to separate PD patients from non-PD controls with high sensitivity and specificity (100% and 90% respectively) (Schwarz et al., 2014). A group in Korea performed 7T MRI T2* weighted sequences on eight PD patients and eight controls and were able to show morphological differences in the lateral border of the substantia nigra between the two groups (Cho et al., 2011). The authors also measured the distance between the midline (measured from anterior-posterior line) to the lateral border of the substantia nigra and calculated the 'sum of absolute

differences' (Cho et al., 2011). A significant difference in this parameter was found when PD patients and controls were compared (Cho et al., 2011). One limitation of this study was that data analysis was not uniform: the mean sum of absolute difference for the affected side was calculated for PD patients, whereas for controls the mean for the right and left side was determined (Cho et al., 2011).

These studies show that ultra-high field MRI has potential for use as a diagnostic tool, but studies have been limited by small numbers. In addition, these techniques have been used in individuals with well-established disease, therefore utility of these tests in very early disease is uncertain. Further work in larger cohorts with early disease is required to confirm these findings.

Using Multiple MRI techniques

Combining various MRI techniques described above may increase the reliability and utility of MRI as a potential biomarker in PD (Peran et al., 2010). In a study which used 3T MR imaging including fractional anisotropy and mean diffusivity and R2* sequences, Peran et al (2010) reported a high discriminating power exceeding 95% between PD patients and controls (Peran et al., 2010). When used in isolation each of the parameters had much lower discriminating power (Peran et al., 2010). Similarly, Menke et al. (2009) used driven equilibrium single pulse observation of T1 (DESPOT1) which accurately and quickly determines relaxation time and DTI on a 3T MR scanner to obtain data for 10 PD patients and an equal number of controls (Menke et al., 2009). Combining the two parameters increased the sensitivity and specificity of distinguishing PD patients and controls to 100% and 80% respectively (Menke et al., 2009).

In conclusion, MRI in PD is a promising biomarker but the field is still developing. Studies using different MRI techniques have shown changes which correspond to pathological

processes and structural changes associated with neurodegeneration in PD. Each technique has been used to measure different aspects of PD (e.g. iron accumulation, microstructural alterations and atrophy), but these techniques remain suboptimal when used in isolation. There is a suggestion of increased sensitivity with using multimodal MRI including ultra-high field strength. More work is required to validate these techniques before they can be applied into routine clinical practice.

1.6.3.2. Radionuclide imaging in Parkinson's disease

Role of PET and SPECT as diagnostic biomarker

As discussed in the section above, PET and SPECT imaging demonstrates striatal hypofunction in PD patients but not in healthy controls (Lin et al., 2014, Bruck et al., 2009, Rinne et al., 2001, Vermeulen et al., 1995, Booij et al., 1997). But controversy surrounds sensitivity of these imaging tools as diagnostic markers in early PD. For example, SPECT ^{123}I -F-CIT showed dopaminergic deficit in only 87% (n=33) of 38 patients who fulfilled the UKPD diagnostic criteria for PD at base line (Benamer et al., 2003). Similarly, in a multicentre prospective study (REAL PET study) to compare the progression in dopaminergic deficit progression in patients on levodopa and ropinirole using [^{18}F]-DOPA PET, 11.3% of the 186 patients had normal [^{18}F]-DOPA striatal uptake and this remained normal in 19 of these patients after a 2 year follow up period (Whone et al., 2003). In another large treatment trial, 14.7% of patients with a clinical diagnosis of PD had normal B-CIT SPECT and this remained unchanged at 4 years (The Parkinson Study Group, 2004). This group of patients with an apparent parkinsonian syndrome but no evidence of nigrostriatal deficit are now referred to as 'Scans Without Evidence of Dopaminergic Deficit' or SWEDDS (The Parkinson Study Group, 2004). 150 of the patients who had a clinical diagnosis of PD based on the current clinical criteria and normal scans were followed up for 3 years and only 3%

retained the initial PD diagnosis and their scans remained normal (Marshall et al., 2006). There are two possible explanations for the discrepancy between the clinical and radiological diagnosis. It is possible that the SWEDDS were incorrectly labelled as having PD (Marshall et al., 2006). Support for this notion comes from results of the PRECEPT study, a RCT trial to assess the safety and effect of CEP-1347 as a disease modifying treatment for PD. The trial enrolled 806 early PD patients of which 799 had ^{123}I -CIT SPECT performed at baseline (Marek et al., 2014). 11% (n=91) of the latter group were SWEDDS (Marek et al., 2014). 72 of the SWEDDS had follow up scans at 22 months and of these 92% (n=66) remained in the SWEDDS category (Marek et al., 2014). In terms of clinical progression, those who had abnormal scans at baseline had a greater change in both the total and motor UPDRS scores compared to the SWEDDS (Marek et al., 2014).

A recent systematic review of SWEDDS follow up studies showed that a large proportion have diagnoses other than PD, including adult onset dystonia (Erro et al., 2016). This was supported by the findings that they had no bradykinesia, similar electrophysiological findings to those with other dystonias, no non-motor symptoms, and a lack of progression characteristic of PD (Erro et al., 2016). Other conditions such as normal pressure hydrocephalus, age-related subtle extrapyramidal signs, essential tremor, fragile X syndrome, and depression may also mimic PD (Erro et al., 2016). Alternatively, it is possible that PET and SPECT imaging have limited sensitivity in early PD (The Parkinson Study Group, 2004). A small proportion (3-6%) of SWEDDS showed clinical progression and have abnormal scans on follow up (Marek et al., 2014, Marshall et al., 2006). In addition another study which assessed MIBG cardiac uptake in PD patients, SWEDDS and controls identified two SWEDDS who showed clinical progression, levodopa responsiveness, had olfactory dysfunction, and reduced MIBG uptake (Jang et al., 2013). This finding suggests that this

subgroup of SWEDDS patients probably had false negative DAT SPECT imaging, which calls into question the sensitivity of dopamine transporter scan in early PD. De La Fuente-Fenandez (2012) used a mathematical formula to calculate the diagnostic accuracy of DAT SPECT imaging in PD and reported similar diagnostic accuracy to that of a clinical diagnosis in early and later disease: 84% and 98% respectively (de la Fuente-Fernández, 2012). The accuracy of DAT SPECT imaging in detecting degenerative Parkinsonism was assessed using post-mortem data and sensitivity and specificity of 85% and 86% was reported (US FDA PCNS Advisory Committee Briefing document, 2009). Since the diagnostic accuracy of both DAT SPECT and clinical diagnosis in early disease is not 100%, neuropathological confirmation is still required to provide a definitive diagnosis. A more reliable diagnostic biomarker for early disease is therefore needed.

Role of PET and SPECT in pre-motor diagnosis

Radionuclide imaging has also been used to detect premotor PD. Adam et al. (2005) used PET tracer [¹⁸F]-DOPA and SPECT tracers: ¹¹C- DTBZ and ¹¹C-MP and compared imaging findings of 15 family members of a *LRRK2* mutation (four symptomatic and 12 asymptomatic) versus 33 healthy controls and 67 patients with sporadic PD (Adams et al., 2005). The pattern of SPECT and PET tracer reduction in the sporadic PD group were similar to those with symptomatic *LRRK2* mutation (Adams et al., 2005). The study also showed reduced DBTZ and MP binding in four asymptomatic *LRRK2* mutation carriers (Adams et al., 2005). Similarly, PET and SPECT abnormalities were reported in asymptomatic *LRRK2* carriers, in another study (Nandhagopal et al., 2008). When compared to healthy controls, two of the asymptomatic *LRRK2* carriers showed progression in PET findings on follow up

assessment, and one of these subjects developed clinical PD (Nandhagopal et al., 2008). These findings suggest a potential role of radionuclide imaging in prodromal disease.

Role of PET and SPECT in the differential diagnosis of PD.

[¹²³I] FP-CIT has been employed to differentiate PD from essential tremor with sensitivity of 96.5% and 100% specificity for essential tremor (Benamer et al., 2000). Similarly, Asenbaum et al. (1998) reported normal striatal uptake in essential tremor patients and was able to separate PD from essential tremor patients with 93% specificity and 100% sensitivity using [¹²³I] B-CIT (Asenbaum et al., 1998). Dystonic tremor may be mistaken for the resting tremor of early PD, and [¹²³I] FP-CIT has also been used in differentiating the two, by showing normal striatal uptake in the earlier group (Schneider et al., 2007).

In differentiating idiopathic PD from atypical Parkinsonism, SPECT imaging has poor sensitivity (Burn et al., 1994, Pirker et al., 2000). The characteristic feature of all these parkinsonian syndromes is neuronal degeneration in the substantia nigra. [¹⁸F]DOPA and [¹²³I]B-CIT imaging reliably discriminates these syndromes from healthy controls by demonstrating reduced striatal uptake (Pirker et al., 2000, Burn et al., 1994). However, there is no difference in [¹²³I]B-CIT binding between PD and other parkinsonian syndromes (Pirker et al., 2000). Although Burn et al. (1994) showed that by using discriminant function analysis, PD patients could be separated from PSP patients 90% of the time using ¹⁸F-DOPA imaging, the study showed that the test was less reliable in differentiating PD from MSA (Burn et al., 1994). Others have shown that quantification of midbrain [¹²³I]B-CIT uptake improved accuracy in discriminating PD from atypical PD (PSP and MSA-P) (Seppi, 2006). In conclusion, PET and SPECT have a limited role in differentiating PD from other degenerative forms of Parkinsonism.

Role of PET and SPECT in monitoring disease progression

Radionuclide imaging has been used to assess disease progression in several clinical trials with conflicting results. As mentioned above, the REAL-PET study used [¹⁸F]-DOPAPET to assess the rate of disease progression in PD patients treated with ropinirole versus levodopa treatment (Whone et al., 2003). Disease progression was measured as the rate of reduction in [¹⁸F]-DOPA uptake. Results showed that the rate of reduction in tracer uptake was more marked in the levodopa treated group compared to ropinirole group implying that levodopa treatment increased disease progression (Whone et al., 2003). However, clinical severity measures did not correlate with this finding: clinical outcome was better in the levodopa than ropinirole treated group (Whone et al., 2003). The Parkinson's Disease Study Group also compared the disease progression measured by rate of reduction in SPECT [¹²³I] B-CIT uptake between a subset of PD patients who had levodopa as initial therapy versus pramipexole (Parkinson Study Group Datatop Investigators, 2000). Results from the study implied a slower disease progression with pramipexole, but again clinical and imaging findings were discordant (Parkinson Study Group Datatop Investigators, 2000). Another large multicentre trial, the Earlier versus Late Levodopa therapy in Parkinson's disease (ELLDOPA) assessed the effect of three different doses of levodopa (150 mg, 300 mg and 600 mg) on UPDRS scores compared with placebo and evaluated the effect of levodopa on disease progression using SPECT [¹²³I]B-CIT (The Parkinson Study Group, 2004). Levodopa significantly reduced worsening of motor symptoms in a dose dependent manner whereas the placebo arm showed progressive deterioration in clinical symptoms during the study period. There was a significant reduction in [¹²³I]B-CIT uptake in the levodopa arm compared to placebo group (The Parkinson Study Group, 2004).

Two possible explanations have been put forward to explain the accelerated decline in radiotracer uptake with levodopa. Firstly, levodopa accelerates dopaminergic neuron

degeneration (Parkinson Study Group Datatop Investigators, 2000, Whone et al., 2003). Against this notion, was the finding that levodopa treated patients had better clinical outcomes when compared to patients on dopamine agonists and placebo (Whone et al., 2003, Parkinson Study Group Datatop Investigators, 2000, The Parkinson Study Group, 2004). In addition after drug washout, patients on placebo had a greater clinical deterioration than the levodopa group (The Parkinson Study Group, 2004). If levodopa caused neurotoxicity a deterioration in symptoms would have been observed after the drug was stopped (The Parkinson Study Group, 2004). Secondly levodopa induces DAT down regulation and hence the apparent reduction in tracer uptake (The Parkinson Study Group, 2004, Pavese and Brooks, 2009). Against this was the observation that the SWEDDS subgroup in the study who were treated with levodopa did not show a similar reduction B-CIT uptake (The Parkinson Study Group, 2004). Nevertheless, there remains controversy as to whether SWEDDS actually have PD. [18F]-DOPA PET has also been used to assess survival of embryonic dopaminergic cell transplant and its effect on PD symptoms (Freed et al., 2001). [18F]-DOPA studies in these patients showed increased tracer uptake confirming graft survival and function, but there was no corresponding clinical improvement (Freed et al., 2001).

Limitations of PET and SPECT as biomarker in PD

PET and SPECT remain valuable diagnostic tools but sensitivity in early disease is not optimal. There is also a possibility that symptomatic treatments for PD can regulate DAT expression and AADC activity, (Cummings et al., 2011) which potentially interferes with radiotracer uptake and therefore the imaging results (Ravina et al., 2005). The utility of PET and SPECT in monitoring disease progression and evaluating the effects of treatment remains uncertain as the results from clinical trials so far have been inconclusive. These techniques

use radioactive ligands and are also more expensive compared to MRI. In conclusion, there remains a need for a sensitive and affordable imaging biomarker that can be used for diagnosis, following disease progression, and assessing the effects of treatments.

1.7. Treatment

Although there is no cure for the disease, available symptomatic treatments effectively control motor symptoms and improve quality of life (Miyasaki et al., 2002, Ferreira et al., 2013). Levodopa is the main precursor in dopamine synthesis and has been the mainstay of treatment for 50 years (Lees et al., 2014). Several other drug classes are licenced for use as monotherapy in early Parkinson's disease and/or adjuvant therapy in later disease.

Levodopa

Levodopa provides superior benefits in motor function, activities of daily living, and quality of life compared with other drug classes (Miyasaki et al., 2002, Stowe et al., 2009, Ferreira et al., 2013). Its effectiveness was confirmed in a randomised controlled trial of 361 untreated patients given levodopa 150 mg, 300 mg, 600 mg daily, or placebo. The Unified Parkinson's Disease Rating Scale (UPDRS) is the standard investigator-completed outcome measure used in Parkinson's disease trials. After 40 weeks of treatment, total UPDRS scores in the levodopa-treated groups remained significantly better than those of the placebo group (1.9 ± 6.0 , 1.9 ± 6.9 , and 1.4 ± 6.9 for levodopa groups respectively and placebo 7.8 ± 9 ; $P < 0.001$) (The Parkinson Study Group, 2004). A more recent large open-label randomised trial (PD MED) in early Parkinson's disease demonstrated a small benefit of levodopa in patient-rated quality of life over dopamine agonists and monoamine oxidase-B inhibitors (PDQ-39 mobility score mean difference 1.8, 95% CI 0.5-3.0, $p = 0.005$; EuroQol EQ-5D mean difference 0.03, 95% CI 0.01-0.05, $p = 0.002$) (PD MED Collaborative Group, 2014). Although

the predefined minimum clinically important difference (6 points in the PDQ-39 mobility section) was not reached, the small benefits from levodopa were still observed at 7 years follow up (PD MED Collaborative Group, 2014). Furthermore, study participants on either dopamine agonists or monoamine oxidase B inhibitor therapy had a higher probability of requiring add-on treatment than those on levodopa only at 2 years (40% , 64% and 20% respectively; $P < 0.0001$) (PD MED Collaborative Group, 2014). Levodopa is the preferred drug for patients with significant motor impairment as it provides superior motor benefits compared to other treatments (Miyasaki et al., 2002, Ferreira et al., 2013). Levodopa is also the first-line treatment in older patients (above 60 years), particularly when there is cognitive impairment, as other dopaminergic treatments increase the risk of neuropsychiatric complications (Olanow et al., 2009).

Levodopa is often well tolerated but chronic use is associated with dyskinesias and motor fluctuations which include wearing-off phenomenon and unpredictable on/off fluctuations. (Stowe et al., 2009, Ahlskog and Muentner, 2001). These occur in up to 40% of patients after 5 years of treatment (Ahlskog and Muentner, 2001).

Dopamine agonists

Dopamine agonists simulate dopamine by binding directly to post-synaptic dopamine receptors in the striatum (Olanow et al., 2009). Non-ergot dopamine agonists include orally administered pramipexole and ropinirole, along with transdermal rotigotine. Ergot-derived dopamine agonists (cabergoline, bromocriptine and pergolide) are associated with heart-valve and retroperitoneal fibrosis (NICE guidelines [CG35], 2006). Their use requires frequent monitoring for fibrotic complications, so the National Institute for Health and Care Excellence guidelines recommend non-ergot dopamine agonists (NICE guidelines [CG35], 2006).

Dopamine agonists are used as initial therapy in young patients and those with mild symptoms to delay levodopa use and hence the onset of motor complications (Ferreira et al., 2013, Olanow et al., 2009).

The effectiveness of dopamine agonists in ameliorating motor symptoms has been reported in several randomised controlled trials and systematic reviews (Ferreira et al., 2013, Stowe et al., 2009, NICE guidelines [CG35], 2006, Poewe et al., 2011, Sethi et al., 1998, Watts et al., 2007). A systematic review which sought to assess the effectiveness and tolerability of dopamine agonists in the treatment early PD, included randomised controlled trials (RCTs), comparing dopamine agonist to placebo, levodopa or both (Stowe et al., 2009). Results showed that dopamine agonists had superior symptomatic effects when compared to the placebo (Stowe et al., 2009). Fewer motor complications were reported in the dopamine agonist treated groups compared to levodopa: motor fluctuation [Odds ratio (OR) 0.75, 95% CI 0.63-0.90; $p=0.02$] and dyskinesias [OR 0.15, 95% CI 0.43 -0.59; $p< 0.00001$], but levodopa showed greater improvements in symptoms control (Stowe et al., 2009).

In addition to their use in early disease, dopamine agonists have also been shown to be effective adjuncts to levodopa in advanced disease (LeWitt et al., 2007, Pahwa et al., 2007, Schapira et al., 2011). Dopamine agonists also provide greater motor control when compared to other adjuvant therapies in advanced PD (Stowe et al., 2011). For example UPDRS scores improved by 4.8, 2.9, and 2.0 points in patients treated with dopamine agonists, MAOBI and COMTI respectively, $p<0.001$ (Stowe et al., 2011).

A major issue facing clinicians, is the management of impulse control disorders which occur in approximately 14% of dopamine agonist-treated people (Weintraub et al., 2010). These include hyper-sexuality, pathological gambling, excessive shopping, and excessive eating

(Weintraub et al., 2010, Weintraub et al., 2015). In a cross sectional study of 3090 idiopathic Parkinson's disease patients, these disorders were more common in dopamine agonists-treated than patients not on dopamine agonists (17% versus 6.9% $P < 0.001$), (NNH -10) (Weintraub et al., 2010). Other impulse control behaviours which occur with levodopa and apomorphine include punding (frequency: 1.4-14%), where patients perform repeated, pointless actions such as sorting or disassembling objects, and the dopamine dysregulation syndrome, which is characterised by a compulsion to overuse dopaminergic medication (Weintraub et al., 2015).

Monoamine oxidase B inhibitors (MAOBI)

MAOBI are recommended first line treatment in those with mild symptoms, and are also used as adjuncts to levodopa in advanced disease (NICE guidelines [CG35], 2006). MAOBI (rasagiline and selegiline) selectively inhibit monoamine oxidase type B enzyme which metabolises dopamine and thus increase dopamine availability (Olanow et al., 2009).

A systematic review of the efficacy of MAOBI in early Parkinson's disease demonstrated a small but significant improvement in UPDRS motor scores with MAOBI compared to placebo (mean weighted decrease in UPDRS motor scores 3.79 points in favour of MAOBI; 95% CI -5.30, -2.27; $P < 0.00001$) (Macleod et al., 2005).

In a systematic review, more participants in the MAOBI group required add-on therapy compared to levodopa and dopamine agonist-treated groups (OR 12.02, 95% CI 6.78-21.31; $P < 0.00001$ and OR2, 95% CI 1.05-3.81; $p = 0.04$, respectively) (Caslake et al., 2009). When compared to placebo, MAOBI adjunctive therapy in advanced PD led to a significant reduction in off time compared to placebo: -0.93 hours/ day, 95% CI -1.25, -0.62; $p < 0.00001$)

(Stowe et al., 2010). MAOBI use, also resulted in greater mean daily levodopa dose reduction: 29.11mg/day (95% CI -43.18, -15.04; $p < 0.00001$) (Stowe et al., 2010).

Catechol-o-methyl transferase inhibitors (COMTI)

COMTI such as tolcapone and entacapone inhibit the enzyme COMT which metabolises dopamine and thus increase the plasma levels of levodopa (Mizuno et al., 2007). Entacapone is a peripheral inhibitor of COMT, whereas tolcapone is thought to have central effects (Olanow et al., 2009). COMTI are used as adjunctive treatment to levodopa in advanced disease (Olanow et al., 2009) when motor fluctuations have developed. A Cochrane review compared COMTI and placebo and showed significant improvements in UPDRS motor scores, reduction in off- time and daily levodopa requirements when COMTI were used as an adjunct to levodopa: -2.02 points, 95% CI -2.68, -1.37; $p < 0.00001$, -0.83hours/day, 95% CI -1.04, -0.62; $p < 0.00001$, and 52.07mg/day, 95% CI -61.09, -43.05; $p < 0.00001$) respectively (Stowe et al., 2010). Side effects which are common to all dopaminergic drugs, such as nausea, vomiting, somnolence, hallucinations, and hypotension, can occur with COMTI treatment. In addition urine discoloration has only been reported with COMTI treated patients (Stowe et al., 2010).

Recently, a new COMTI, opicapone, has been licenced for use as adjuvant therapy in advanced PD. Opicapone increases levodopa bioavailability and its efficacy and safety was shown in an RCT (Ferreira et al., 2015). Another study to assess the safety and efficacy of opicapone demonstrated tolerability, reduction in off-time, and increase in on-time without troublesome dyskinesias, in patients treated with opicapone 50 mg when compared to placebo (Ferreira et al., 2016). In the same study, opicapone 50 mg was non-inferior to entacapone but had superior effects when compared to placebo (Ferreira et al., 2016). In addition to

dopaminergic side effects, entacapone can cause diarrhoea and tolcapone can potentially cause liver toxicity and therefore requires regular monitoring of liver function when the treatment is initiated (Ferreira et al., 2013). Opicapone has the advantage of a once daily dosing, so far has not shown any effects on liver function, and does not cause urine discoloration (Ferreira et al., 2016).

Advanced disease treatments

Pulsatile stimulation of striatal dopaminergic receptors by the short acting levodopa, is thought to be the mechanism by which motor complications occur (Olanow et al., 2009). When motor fluctuations become refractory to oral therapies, in advanced PD, treatments such as apomorphine, levodopa intestinal gel, and subthalamic nucleus (STN) deep brain stimulation (DBS) are considered. These treatments provide continuous dopaminergic stimulation, (Olanow et al., 2006) and allow a reduction in oral medications which may reduce dyskinesias. Factors such as patient's age, disease duration, the presence of cognitive impairment, speech difficulties, and patient preference need to be taken into consideration before choosing any of these three treatments (Volkman et al., 2013). Younger patients probably attain greater benefits from STN- DBS compared to older patients above the age 70 years, but levodopa infusion is effective in both groups, and is also tolerated well by older patients, even those with mild cognitive impairment (Volkman et al., 2013). Tolerability of apomorphine in the latter group is uncertain (Volkman et al., 2013). DBS is contraindicated in individuals with cognitive impairment and depression (NICE guidelines [CG35], 2006). Apomorphine is a short acting dopamine agonist which is commonly given subcutaneously, either as intermittent injections (rescue therapy for off-episodes), or as continuous infusion through a syringe driver during the waking day (Clarke et al., 2009, Volkman et al., 2013). Subcutaneous apomorphine has a rapid onset of action, and is also advantageous in that it

bypasses first pass metabolism, and when given as an infusion allows steady plasma and central nervous system levels to be reached (LeWitt, 2004). A recent phase two double blind RCT which compared subcutaneous apomorphine to placebo in advanced PD, included 16 patients from four Japanese institutions (Nomoto et al., 2015). When baseline and 20-minute post-treatment UPDRS scores were compared, patients treated with apomorphine had a significantly greater change in UPDRS motor scores compared to the placebo group: -24 vs -4.1; $p < 0.002$ (Nomoto et al., 2015). Further evidence for use of apomorphine is from small non-controlled studies. From these, a 44% median reduction in off time, and 40% increase in on-time without dyskinesias (Volkman et al., 2013), and improvement in UPDRS motor scores was also reported (Clarke et al., 2009). Side effects include sedation, nausea, injection site reactions and neuropsychiatric symptoms e.g. confusional states, psychosis, hallucinations, and impulse control disorders (Volkman et al., 2013, Clarke et al., 2009).

Levodopa gel is administered as a continuous infusion into the jejunum or duodenum, via a gastrostomy tube connected to a portable infusion pump (Clarke et al., 2009). This reduces erratic absorption which can occur when levodopa is given orally. Levodopa infusion was shown to be superior to oral therapies and improved both UPDRS scores and patient quality of life in a randomised controlled study (Nyholm et al., 2005). A more recent multicentre randomised placebo controlled trial also showed similar improvements in patients with advanced PD (Olanow et al., 2014). The study randomised 71 patients to either levodopa-carbidopa 25/100mg capsules and placebo levodopa-carbidopa infusion gel or placebo levodopa capsules and levodopa-carbidopa infusion gel, and followed up the patients for 12 weeks (Olanow et al., 2014). Study results showed a significant reduction in off-time (-1.91 ± 0.57 hours; $p = 0.0015$) in the active levodopa infusion gel arm compared to active oral levodopa-carbidopa arm. 'On' time without dyskinesias was also significantly better with

active levodopa infusion compared to oral levodopa-carbidopa treatment: 2.2 ± 0.90 hours; $p=0.0142$ (Olanow et al., 2014). A review of levodopa infusion studies also demonstrated a reduction in off-periods by 40-80%, dyskinesias by 60-90%, and also beneficial effect on non-motor symptoms (Volkman et al., 2013). Dopaminergic side effects including confusion, somnolence, dyskinesias, agitation, were reported, but these were more frequent in the oral therapy group (Nyholm et al., 2005). Continuous levodopa infusion strategy provides steady plasma levodopa levels, hence constant striatal dopamine levels, which prevents pulsatile dopaminergic stimulation, a potential mechanism by which motor complication develop (Olanow et al., 2006).

Bilateral STN DBS involves electrical stimulation of the subthalamic nuclei via electrodes inserted stereo-tactically and connected to a neurostimulator implanted in a subclavicular pocket (Clarke et al., 2009). There is good quality evidence to support use of DBS in the management of advanced PD. RCTs have demonstrated DBS superiority over medical therapies in terms of motor scores and quality of life in patients with advanced PD (Williams et al., 2010, Deuschl et al., 2006, Weaver et al., 2009). In a literature review including class one (RCT of good quality, providing the highest level of evidence) to class three (case-control) studies, Volkman et al. (2013) reported 30-60% improvement in UPDRS motor scores, reduction in off time (median 68%), and increase in on time (median 71%), in patients treated with STN DBS (Volkman et al., 2013). There was also a reduction in dyskinesias in those treated with STN DBS (Volkman et al., 2013).

There have been no direct head-to-head RCTs of advanced disease therapies. An un-randomised observational study of the three treatments suggested that STN DBS provided better motor control and no dyskinesias, whereas patients on levodopa gel had good motor control but with mild to moderate dyskinesias (Elia et al., 2012). The apomorphine group had

the worst motor scores when compared to STN DBS and levodopa gel groups (Elia et al., 2012). STN DBS may also improve non-motor symptoms as shown in a recent study which demonstrated significant reduction in both clinician and patient rated non-motor symptoms scores (Dafsari et al., 2016). Side effects reported in the RCTs included depression, gait disturbances, procedure related adverse effects including infection, intracranial bleed, and lead migration (Deuschl et al., 2006, Weaver et al., 2009).

Rehabilitation

In the UK, NICE guidance recommends that all patients with PD should have access to occupational therapy, physiotherapy and speech and language therapy (NICE guidelines [CG35], 2006). These therapies are aimed at helping patients maintain their independence, support them to adapt to changes and disability that occur as the disease progresses, (Dixon et al., 2007), and also improve quality of life. Available evidence suggests that effectiveness of these therapies may be limited. For example a review which assessed effectiveness of occupational therapy in PD reported small benefits (Dixon et al., 2007). Results were inconclusive since the included studies were limited by small sample sizes and methodological flaws (Dixon et al., 2007).

A systematic review included 39 RCTs which compared physiotherapy to no intervention reported a significant improvement in walking speed, freezing of gait, timed up and go test, functional reach test, balance, and UPDRS motor and ADL scores, in the physiotherapy group (Tomlinson et al., 2013). Physiotherapy had no effect on falls rate, quality of life, and mobility as measured by the PDQ-39 (Tomlinson et al., 2013). Another systematic review and meta-analysis which included only eight RCTs, assessed the effect of physiotherapy techniques such as aerobics, stretching, treadmill walking, and stretching exercises on balance and postural instability (Yitayeh and Teshome, 2016). When using the falls efficacy scale, a 6.7,

95% CI, -14, 0.54; $p=0.07$ reduction in the incidence of falls with physiotherapy, was demonstrated but this did not reach statistical significance (Yitayeh and Teshome, 2016). Combining balance training and other physiotherapy techniques improved balance and postural instability (Yitayeh and Teshome, 2016). Further, a recently published RCT which included 762 patients with mild to moderate PD compared the effectiveness of individualised physiotherapy and occupational therapists versus no therapy on quality of life (QOL) and activities of daily living (ADL).(Clarke et al., 2016). The primary outcome was the change in NEADL score at three months and secondary outcomes included QOL measured by the PDQ39. This study showed no significant benefit of these therapies in patients with mild to moderate PD (Clarke et al., 2016).

A Cochrane review included six RCTs, which assessed two different types of speech and language therapy interventions (Herd et al., 2012). Interventions compared in these studies included the standard Lee Silverman Voice therapy (LSVT-LOUD), its modified version LSVT-ARTIC, and respiration therapy (Herd et al., 2012). Methods of delivering LSVT were also compared: face to version versus online delivery. Results of these studies showed greater improvements in loudness: 5dB, 95% CI -8.3,-1.7; $p=0.003$, 5.5dB, 95% CI 3.4, 7.7; $p<0.00001$, and voice monotonicity (0.45 semitones, 95% CI 0.01, 0.93; $p=0.04$), in patients treated with LSVT-LOUD compared to respiration therapy (Herd et al., 2012). When LSVT-LOUD and LSVT-ARTIC were compared, the earlier group demonstrated greater improvements in speech intelligibility (Herd et al., 2012). The review also showed non-inferiority of online LSVT delivery compared to face-face intervention. Definitive conclusions on the effectiveness of a speech and language therapy techniques over another could not be drawn from this review due to the limited number of studies with large sample sizes (Herd et al., 2012).

There is evidence to suggest that multidisciplinary care results in better patient outcomes. A randomised controlled trial included 122 PD patients, and randomly assigned them to either multidisciplinary team care or general neurologist care (Van der Marck et al., 2013). The primary outcome was a change in quality of life at 8 months follow up, measured by the PDQ 39. The change in UPDRS motor subscale was also measured as a secondary outcome. Study authors reported an improvement in quality of life from baseline in the intervention group (Van der Marck et al., 2013). There was a significant difference in the change in PDQ-39 summary index scores between intervention and control group 3.4, 95% CI 0.5-6.2; P= 0.02 (Van der Marck et al., 2013). The study also showed an improvement in UPDRS motor scores in the intervention group, which was significantly different from the control group: 4.1 points 95% CI 0.8-7.3; p=0.01 (Van der Marck et al., 2013). Multidisciplinary teams should include specialists who can deal with the various motor and non-motor aspects of the disease, which may not respond to dopaminergic therapies. Team members may include a PD specialist (neurologist or geriatrician), PD nurse specialist, psychiatrist, speech and language therapist, physiotherapist, occupational therapist, dietician, social worker, and a urologist (van der Marck et al., 2009). In advanced stages of the disease a palliative care consultant may be required to manage the terminal aspects of the disease. In the UK the PD nurse specialists have a crucial coordinating role among the team members and also act as the first point of contact for the patient in both the community and secondary care. The effectiveness of PD nurse specialist nurses in PD management was assessed in an RCT which demonstrated better patient well-being, with no added costs, when PD nurses were involved in PD care (Jarman et al., 2002). The study compared costs, patient well-being (measured by PDQ39, Euro-QOL, global subjective wellbeing questionnaire), and health outcomes (mortality, fracture rates, stand up test, dot in square test) in patients receiving care from a general practitioner versus

PD nurse specialist care (Jarman et al., 2002). After a two year follow up period, there were no differences in the measured health outcomes, costs, and patient well-being between the two groups. But, when global subjective well-being scores were compared between the two groups, patients receiving care from the PD nurse specialist had better scores: mean difference -0.23, 95% CI -0.4,-0.06; p=0.008 (Jarman et al., 2002).

1.8. Hospitalisation in Parkinson's disease

1.8.1. Background

Since there is no cure or disease modifying treatment for PD at present, progressive neurodegeneration occurs resulting in worsening of motor and non-motor symptoms overtime. Disability due to worsening symptoms ensues with advancing disease (Gershanik, 2010). When gait and postural instability develops complications such as fall as fractures become more frequent (Gershanik, 2010). Although symptomatic treatments for the disease have been shown to improve functional independence and quality of life of patients, (Olanow, 2008) side effects may become problematic especially when treatment regimens become complicated in advanced disease. Long-term levodopa use causes motor complication such as dyskinesias and on and off fluctuations in up to 80% of patients after 5-10 years (Bhidayasiri and Truong, 2008). Such complications add to the disability which occurs with advancing disease and also impair quality of life (Gershanik, 2010). Other side effects of dopaminergic medications include drowsiness, dizziness, postural hypotension, and hallucinations (Stowe et al., 2011). In addition to these problems, non-motor symptoms of the disease such as depression and dementia, dysautonomia, dysphagia and speech difficulties

progress with time and increase the patient's reliance on others for assistance with activities of daily living (Gershanik, 2010) and may result in institutionalisation.

It is therefore not surprising that the use of healthcare resources and hospitalisations increase with advancing disease. The resulting economic burden is substantial (Low et al., 2015). In the UK, the annual cost of the disease was estimated to be approximately £49 million per annum (Findley, 2007). A cross sectional survey demonstrated that the highest proportion of direct costs for the disease were from NHS cost (38%) followed by social services 35% (Findley et al., 2003). Increasing disease severity and patient age were associated with higher disease costs (Findley et al., 2003). PD drug costs, rehabilitation (Dodel et al., 1998, Von Campenhausen et al., 2011), loss of employment, and early retirement due to the disease also contributes to indirect disease costs (Von Campenhausen et al., 2011, Dodel et al., 1998, Guttman et al., 2003). In other studies, inpatient care and institutionalisation were shown to be the main drivers for direct health care costs (Huse et al., 2005, Findley, 2007, Dodel et al., 1998).

When compared to age matched counterparts with other diseases, PD patients have higher hospitalisation rates (Guttman et al., 2003, Hobson et al., 2012). In one study, PD patients were 1.45 times more likely to be hospitalised than age matched controls without PD (Guttman et al., 2003). Similarly, another study showed that patients with PD utilised healthcare resources more than their counterparts without the disease: median utilisation of controls versus PD patients for emergency room visits (0.6 versus 0.4 $p=0.05$) and physician's visits (5.9 versus 7.9, $p=0.001$), respectively (Parashos and O'Brien, 2002, Huse et al., 2005). Further, a retrospective study which included 1 469 PD cases and 2 439 controls reported higher healthcare resource utilisation including hospitalisation and physician visits among PD patients compared to the reference group (Hobson et al., 2012).

Once admitted to hospital, PD patients are 2.05 times more likely to have a repeat hospitalisation (Hassan et al., 2013). Disease duration, cognitive impairment, drug induced psychosis, and co morbidities were shown to increase the risk of readmission to hospital (Hassan et al., 2013, Klein et al., 2009).

1.8.2. What are the reasons for PD patient hospitalisation?

Several factors account for increased hospitalisation in PD. In as many as 50% of cases, the reason for emergency hospital admissions can be linked to the disease (Martignoni et al., 2004).

Falls

Falls and fractures account for 13-17% of PD hospital admissions (Temlett and Thompson, 2006, Woodford and Walker, 2005, Tan et al., 1998). A strong association between falls and the rate of hospital encounters has been reported (Hassan et al., 2013, Klein et al., 2009). Falls tend to occur more frequently in PD patients compared to the general population (Wood et al., 2002). A falls incidence as high as 68% was reported in one prospective study among 109 PD patients (Wood et al., 2002).

Several factors explain the increased risk of falls seen in patients with PD. These include old age, cognitive impairment, and disease severity (Wood et al., 2002, Contreras and Grandas, 2012, Wielinski et al., 2005, Kerr, 2010, Allcock et al., 2009). Tan et al showed that 49% of PD patients who fell had Hoehn and Yahr (H&Y) stage 4 disease (Tan et al., 1998). When PD patients progress to H&Y 3 postural instability and balance impairment emerges which often results in the increased risk of falls (Contreras and Grandas, 2012, Tan et al., 1998).

Cholinergic degeneration may also play a part in the causation of falls in PD as demonstrated by PET studies (Bohnen et al., 2009). PD patients with falls had reduced cortical and thalamic cholinergic activity compared to controls and PD non-fallers (Bohnen et al., 2009). In addition, another study reported a significant correlation between balance and gait instability with a reduction in acetylcholinesterase activity (Gilman et al., 2010). PD fallers were found to have worse cognitive scores which suggests that cognitive impairment may contribute to the risk of falling (Wielinski et al., 2005, Wood et al., 2002). A small study including 22 PD patients demonstrated a correlation between greater postural instability and cognitive performance (Nocera et al., 2010). Allcock et al. (2009) also showed an association between attention scores and increased frequency of falling (Allcock et al., 2009).

In advanced PD, treatment related motor fluctuations and freezing episodes may also increase the risk of falls (Wood et al., 2002). An association between use of high doses of levodopa, COMTI, cholinesterase inhibitors and neuroleptics with falls, was reported in a retrospective study (Contreras and Grandas, 2012). This finding may be attributed to treatment complications such as dyskinesias or on and off fluctuations. These treatments are used in advanced disease and the apparent association may reflect a group of patients who are at a higher predisposition to falls due to other disease related factors such as postural instability and cognitive impairment. Dopamine agonists have also been implicated in the causation of falls, and postulated mechanisms include sudden onset of sleep and freezing episodes (Allcock et al., 2009), and postural hypotension.

Consequences of falls in patients with PD can be significant. Injuries including lacerations and fractures occur and these often result in hospitalisation (Contreras and Grandas, 2012, Tan et al., 1998). In one retrospective study examining falls risk factors, frequency, and injuries in 1092 PD patients, 54.6% (n=597) of the patients reported a fall. 65% of these

sustained an injury of which 33% were fractures and 75.5% required health care resource use as a result of the fall (Wielinski et al., 2005).

Fractures

Fractures are common in patients with PD compared to age-matched controls (Genever et al., 2005, Walker et al., 2013, Di Monaco et al., 2006, Bhattacharya et al., 2012). Hip fractures are by far the commonest site of fracture in these patients (Genever et al., 2005, Wielinski et al., 2005). In individuals above the age of 60 years, the incidence of hip fractures in PD patients was shown to be six times higher than in controls without the disease (Walker et al., 2013).

Falls are a significant risk factor for fractures in PD. Low bone mineral density is reported in PD patients, (Van den Bos et al., 2013a, Schneider et al., 2008, Sato, 1997, Taggart and Crawford, 1995) and increases the risk of fractures (Schneider et al., 2008). A recent meta-analysis of studies relating to osteoporosis and bone mineral density in PD pooled results from 15 studies and reported lower bone mineral density in PD patients compared to the general population (OR 1.18, 95% CI 1.09-1.27; $p=0.036$) (Zhao et al., 2013). Bone density has also been shown to correlate inversely with disease duration and H&Y stage (Di Monaco et al., 2006), which may also explain the increased risk of fracture with advancing disease. Low vitamin D levels, weight loss, and immobility (Van den Bos et al., 2013a, Sato, 1997) in PD may explain why PD patients develop low mineral bone density and osteoporosis.

Pneumonia

Pneumonia is also another common reason for hospitalisation in PD. A retrospective study to assess the reasons for PD admissions to an Australian large teaching hospital reported 761 PD admissions over a 5 year period (Temlett and Thompson, 2006). Among the admissions where

PD was recorded as a secondary diagnosis (n=645), 12% had pneumonia recorded as the primary reason for admission (Temlett and Thompson, 2006). Similar figures were reported in a UK study where 11% of the PD admissions were due to pneumonia (Woodford and Walker, 2005). Another large retrospective study which evaluated reasons for non-elective PD admissions in England using the Hospital Episodes statistics (HES) data showed that 14% of the 232 905 non-elective PD admissions were for pneumonia (Low et al., 2015).

Various non-motor and motor features of PD increase the predisposition to lower respiratory infections. Swallowing and the cough reflex provide important protective mechanisms for preventing aspiration and consequently, respiratory tract infection (Melo and Monteiro, 2013). In PD, up to 35% of patients complain of swallowing difficulties and this correlates with disease severity (D'Amelio et al., 2006, Kalf et al., 2012). Several potential mechanisms for how dysphagia develops in PD have been proposed. Firstly, akinesia and rigidity of the pharyngeal muscles as a direct consequence of dopaminergic neuron degeneration in the substantia nigra has been put forward as the main explanation for swallowing in PD (Mu et al., 2012). Since PD dysphagia does not respond to dopaminergic therapy, it is likely that other mechanisms contribute to dysphagia in PD (Melo and Monteiro, 2013). Histochemical and histological studies of pharyngeal muscles of autopsied PD patients and controls found evidence of muscle fibre denervation, atrophy, and abnormal distribution of fibre type in PD patients compared to controls (Mu et al., 2013b). When PD patients with dysphagia were compared to their unaffected PD counterparts, those with dysphagia were found to have more atrophied myofibrils (Mu et al., 2012). Peripheral denervation due to alpha-synuclein deposition may also contribute to the pathophysiology of dysphagia in PD (Mu et al., 2012). Alpha-synuclein deposition has been demonstrated in the sympathetic ganglia, gastrointestinal tract enteric plexus, vagus nerve (Beach et al., 2010, Braak et al., 2006, Mu

et al., 2013b), and in both sensory and motor nerves of the pharyngeal musculature. (Mu et al., 2013a).

Swallowing problems result in pooling of saliva which further increases the risk of aspiration pneumonia (Nobrega et al., 2008, Chou et al., 2007). PD patients also experience silent aspiration and laryngeal penetration which contribute to the risk of respiratory tract infections (Nobrega et al., 2008, Rodrigues et al., 2011). Airway penetration has also been demonstrated in 22% of PD patients without any swallowing complaints (Monteiro et al., 2014).

The cough reflex aids in the removal of mucus and other foreign bodies from the airways and impairment of this reflex may contribute to the risk of respiratory infections in several conditions including PD (Ebihara et al., 2003, Hammond, 2001). Cough efficacy is also impaired in PD patients with both early and advanced disease when compared to controls, (Ebihara et al., 2003) which may contribute to the high risk of respiratory tract infection in PD. Respiratory dysfunction in PD is frequent but patients are often asymptomatic (Sabaté M et al., 1996, Pal et al., 2007). An association between swallowing impairment and respiratory dysfunction has been reported (Monteiro et al., 2014). Coordination of the respiratory cycle and swallowing is important in facilitating safe swallowing (Gross et al., 2008). In a normal state, the sequence of events between swallowing and respiration starts with inspiration followed by deglutition apnoea, then post-swallow expiration, and this is thought to form a protective mechanism which prevents airway aspiration (Selley et al., 1989, Martin-Harris et al., 2003). When compared to healthy controls, PD patients were reported to swallow during inhalation and were more likely to inhale after swallowing increasing the risk of aspiration (Gross et al., 2008).

Urinary tract infection (UTI)

Urinary dysfunction is a key non-motor manifestation of PD, occurring in up to 39% of PD patients (Campos-Sousa et al., 2003). Symptoms range from urinary incontinence, urgency, nocturia, and frequency with the latter two symptoms being the most commonly reported (Campos-Sousa et al., 2003). Urinary dysfunction especially detrusor hyperactivity in PD may be related to the loss of inhibitory effect on the micturition reflex which occurs as a result of dopaminergic neurons in the substantia nigra (Cersosimo et al., 2013, Yeo et al., 2012). Presence of alpha-synuclein in the autonomic ganglia, is another plausible pathogenic mechanism for these symptoms in PD (Beach et al., 2010). Urinary incontinence in PD may be exacerbated by mobility problems in advanced disease. One study showed that urinary incontinence was associated with an increased risk of hospitalisation and admission to nursing homes (Thom et al., 1997). The association persisted after adjusting for age and the presence of several comorbid conditions (Thom et al., 1997). UTI is amongst the commonest reasons why PD patients attend the emergency department, occurring in up to 20% (Tan et al., 1998, Guneyssel, 2008), and is a frequent complication in those who are hospitalised (Martignoni et al., 2004). With progression of motor disability, PD patients become increasingly immobile which can lead to urinary stasis, bacterial proliferation and UTI (Rogers et al., 2008). The link between poor mobility and UTIs was shown in a retrospective study which explored the risk factors for hospitalisation for UTI (Rogers et al., 2008). Authors assessed records of 408 192 elderly subjects who were admitted to skilled nursing facilities in five states in the USA. 89 538 were eventually admitted to hospital and of these patients, 4.4% (n= 3 961) were admitted for urinary tract infections (Rogers et al., 2008). Ability to walk conferred a significant reduction in hospitalisation due to UTI: 69% reduction in risk of hospitalisation in those who walked independently, and 45% in those who required either supervision or limited assistance (Rogers et al., 2008). In this study the presence of other chronic medical conditions including

PD, dementia, diabetes mellitus, renal failure and stroke, and indwelling catheters were some of the factors that were associated with an increased risk for hospitalisation for UTI (Rogers et al., 2008).

Problems related to blood pressure (postural hypotension and syncope)

Postural hypotension is a common non-motor symptom in PD occurring in up to approximately 30% of patients (Velseboer et al., 2011). The frequency of symptomatic orthostatic hypotension (OH) increases with advancing disease and disease duration and age (Ha et al., 2011). Postural hypotension may occur as a result of sympathetic denervation in PD. Dopaminergic medication may also exacerbate OH and in the elderly patients, multiple comorbidities and use of other medications such as antihypertensives and diuretics can also exacerbate OH (Mussi et al., 2009). Patients with OH complain of dizziness, light headedness, fatigue, and even syncope on standing up from a supine or sitting position (Ha et al., 2011, Jamnadas-Khoda et al., 2009) due to central nervous system hypoperfusion (Mussi et al., 2009). Postural hypotension is associated with an increased risk of falls and consequently, serious injuries such as fractures and head trauma ((Lahrman et al., 2006). Up to 50% of patients with OH do not have symptoms and this may also increase the risk of falls (Jamnadas-Khoda et al., 2009)(Jamnadas-Khoda et al., 2009), which may lead to hospitalisation in PD.

Neuropsychiatric symptoms

Anxiety, depression, psychosis, and cognitive impairment are common in PD, with up to 71% of patients reporting at least one symptom (Riedel et al., 2010). The majority of these symptoms can be managed in the outpatient department, but when severe and carers are unable to cope, hospitalisation may be warranted. Klein et al. (2009) showed that amongst 143 PD patients who contributed to 243 admissions to a neurological unit, 24% of the admissions were due to psychosis (Klein et al., 2009). Medication-related psychosis was the

most significant factor contributing to repeated admissions (Klein et al., 2009). In a survey of National Parkinson Foundation (NPF) centres, up to 63% of the international centres and 49% of US NPF centres reported that that would admit patients for behavioural management (Chou et al., 2011).

Other reasons for PD hospitalisation

10-37% of PD hospital admissions are primarily for management of motor symptoms of PD (Tan et al., 1998, Temlett and Thompson, 2006, Klein et al., 2009). Infection of deep brain stimulation leads, battery replacement and adjustment of DBS settings also increase the risk of hospitalisation in PD (Hassan et al., 2013). Gastrointestinal, cardiovascular disorders, (Temlett and Thompson, 2006, Woodford and Walker, 2005) and cerebrovascular emergencies (Guneysel, 2008) also lead to hospitalisation in PD. Marakoff et al. (2011) showed that PD patients who had gastrointestinal disorders were 1.42 times more likely to visit the emergency rooms and higher total health care costs compared to those without these symptoms (Makaroff et al., 2011)(Makaroff et al., 2011). A small proportion of PD patients are admitted electively for DBS surgery, rehabilitation, and confirmation of diagnosis (Chou et al., 2011).

1.8.3. PD hospitalization outcome

When patients with PD present to the emergency department and are hospitalised, the outcome is often poor. Patients have prolonged hospital stays up to 1.19 times longer than controls (Guttman et al., 2003). Another study estimated that PD patients spend 2 more days longer in hospital per year (Huse et al., 2005). They are often discharged to rehabilitation units and a high rate of institutionalisation has been reported (Parashos and O'Brien, 2002, Tan et al., 1998). This adds to the huge burden of care associated with the condition and also

impairs the quality of life. Low et al. (2015) reported high mortality in PD patients during hospitalisation when compared to controls (OR 2.46, 95% CI 2.42, 2.49; $p < 0.00001$ (Low et al., 2015).

In hospital, PD patients are often looked after by teams other than their own PD specialists, (Gerlach et al., 2011b, Martignoni et al., 2004) which may have an impact on the inpatient PD care patients receive. This is compounded by the fact that in some hospitals PD experts are unaware or are not informed about PD admissions (Gerlach et al., 2011a). Out of 51 National Parkinson foundation (NPF) centres taking part in an online survey to evaluate practises and opinions on hospital admission of PD patients, only 25% had mechanisms in place to notify neurologists about PD patient's admission (Chou et al., 2011). In another study, a PD nurse specialist was involved in only 2% of the 59 PD admissions to a surgical ward (Derry et al., 2010). Similarly, in The Netherlands, PD nurse specialists were involved in only 2.2% of the admissions (Gerlach et al., 2013).

Cheng et al. (2007) showed that involvement of specialists who have knowledge and experience in managing PD and its complications is associated with better adherence to PD quality of care indicators, when compared to non-movement disorders specialists (Cheng et al., 2007). Most NPF centres were not confident of the quality of care received by hospitalised PD patients (Chou et al., 2011). The same perception was held by patients and their caregivers in a New Zealand survey (Buetow et al., 2010). In a UK hospital audit, authors surveyed junior doctors and reported that, although a significant proportion of doctors knew the complications of omitting PD medications, only 25% were confident in changing PD medication formulation if this was needed (Sathyababu et al., 2012). In another hospital audit, only 25% of junior doctors knew medications that are contraindicated in PD (Magdalinou et al., 2007). This results in poor medication management when PD patients are admitted to

hospital. Suboptimal PD care during hospitalisation also results in several complications including worsening of motor symptoms, infections, prescription errors, (Gerlach et al., 2012, Gerlach et al., 2013, Mueller et al., 2009) poor surgical outcomes (Walker et al., 2013), and prolonged hospital stays (Huse et al., 2005).

Prescription errors are common during hospitalisation and have been reported in between 17% and 74% of PD admissions (Gerlach et al., 2012, Gerlach et al., 2013, Derry et al., 2010, Magdalinou et al., 2007, Hou et al., 2012, Domingo-Echaburu et al., 2012). These may include dose omissions, sudden medication withdrawal, incorrect dose timing, dosage or formulations, and use of medications which are contraindicated in PD (Gerlach et al., 2012, Gerlach et al., 2013, Derry et al., 2010, Domingo-Echaburu et al., 2012). Poor medication management is associated with worsening of motor symptoms (Gerlach et al., 2013, NICE guidelines [CG35], 2006) and has been shown to correlate with the amount of levodopa dose missed (Gerlach et al., 2013). PD medication errors also increase the risk of complications such as falls, confusion, and hallucinations (Magdalinou et al., 2007, Derry et al., 2010, Barber et al., 2001). Use of contraindicated medication such as anticholinergics can worsen cognition and cause confusion (Ehrt et al., 2009, Bédard et al., 1999). A serious life threatening complication that can occur following abrupt withdrawal of PD medication is Neuroleptic Malignant Syndrome, otherwise known as Parkinson Hyperpyrexia Syndrome (Newman et al., 2009). The syndrome is characterised by altered level of consciousness, fever, rigidity of muscle, dysautonomia, excessive sweating, and raised serum creatine kinase levels (Newman et al., 2009). Such potentially life threatening complications highlight the need for strict adherence to medication doses and timing when PD patients are hospitalised.

1.9. Aims

The previous section highlighted a number of unmet needs in the management of PD. Firstly, the lack of neuroprotective treatments. Secondly, the need for a sensitive tool for use in identifying individuals with early or prodromal disease, which provides a window for use of potential neuroprotective therapies. The extensive scale of PD hospitalisations and poor inpatient outcomes has also been highlighted. As long as there is no cure for the disease, hospitalisation rates and associated costs are likely to grow exponentially with the aging of the population. Interventions to reduce these hospital admissions are therefore urgently required.

The objectives of this project sought to address these pertinent issues and were fourfold:

1. To systematically review literature for interventions aimed at reducing PD hospitalisation.
2. To describe the extent of hospitalisation in both early and advanced PD patients who participated in a large PD oral medication trial in England (PD MED trial). To compare hospitalisation rate, admission duration, and time to first admission in patients treated with the three main groups of symptomatic treatments for early and late disease.
3. To assess the accuracy of reporting PD hospital admissions using data from a large tertiary hospital in England and discuss the impact on PD resource allocation.
4. To develop an MRI based biomarker for potential use as a diagnostic and prognostic marker in PD.

**CHAPTER 2: A SYSTEMATIC REVIEW OF INTERVENTIONS TO REDUCE
HOSPITALISATION IN PARKINSON'S DISEASE**

2.1. Background

PD hospitalisation, its associated high morbidity, mortality, and costs, which are likely to rise in the coming decades due to the aging population and consequent increase in disease prevalence. Therefore, there is a need for interventions aimed at reducing PD hospital admissions. Such interventions should be cost-effective and focussed on reducing known risk factors for hospitalisation. Evidence based initiatives for reducing unplanned hospital admissions in other chronic conditions, are already in existence. These include use of acute clinics, multidisciplinary teams and patient education (Gibson et al., 2002, Thomas et al., 2013, Viswanathan et al., 2015). It is uncertain if these measures are effective for patients with PD. The purpose of this study was to perform a systematic review of the available literature for effective interventions aimed at preventing unplanned hospital admissions in individuals with PD.

2.2. Methods

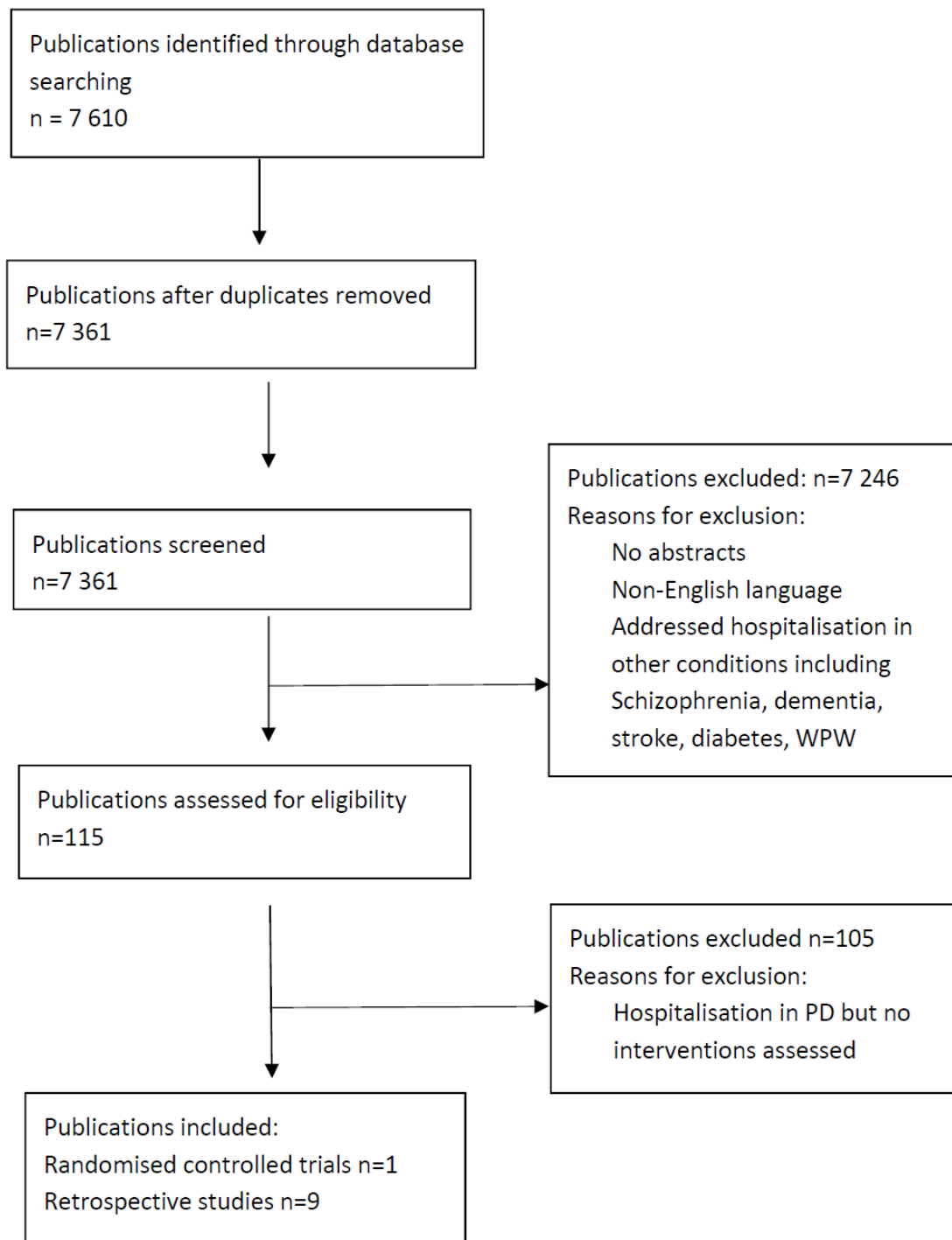
A search for publications from EMBASE, MEDLINE and CINAHL databases up to September 2014 was performed. Eligibility criteria included RCT evaluating effectiveness of intervention versus no intervention in reducing hospital admissions and/or emergency department visits in patients with PD. Index terms and free text terms for ‘Parkinson’s disease’, Parkinson*, ‘hospitalisation’, and hospital* were used, but this yielded no relevant RCTs. The search criteria were therefore broadened to include other study types. This included all studies where a specific measure was employed and led to a reduction in number of hospital admissions or emergency department visits in PD. Interventions included those that have been used in other chronic diseases to reduce unplanned hospital admissions such as patient educational programmes, urgent assessment clinics, specialist’s clinics, and specialist nurses. Outcome measures included a reduction in either hospital admission, non-

elective (unplanned) admissions, or emergency room visits. Study participants were individuals with PD of all disease stages and duration. No age restrictions were applied. Abstracts and title headings were screened by one person and only publications with full abstracts, published in the English language and addressing interventions to reduce hospital admissions in PD were included.

2.3. Results

7 610 abstracts obtained from the three data sources were screened (figure 6). Duplicates and publications addressing hospitalisation in conditions other than PD were excluded. 115 articles and abstracts were assessed for eligibility. From these, 106 excluded articles discussed issues pertaining to PD hospitalisation such as frequency and reasons for admissions, inpatient care and medication errors, but did not assess the effect of specific interventions on reducing hospital admissions in PD. One RCT assessed the effectiveness of two dysphagia management techniques in reducing aspiration pneumonia and also measured hospitalisations as an adverse event. Nine retrospective studies reported a change in healthcare utilisation including the number of emergency visits and or

Figure 6: Flow diagram showing publications identified through database searching and reasons for inclusion and exclusion from the review.



hospitalisation after an intervention and these were included (table 3)(Klein et al., 2009, Willis et al., 2012, Grubb et al., 2012, Grubb et al., 2013, Delea et al., 2011, Wei et al., 2014, Kulkarni et al., 2008, Davis et al., 2010, Ney et al., 2013) .

One RCT compared the incidence of pneumonia among 515 patients with dementia and PD who were randomised to either chin-down posture technique or two types of thickened fluids (nectar- and honey-thick consistencies) over a 3 month follow up period (Robbins et al., 2008). There was no difference in the incidence of pneumonia (primary outcome measure) between the two interventions. When the two types of thickened fluids were compared the cumulative incidence for pneumonia was less in the nectar-thick fluid compared to honey-thick fluid (0.08 and 0.15 respectively; HR 0.50 95% CI 0.23, 1.09; p=0.083), but the difference was not significant (Robbins et al., 2008).

Table 3: Proposed interventions for reducing Parkinson's disease hospital admissions

Proposed intervention Author, year	Study type	Study Group	Study intervention	Outcome	Effect of proposed intervention on hospitalisation and emergency room visits
Dysphagia management					
Robbins et al (Robbins et al., 2008)	Randomised Controlled Trial	515 (PD and dementia patients)	Chin down posture versus honey/nectar thick fluid	Pneumonia incidence, Hospitalisation measured as an adverse event	No difference in serious adverse events (including hospitalisation) between the two intervention groups
Frequent Neurologist involvement					
Willis et al (<i>Willis et al., 2012</i>)	Retrospective cohort	2 4929 PD patients	Neurologist versus no neurologist care	PD-related and general medical related hospitalisation	Neurologist care associated with ↓ hospitalisation for traumatic injury (HR 0.56, 95% CI 0.40-0.78), psychosis (HR 0.71, 95% CI 0.59-0.86) and urinary tract infection (HR 0.74 95% CI 0.63-0.87).
Ney et al (<i>Ney et al., 2013</i>)	Survey	3 883 Parkinsonism, multiple sclerosis, epilepsy, dementia.	Neurologist ambulatory care	Utilisation and cost of non-ambulatory care : inpatient, emergency , and home care	↓ in condition related emergency, inpatient, and homecare care events (OR 0.64, P=0.001) and costs (53% ,p<0.001)
Open access clinic					
Klein et al (Klein et al., 2009)	Retrospective	143 PD patients	Open access clinic	Mean number of hospitalisations per year, mean length of stay	50% ↓ in average number of PD admissions over 2 years : 36 to 18
PD medication choice					
Grubb et al (<i>Grubb et al., 2012</i>)	Retrospective	7 230 PD patients	Rasagiline versus dopamine agonists	Number of hospitalisation, duration of stay and costs	Fewer hospital admissions (OR 0.76, 95% CI 0.66-0.86), shorter length of stay and lower costs in rasagiline group
Grubb et al(Grubb et al., 2013)	Retrospective	3 864 PD patients	Rasagiline versus Selegiline	Hospitalisation, emergency room visits and falls	Lower emergency room visits (OR 0.79; 95% CI, 0.68 to 0.92), lower all hospital admissions (OR 0.82; 95% CI, 0.68 to 0.99), and lower number of falls in rasagiline group

PD Mediation Adherence					
Delea et al (<i>Delea et al., 2011</i>)	Retrospective	1 215 PD patients	Adherence to levodopa/carbidopa/entacopone	All cause and PD- related hospitalisations, length of stay, emergency room and physicians visits, prescriptions and healthcare costs	Satisfactory adherence versus non-adherence: mean number PD related admissions (0.15 vs 0.12 ,p<0.001), emergency visits (0.04vs 0.08, p=0.010), all cause hospitalisation (0.30 vs 0.53, p<0.001)
Kulkarni et al 2008(<i>Kulkarni et al., 2008</i>)	Retrospective Longitudinal cohort	104 PD patients	PD medication adherence	Medication adherence, PD related hospitalisations and emergency room visits.	Suboptimal adherence associated with worsening motor symptoms: Odds for adherers (MPR>0.8) experiencing symptom worsening compared to non-adherers was 67% less (OR 0.33 95% CI 0.13-0.85)
Wei et al (Wei et al., 2014)	Retrospective Crossectional study	7 583 PD patients	PD medication adherence	Hospital visits, emergency room visits , skilled nursing facility, home health agency, physicians visit and health costs	Prevalence and relative risk (low versus high adherers): hospitalisation 57% vs 47% and 1 versus 0.86 (95% CI 0.81-0.90), Emergency room visits 68% vs 59% and 1 versus 0.91,95% CI 0.86-0.96
Davis et al (Davis et al., 2010)	Retrospective	3 119 PD patients	PD medication adherence	Medication adherence, health care utilisation (including hospital admissions and emergency room visits), and costs	Mean hospitalisation per annum, non-compliant versus compliant: 2.3 versus 1.8; p<0.005. Mean number of emergency departments visits were not significantly different 1.9 versus 1.8

The overall incidence of pneumonia in this study was low, but the authors could not attribute the low incidence to the interventions used in this study because no comparison group was included (Robbins et al., 2008). Hospitalisations were reported as an adverse event and 20% of participants had at least one hospital admission in each of the two intervention arms. The number of withdrawals due to adverse events, including hospitalisations, was higher in the thickened fluid group (4%) compared to chin down posture group (2%): 2% difference, 95% CI -0.4%, -5%; $p=0.112$ (Robbins et al., 2008).

A retrospective cohort study among 24 929 Medicare recipients in the United States (US) evaluated the effect of frequent neurologist care on PD hospitalisation and reported a reduction in hospital admissions for traumatic injury (HR 0.56, 95% CI 0.40-0.78; $p<0.001$), psychosis (HR 0.71, 95% CI 0.59-0.86; $p<0.001$), and urinary tract infection (HR 0.74 95% CI 0.63-0.87; $p<0.001$) in patients who had frequent neurologist care compared to those who were not treated by a neurologist (Willis et al., 2012). Another US survey assessed the impact of neurologist care on healthcare costs and utilisation among 3 883 individuals with chronic neurological conditions including Parkinsonism and showed that respondents who had consulted a neurologist had a reduction in disease-related home care, emergency, and inpatient care events compared to those with no neurologist involvement (OR 0.64, $P=0.001$) (Ney et al., 2013). In another retrospective study, PD patients with poor motor symptom control, medication-related complications, or neuropsychiatric symptoms were allowed to access an urgent neurology clinic without prior appointment. This resulted in a 50% reduction in PD admissions over a 2 year period (Klein et al., 2009). In addition there was a reduction in hospital length of stay from 11 to 4.5 days (Klein et al., 2009).

Two retrospective studies reported fewer hospital admissions in PD patients treated with rasagiline when compared to dopamine agonists (Grubb et al., 2012) or selegiline (Grubb et al., 2013). Comparisons of the number, duration, and cost of hospital admissions for 7 230 PD patients who were initially treated with either a dopamine agonist or rasagiline were compared using data from the Medicare Supplemental Database. The authors reported fewer hospital admissions (OR 0.76, 95% CI 0.66-0.86), shorter duration of stay (-0.38 day $p < 0.0001$), and reduced costs in rasagiline-treated patients compared to the DA group: \$12 327 versus \$16 525 respectively (Grubb et al., 2012). In a retrospective study involving 3 864 individuals with PD, Grubb et al showed that, rasagiline use was associated with lower emergency room visits (OR 0.79; 95% CI, 0.68 to 0.92) and all cause hospital admissions (OR 0.82; 95% CI, 0.68 to 0.99) compared to the selegiline-treated group (Grubb et al., 2013). However, no difference was found in the odds of PD-related hospital admissions and fractures (Grubb et al., 2013).

Four retrospective studies assessed the frequency of medication adherence in PD and also measured hospitalisation as an outcome. In a retrospective historical cohort study which included 1 215 from a US health claim database, Delea et al. (2011) showed that patients with satisfactory levodopa/carbidopa/entacapone therapy compliance (proportion of days where a patient had medication supply $\geq 80\%$), had 49% fewer emergency room visits ($p=0.010$) and 39% less PD-related hospitalisations compared to the non-adherent group ($p=0.001$) (Delea et al., 2011). The number of all hospitalisations and overall PD-related costs were also lower in the group of patients with satisfactory treatment adherence (Delea et al., 2011). In another study of 104 PD patients, suboptimal adherence to PD medication was associated with worsening motor symptoms (Kulkarni et al., 2008). PD related hospitalisations and emergency room visits were used as an indicator of worsening motor symptoms, in addition to an increase PD medication dosage. Medication possession ratio

(MPR), a measure of medication adherence was defined as the proportion of days covered by a PD prescription: the numerator was the number of days covered by a PD prescription and the denominator was the number of days between prescription disbursements (Kulkarni et al., 2008). During the first year of follow up, the mean possession ratio, PD related emergency room visits, and hospitalisation were 0.52 ± 0.35 , 0.07 ± 0.29 , and 0.91 ± 2.19 respectively. In the fifth year the MPR was lower 0.42 ± 0.37 with higher emergency room visits and hospitalisation: 0.18 ± 0.70 and 1.40 ± 3.74 (Kulkarni et al., 2008). In a retrospective cross-sectional study, Wei et al. (2014) used Medicare administrative data for 7 583 patients with PD and found that those with a medication possession ratio of more than 90% had lower emergency room visits and hospitalisation rate than poor adherers (less 80% MPR) (Wei et al., 2014). Another US study evaluated the prevalence and cost of poor adherence to PD medication using retrospective administrative and claims data. The study included 3 119 participants who had a PD International Classification of Diseases (ICD) code recorded and were on PD medications during the study period. The mean number of hospitalisations over 12 months was significantly higher amongst individuals who were non-compliant with PD medication in comparison to the compliant group (2.3 versus 1.8; $p < 0.005$) (Davis et al., 2010). However, there was no difference in the mean number of emergency room visits (Davis et al., 2010). Mean health costs were significantly higher in the non-adherent group (Davis et al., 2010).

2.4. Discussion

Hospitalisation in PD is a significant cost-driver (Findley et al., 2003) and patient outcome is often poor (Temlett and Thompson, 2006, Huse et al., 2005, Mueller et al., 2009). This review highlights the lack of evidence-based interventions for reducing PD patient hospitalisation.

Of the few studies in this area, only one RCT of a specific intervention was found. This reported a low overall pneumonia incidence and no difference in the frequency of pneumonia when either chin-down posture or thickened fluids were used to manage dysphagia (Robbins et al., 2008). This study was limited by a short follow up period and the absence of a control group (Robbins et al., 2008). There was no difference in hospitalisation rates between the two interventions. Further, the cumulative pneumonia incidence was less when fluids with nectar compared to honey consistency were used (Robbins et al., 2008). This difference could have been a chance finding because the overall incidence of pneumonia in the study was low. Although speculative, the low pneumonia rates reported in the study suggests that chin-down posture and fluids of nectar/honey consistency may be useful interventions for pneumonia prevention.

There were nine retrospective studies where hospitalisation rates and emergency room visits in PD were measured. These studies suggested that frequent neurologist consultation, open access clinics, and compliance with PD medication may reduce hospital admissions and emergency room attendance by PD patients. The cost effectiveness and the actual number and frequency of neurological consultations required to reduce PD hospitalisation is uncertain. Frequent consultations may provide opportunities for medication adjustments, earlier detection and management of complications which, in turn, reduces the need for hospitalisation (Willis et al., 2012). Open access clinics also provide similar opportunities for symptoms and complication management which potentially prevents hospital admissions (Klein et al., 2009). The effectiveness of open access clinics was based on a report from a single centre with no comparison group. An association between PD medication adherence and reduction in emergency room visits and PD-related hospital admissions was reported in four retrospective studies (Delea et al., 2011, Davis et al., 2010, Wei et al., 2014, Kulkarni et

al., 2008). Sub-optimal adherence to PD medication can have negative implications on motor outcomes and patient quality of life (Grosset et al., 2007). These findings imply that optimising PD symptom control through medication compliance possibly reduces hospital admissions for poor motor control. Interventions such as regular neurologist care and open access clinics may be difficult to implement in countries where some individuals have no health insurance. In addition, some patients may not be able to afford their PD prescriptions which may impact on medication adherence, motor symptom control, and therefore hospitalisation. A surprising finding was the association between rasagiline treatment and fewer hospital admissions reported in two retrospective studies (Grubb et al., 2013, Grubb et al., 2012). Dopamine agonists have superior symptomatic effects compared to monoamine oxidase inhibitor treatment (Caslake et al., 2009). However, dopamine agonist treatment is associated with more side effects (Caslake et al., 2009). This may account for the fewer hospitalisations in rasagiline group. The differences in emergency room visits between selegiline and rasagiline treated patients cannot be explained. These two studies were funded by the rasagiline manufacturer which may have led to bias.

A major limitation of these studies is their retrospective design. Reliability of the reported results is dependent on the accuracy of the databases from which the data were extracted. It is also impossible to control for all confounding factors in such studies. Therefore, definitive conclusions regarding the effectiveness of the above interventions cannot be drawn based on this evidence. Nevertheless, these studies provide some insight into the interventions that require further evaluation.

Falls, fractures, infections, cognitive, and motor decline have been identified as risk factors for unplanned hospital admissions in patients with PD (Hassan et al., 2013).

Implementing preventative measures for these complications may reduce the need for hospital admission. Falls in PD can occur as a result of postural instability, poor motor symptom control, and drug-related side effects (e.g. postural hypotension). Optimising motor symptom control and managing medication side effects may prevent falls and hence hospital admissions. Furthermore physiotherapy is thought to improve PD motor symptoms, mobility, and balance, (Tomlinson et al., 2013) which may also potentially reduce the risk of falls. A recent Cochrane review compared the effectiveness of physiotherapy interventions and no intervention in PD on outcomes such as gait, falls, and clinician-rated measures of impairment and disability. Health resource usage including hospitalisation was not reported as an outcome measure. Although there was a reported trend towards improvement in falls, there was no significant difference in the number of falls between the physiotherapy and non-intervention arms in 6 studies from which data could be abstracted (Tomlinson et al., 2013). The authors reported that the absence of a positive effect from physiotherapy could have been a result of short follow up periods, reliance on falls diaries, and the small number of participants in the included studies (Tomlinson et al., 2013). Fracture prevention measures may reduce hospitalisation as shown in an Italian retrospective study which included 5,167 postmenopausal women who were discharged from hospital with primary or secondary diagnosis of hip fracture (Tarantino et al., 2011). The study compared the effect of treatment or no treatment with fracture prevention medication on mortality, re-fracture rates, and costs of health resource use (including hospitalisation). For those who were treated with bisphosphonates, the effect of compliance with treatment on these outcome measures was also assessed (Tarantino et al., 2011). Only 34% of the included patients were exposed to fracture prevention treatments and 1 044 events (deaths and hospitalisation) were reported, and these were related to re-fractures (Tarantino et al.,

2011). A significantly reduced incidence of death (-55%, $p < 0.001$) and re-fracture related hospitalisations (-40% $p < 0.001$) was reported when fracture prevention treatment was used (Tarantino et al., 2011). In those who were treated with bisphosphonates, adherence of <40% was associated with higher total costs including re-hospitalisation, compared to those with better adherence (Tarantino et al., 2011). It is uncertain if osteoporosis and fracture prevention measures translate into a reduction in hospitalisation rates in PD.

Early speech and language therapy involvement in dysphagia management may potentially reduce the number of hospital admissions for aspiration pneumonia. To date, no studies have systematically assessed whether pneumonia, falls, and fracture prevention strategies discussed above can reduce the number of hospital admissions in PD.

PD nurse specialists, where available, may play a crucial role in identifying PD patients in the community who are at high risk of hospitalisation. By working in liaison with other PD specialists in primary and secondary care, social services, palliative care and rehabilitation services, early treatment can be instituted in the patients' home. Community PD nurse care has been shown to be cost neutral when compared to primary care physicians in a RCT (Jarman et al., 2002). However, effectiveness of this approach in reducing PD hospitalisation rates has not been assessed.

Interventions such as specialist clinics (multidisciplinary or nurse-led), (Thomas et al., 2013) medication management, (Viswanathan et al., 2015) and patient self-management educational programmes (Gibson et al., 2002) have been shown to be effective in reducing unplanned admissions in other chronic conditions. The limited evidence available in PD suggests that specialist clinics and medication management may reduce hospitalisation and therefore these interventions require further evaluation. A PRISMA checklist would have highlighted some of the limitations of this study which include the inclusion of

positive studies which were predominantly retrospective and the lack of an assessment of risk of bias for the individual studies.

This review has highlighted a gap in evidence-base for interventions that are effective in reducing PD hospital admissions. Proposed measures are based mainly on retrospective studies and none have been systematically assessed in PD. Nevertheless, these interventions merit further evaluation in well-designed prospective trials. In view of the health costs and morbidity associated with PD hospitalisation, it is imperative that PD experts and policy makers make a joint effort to find ways to reduce hospitalisation in PD as the search for disease modifying treatment continues.

**CHAPTER 3: FREQUENCY OF HOSPITALISATION IN PARKINSON'S
DISEASE USING HOSPITAL EPISODES STATISTICS.**

3.1. Background

At present there is no good quality evidence for effective interventions which can mitigate hospitalisation in PD. There is a suggestion that controlling motor symptoms may potentially reduce healthcare utilisation in PD, but supporting evidence for this is poor. Other studies have demonstrated a difference in hospitalisation rates in individuals treated with different dopaminergic therapies, but these studies are limited by their retrospective nature (Grubb et al., 2012, Grubb et al., 2013). To date there are no randomised controlled trials which assessed the effect dopaminergic treatments on hospitalisation in PD.

The aims of the present study were to compare hospitalisation rates and duration of admission for PD patients on the different classes of oral medications used for treating early and advanced PD. The patients took part in an open label randomised controlled trial of oral medications used for treating Parkinson's disease (acronym PD MED):

- PD MED EARLY: levodopa versus dopamine agonists versus MAOBI
- PD MED LATER: dopamine agonists versus MAOBI versus COMTI

The current study also sought to describe the demographic characteristics and patterns of hospitalisation of the PD MED participants.

3.2. Methods

The PD MED EARLY trial is the second largest oral medication trial conducted in patients with PD. The study evaluated the cost and clinical effectiveness of the three different classes of oral medications used for treatment of early PD: levodopa, dopamine agonists, and MAOBI inhibitors. Patients were initially drug naïve or treated for less than 6 months (PD MED Collaborative Group, 2014). The study design and results for the PD MED EARLY trial were published elsewhere (PD MED Collaborative Group, 2014).

1 620 patients diagnosed with idiopathic PD based on the UKPD brain bank criteria, were recruited into the early disease trial (PD MED EARLY) and 500 patients to the later disease trial (PD MED LATER). There were 91 recruiting centres majority of which were in the UK: UK (89 centres), Russia (1 centre), and Czech Republic (1 centre) (PD MED Collaborative Group, 2014).

PD MED EARLY showed a small benefit of levodopa over levodopa sparing treatments in patient-rated quality of life and this was sustained at 7 years follow up (PD MED Collaborative Group, 2014).

In PD MED LATER, patients with advanced disease requiring adjunctive therapy were randomised to COMTI, dopamine agonists or MAOBI. The PD MED LATER study results will be published in early 2017. Dopamine agonists' superiority over COMTI and MAOBI in advanced disease was reported in systematic reviews (Stowe et al., 2011, Stowe et al., 2010, Caslake et al., 2009). It was therefore postulated that dopamine agonist treated patients would fare better than MAOBI and COMTI treated patients in terms of PD-related hospitalisations.

Hospital Episodes Statistics (HES) data for study participants from English and Welsh PD MED study centres, were obtained from the Health and Social Care Information Centre (HSCIC). The HES data included inpatient episodes from 1st April 1999 to 31st March 2013. HES data were linked to the PD MED trial database on the following variables: patient PD MED trial number (unique identifier); month and year of birth; gender; first part of patient postal code.

All HES inpatient episodes for PD MED study participants from England and Welsh centres, with exact matches on the first three variables (PD MED trial number, month and year of birth, and gender) , were included. An inpatient episode was defined as the time

a patient spent under the care of one consultant during a hospital admission (The NHS Information Centre for health and social care, 2010). A hospital admission or complete spell was composed of one or more finished consultant episodes. For purposes of this study, a hospital admission was defined as a complete inpatient spell.

Duplicate episodes and other inpatient episodes which occurred prior to patient randomisation to the PD MED trial were excluded. First patient randomisation was on 9th November 2000, so admissions prior to this date were excluded. For patients who died during the study period, inpatient episodes recorded after the date of death were also excluded: these were considered as HES data errors. Hospital episodes for PD MED participants from Scottish centres were excluded as these are stored in a separate database from that of England and Wales. Hospitalisation data for non-UK PD MED participants were not available.

Descriptive analyses were performed and summary tables produced for the following: baseline demographic characteristics including age, sex, disease duration, H&Y, and co-morbidity. Hospitalisation data included the number of hospital admissions per patient during the study period, type of admission (elective or non-elective), admission duration, and primary reasons for admission using the ICD-10 codes. Zero bed days were defined as admissions where a patient was admitted and discharged within 24 hours (Low et al., 2015). Primary diagnoses were divided into PD-related (complications of PD and its treatment; table 4) and other (all other primary diagnoses).

Table 4: List of PD-related primary diagnosis categories

PD-related categories	Examples of included Primary diagnoses
Parkinson's disease	Parkinson's disease
Neuropsychiatric symptoms	Disorientation Hallucinations Senility Restlessness and agitation
Non motor symptoms	Nausea and vomiting
	Constipation
	Somnolence
	Hyperhidrosis
	Malaise and fatigue
	Dysphagia
Problems related to blood pressure	Hypotension
	Dizziness
	Orthostatic hypotension
	Syncope and collapse
Infections	Urinary tract infection
	Pneumonia
Falls, fractures, injuries	Tendency to falls Fractures

Table 4 above shows examples of some of the PD-related primary diagnoses which were included. All fractures, falls and injuries were included. (Non-traumatic subdural haemorrhages were excluded)

Outcome measures included: number of admissions, time to first hospital admissions, time to the first PD-related event, and admission duration. The outcome measures were analysed by using the same two comparisons used for PD MED EARLY: levodopa versus levodopa-sparing (dopamine agonists and MAOBI arms combined), and dopamine agonist versus MAOBI. For PD MED LATER study comparisons were made between dopamine agonists versus combined COMTI and MAOBI then MAOBI versus COMTI. A negative binomial model was used to compare the number of admissions and the duration of admissions between the treatment arms. This model was used for the analysis, as this is an appropriate model for over-dispersed count data. Survival analysis was used to compare the risk of events (time to first admission and time to first PD-related admissions).

All models were run without covariates and then including significant covariates. Covariates used include: H&Y stage (categorised as 1-1.5, 2, 2.5-5), disease duration (categorised as <0.5 years; \geq 0.5 years), age (categorised as <70 years; \geq 70 years), and gender.

Data were presented as mean \pm standard deviation (SD) or median (interquartile range - IQR) and 95% CI where appropriate. A significance level of 5% was used to assess for statistical evidence between groups. SAS software version 9.2 & 9.4 and Stata SE version 14 were used for data analysis.

3.3. Results

PD MED EARLY

Patient demographics

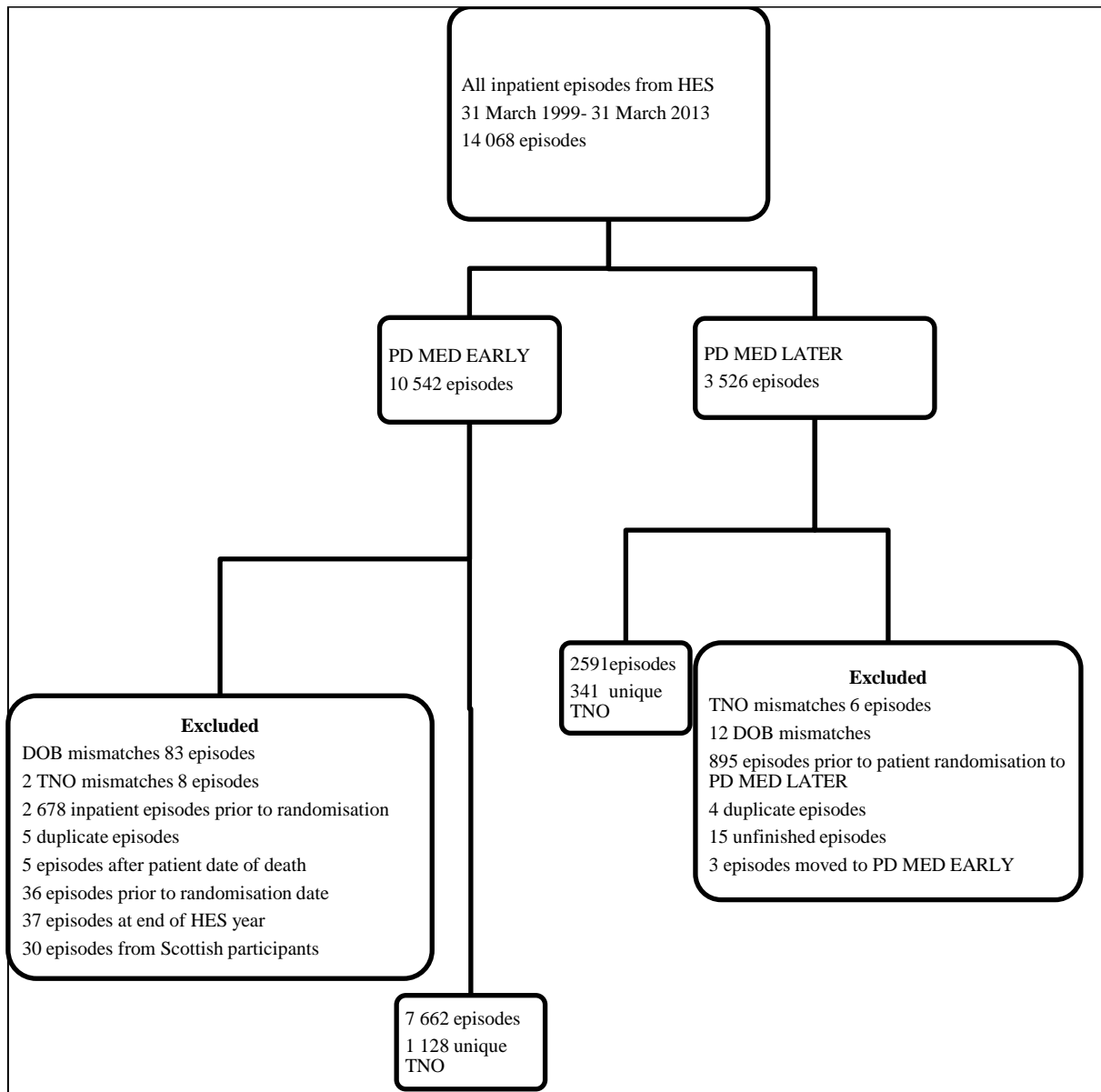
1 548 of the 1 620 who participated in the early disease PD MED trial were from English and Welsh study centers: mean age 70 ± 8.8 years, 65% male. 97% of the participants were above age 50 years. Mean disease duration was 7.8 ± 13.3 months at study entry (table 5).

73% (n=1 128) of participants were admitted to hospital during the study period 1st April 1999 to 31st March 2013: mean age 71 ± 8.5 years, 66% male, mean disease duration 7.1 ± 13.1 months, and median follow up time was 5.7 (4.2-8.1) years, range 0-12.3 years.

93% of the 1 128 patients had mild disease: H&Y stage 1 to 2.5 (table 5). Only 2% (n=23) of the patients were below age 50 years. 374 patients were on levodopa only, 431 on dopamine agonist and 323 on MAOBI.

Data cleaning

Figure 7: Flow diagram showing all inpatient episodes from Hospital Episode Statistics (HES) data.



Included and excluded episodes. DOB: Date of Birth, TNO: Trial number, HES: Hospital Episode Statistics

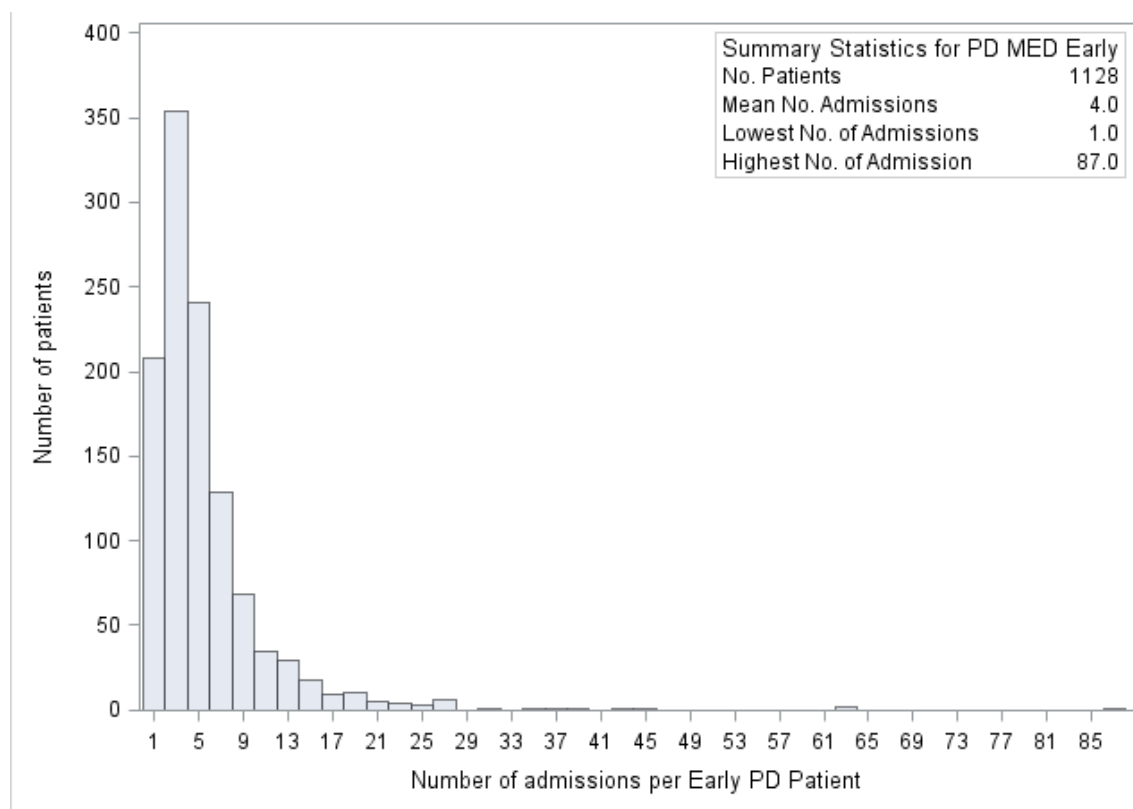
Table 5: Baseline demographic data for PD MED EARLY patients from England and Wales and the subset of patients who had at least 1 admission

Variable	All early PD MED patients included in dataset	Early PD MED Patients who had an admission	Early PD MED Patients who did not have an admission
	N=1 548	N=1 128	N=420
Mean Age(SD)	70 (8.8)	71 (8.5)	67 (9.0)
Age N (%)			
<50	39 (3)	23 (2)	16 (4)
50-59	144 (9)	83 (7)	61 (15)
60-69	499 (32)	339 (30)	160 (38)
70-79	685 (44)	528 (47)	157 (37)
≥80	181 (12)	155 (14)	26 (6)
Sex N (%)			
Male	1 003 (65)	740 (66)	263 (63)
Duration of PD in Months(Mean[SD])	7.8 (13.3)	7.1 (13.1)	9.7 (13.7)
Hoehn & Yahr Stage N (%)			
1	468 (30)	337 (30)	131 (31)
1.5	314 (20)	217 (19)	97 (23)
2	442 (29)	328 (29)	114 (27)
2.5	223 (14)	162 (14)	61 (15)
3	97 (6)	80 (7)	17 (4)
4-5	4 (1)	4 (1)	0 (0)

Hospital admissions

A total of 10 542 inpatient episodes were obtained from the HES data. After excluding duplicate records, inpatient episodes occurring before the PD MED randomisation and other HES data errors, 7 662 inpatient episodes were obtained (figure 7 above), and these were accounted for by 1 128 patients. These episodes made up 5 734 hospital admissions for the period 1st April 1999 to 31st March 2013. 5 725 were completed admissions and the remaining 9 admissions had no discharge date. 31st March was considered as the discharge date since data from 1st April 2013 onwards were not available: HES data years end on 31st March. The median (IQR) number of admissions per patient were: 4 (2-6), range 1-87 admissions (figure 8). Median duration of admission: 15 (2-54.5) days, range 0-355 days. 49% (n=2 809) of the admissions were zero bed days. There were 3114 (54%) elective and 2 413 (42%) non-elective (emergency) admissions, 203 transfers between hospitals, and 1 maternity admission. The method of admission was not recorded for 3 admissions.

Figure 8: Number of admissions per patient for PD MED EARLY patients who were hospitalised



Elective admissions

844 patients had 3 114 elective admissions: median number of admissions per patient was 1(0-2), range 1 - 82 admissions. Median duration of admissions was 0 (0-6) days (range 0 - 355 days). 80% (n=2 481) of all elective admissions were zero bed days. Majority of zero bed day admissions were for reasons not related to PD: neoplasms (16%), disease of the eye and adnexa (12%), gastrointestinal disorders (11%), musculoskeletal and connective tissue disorders (7%), symptom, signs, laboratory and clinical findings not classified elsewhere (6%), cardiovascular and circulatory disorders (3%), and genitourinary disorders (4%). Only 7% (n=205) of all elective admissions had a PD-related primary diagnosis on discharge. Of these PD was recorded as the primary discharge diagnosis in 75% of the admissions.

Non-elective admissions

There were 2 413 non-elective admissions from 854 patients. 292 of these patients were on levodopa treatment only, 326 on a dopamine agonist and 236 on a MAOB inhibitor. The median number of admissions per patient was 1(0-2): range 1 - 20 admissions (figure 9). 13% of non-elective admissions were zero bed days. Median duration of admissions was 6.0(1-16) days and range was 0 - 256 days. 42% (n=1 033) of all non-elective admissions had a PD-related primary diagnosis on discharge from hospital in 547 patients. Table 6 and 7 show the commonest PD-related non PD-related reasons for admissions respectively.

Figure 9: Number of non-elective admissions per patient for PD MED EARLY patients who were hospitalised

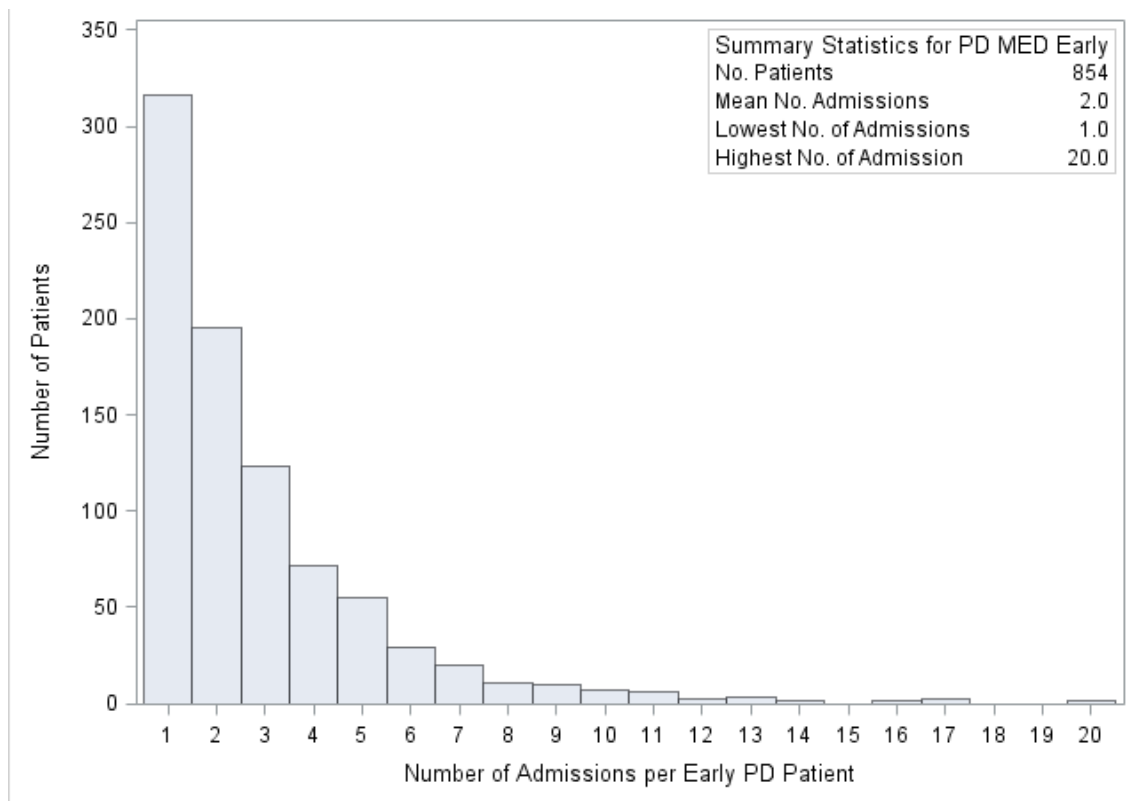


Table 6: PD-related primary diagnoses (reasons for admission) for PD MED EARLY trial patients who had non-elective admissions

Primary PD-related reason for admission	Number of admissions	%
Infections	324	29.8
Fall/Fracture/Injuries	318	29.3
Parkinson's disease	184	16.9
Problems related to blood pressure	132	12.2
Neuropsychiatric disorders	64	5.9
PD non-motor symptoms	62	5.7
PD drugs adverse effects	2	0.2

Table 7: Non PD-related primary diagnoses (reasons for admission) for PD MED EARLY trial patients who had non-elective admissions.

Diagnosis Category	Frequency	%
Symptoms, signs, clinical and laboratory findings not classified elsewhere	429	32.3
Cardiovascular and circulatory disorders	241	18.2
Gastrointestinal disorders	131	9.9
Musculoskeletal and connective tissue disorders	105	7.9
Respiratory tract disorders	64	4.8
Neoplasms	53	4.0
Diseases of the nervous system	45	3.4
Diseases of the skin and subcutaneous tissues	45	3.4
Complications of surgical and medical care	44	3.3
Genitourinary tract disorders	32	2.4
Endocrine nutritional and metabolic disorders	28	2.1
Infectious diseases	23	1.7
Anaemia	19	1.4
Mental and behaviour disorders	18	1.4
Infectious diseases	17	1.3
Poisoning by drugs medicaments and biological substances	12	0.9
Diseases of the eye and adnexa	7	0.5
Factors influencing health status and contact with health services	5	0.4
Foreign bodies	3	0.2
Burns and corrosions	2	0.2
Toxic effects of nonmedicinal substances	2	0.2
Disease of the ear and mastoid	1	0.1
Disorders of immune mechanisms	1	0.1

Hospital admissions by treatment arm.

Elective and non-elective admissions

When both elective and non-elective admissions for the 1 128 patients were considered, there was no difference in the number of admissions during the study period, between

the levodopa arm and the levodopa sparing arms (RR 0.90, 95% CI 0.81, 1.06), time to first admission, admission duration between the treatment arms (table 8). When considering the number of PD-related admissions only, no difference between levodopa and levodopa-sparing arms (RR 1.2, 95% CI 0.95, 1.40) was found.

When dopamine agonist and MAOBI treatment arms were compared, no difference in rate of admissions was found (RR 1.0, 95% CI: 0.85, 1.18). The rate of PD-related admissions did not differ between these two arms (RR 0.9, 95% CI 0.75, 1.20).

Table 8: Time to first admission, admission rates and duration for PD MED EARLY patients on levodopa versus levodopa sparing

		LD alone	LD-sparing
N		505	845
Number of admissions	Mean (SD)	3.8 (5.96)	3.5 (4.32)
	Median (IQR)	2.0 (0.0-5.0)	2.0 (0.0-5.0)
N*		374	626
Time to first admission (years)	Mean (SD)	2.6 (2.28)	2.5 (2.1)
	Range	0 – 12	0 – 9
Total Admission Duration (per patient) (Days)	Mean (SD)	40.7 (66.36)	42.9 (62.31)
	Median(IQR)	15.0 (2.0-51.0)	17.5 (2.0-62.0)
N		1915	2969
Admission Duration (Days)	Mean (SD)	8.0 (19.2)	9.0 (20.9)
	Median(IQR)	1.0 (0.0-7.0)	1.0 (0.0-9.0)

*Only including those patients who had an admission. Comparisons in this table exclude individuals who were randomised between the LD-sparing arms. Total admission duration was calculated as the sum of the duration of admissions for all patient divided by the number of patients. Admission duration was average admission duration for all the admissions in each group.

Non-elective admissions

Levodopa versus Levodopa sparing treatment

Significant predictors for the number of non-elective admissions were: older age, H&Y stage, and disease duration at randomisation. A negative binomial model which included these predictors was used, but no difference in admissions rates between levodopa and levodopa-sparing treatment was found (RR 1.0; 95% CI; 0.84, 1.14).

Similarly, there was no difference in the time to the first admission (HR: 1.1 95% CI; 0.9-1.2; figure 10), and mean admission duration (RR 1.1; 95% CI 0.91, 1.23; table 9), between levodopa and levodopa sparing treatment. When assessing PD-related admissions only, no difference in both admission rate (RR 1.0, 95% C.I 0.81, 1.22) or time to first PD-related admissions (HR 1.0 95% CI 0.9, 1.3; figure 11), between levodopa and levodopa-sparing arms, was found. Admissions duration between these treatments did not differ (RR 1.2; 95% CI 0.93, 1.47). Furthermore, time to the first fall, fracture or injury related admission was no different when these treatment arms were compared (HR 1.1; 95% CI 0.8, 1.4; figure 11).

Table 9: Time to first admission, admission rates and duration for PD MED EARLY patients who had non-elective admissions (levodopa versus levodopa sparing arms)

		LD alone	LD-sparing
	N	505	845
Number of admissions	Mean (SD)	1.6 (2.28)	1.6 (2.22)
	Median (IQR)	1.0 (0.0-2.0)	1.0 (0.0-2.0)
	N*	292	489
Time to first admission (years)	Mean (SD)	3.6 (2.56)	3.2 (2.21)
	Range	0 – 12	0 – 10
Total Admission Duration (per patient)	Mean (SD)	40.2 (56.20)	39.0 (49.17)
	Median (IQR)	20.0 (5.0-55.0)	21.0 (7.0-58.0)
	N	831	1366
Admission Duration (days)	Mean (SD)	14.1 (24.14)	14.0 (21.72)
	Median (IQR)	6.0 (1.0-18.0)	6.0 (1.0-17.0)

Table 9 above, * Only including those patients who had an admission. Comparisons in this table exclude individuals who were randomised between the LD-sparing arms. Total admission duration was calculated as the sum of the duration of admissions for all patient divided by the number of patients. Admission duration was average admission duration for all the admissions in each group.

Figure 10: Time to first admission (levodopa versus levodopa-sparing) for PD MED EARLY patients who had non-elective admissions

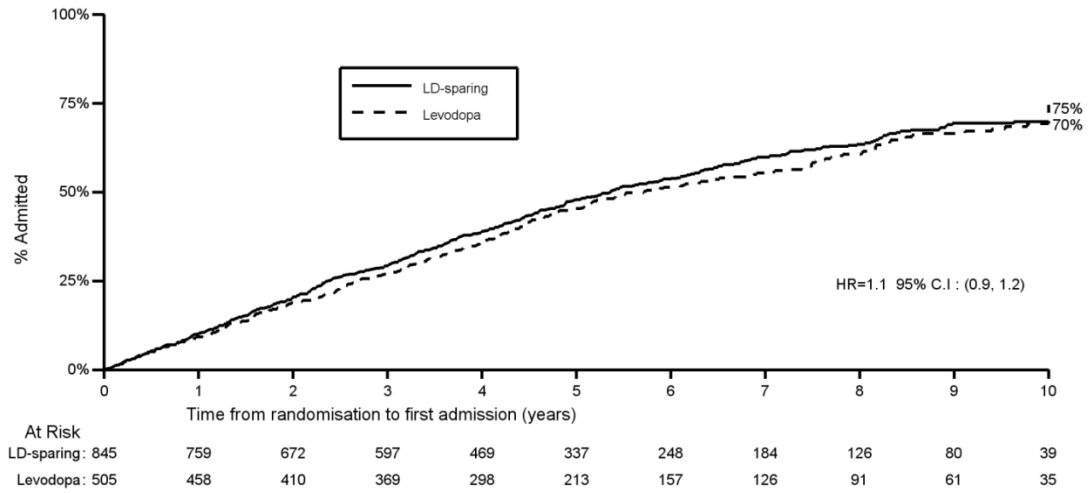
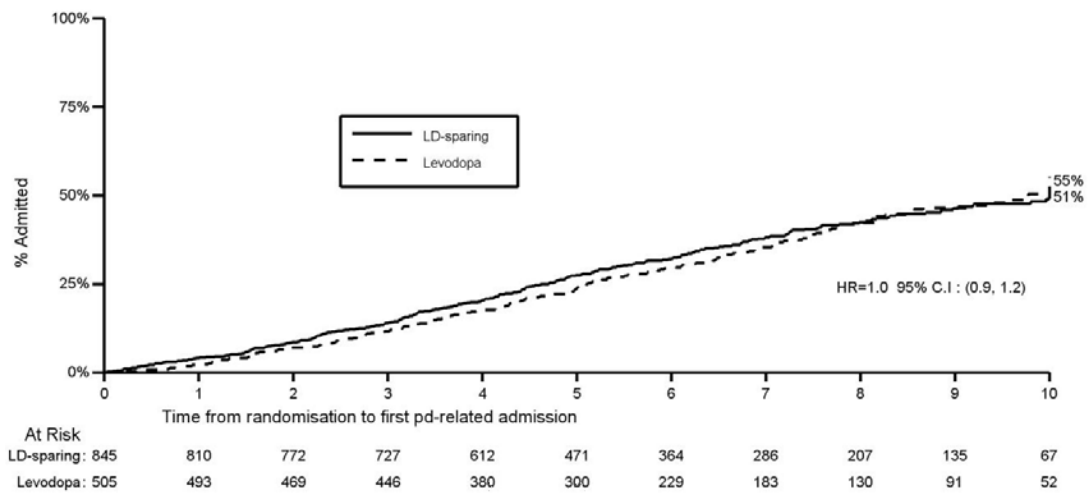
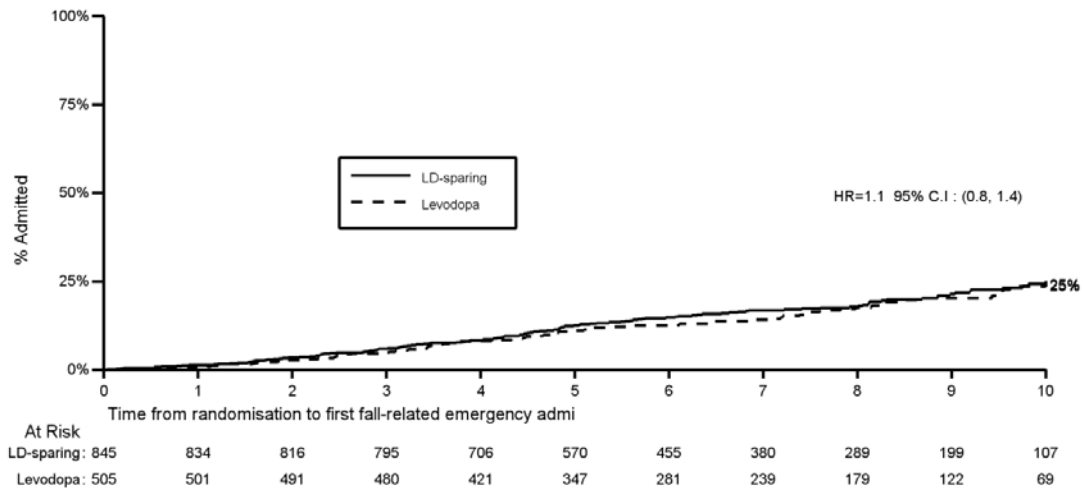


Figure 11: Time to first PD-related admission (levodopa versus levodopa-sparing) for PD MED EARLY patients who had non-elective admissions



There were 227 (17%) patients who had a fall/fracture/injury related admission. There was no difference in the time to first fall related admission (see figure 12)

Figure 12: Time to first fall/fracture/injury related admission (levodopa versus levodopa-sparing) for PD MED EARLY patients who had non-elective admissions



Dopamine agonist versus MAOBI

When comparing dopamine agonist versus MAOBI arms, there was no difference in the rate of admissions (RR 1.1; 95% CI 0.89, 1.31), admission duration (RR 1.1; 95% CI 0.92, 1.43), time to first admission (figure 13), and time to first PD-related event (figure 14).

Figure 13: Time to first admission (dopamine agonist versus MAOBI) for PD MED EARLY patients who had non-elective admissions

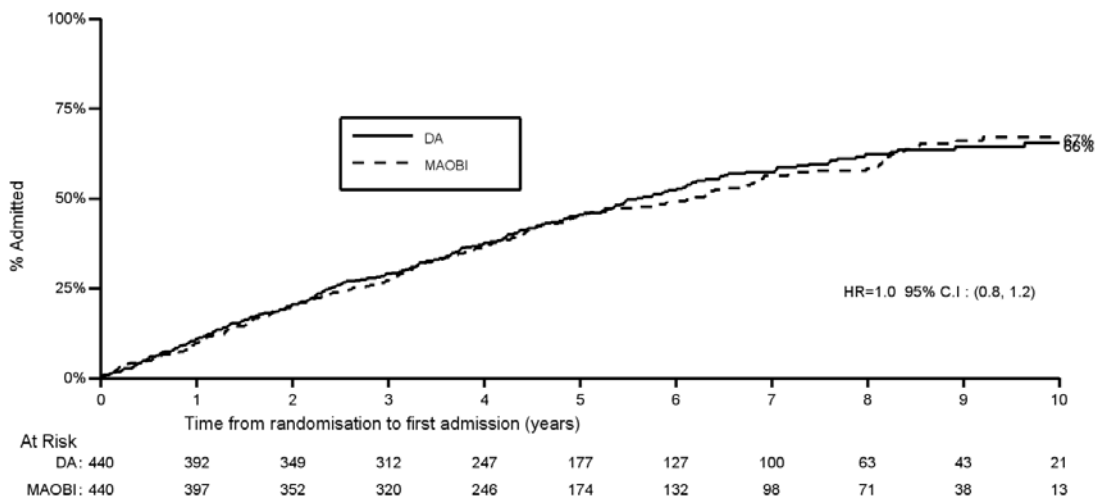
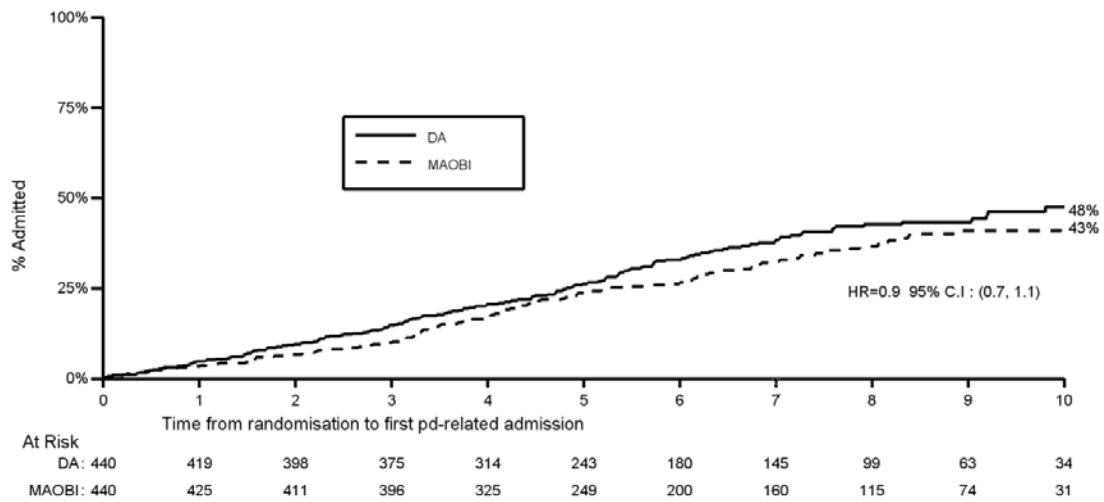


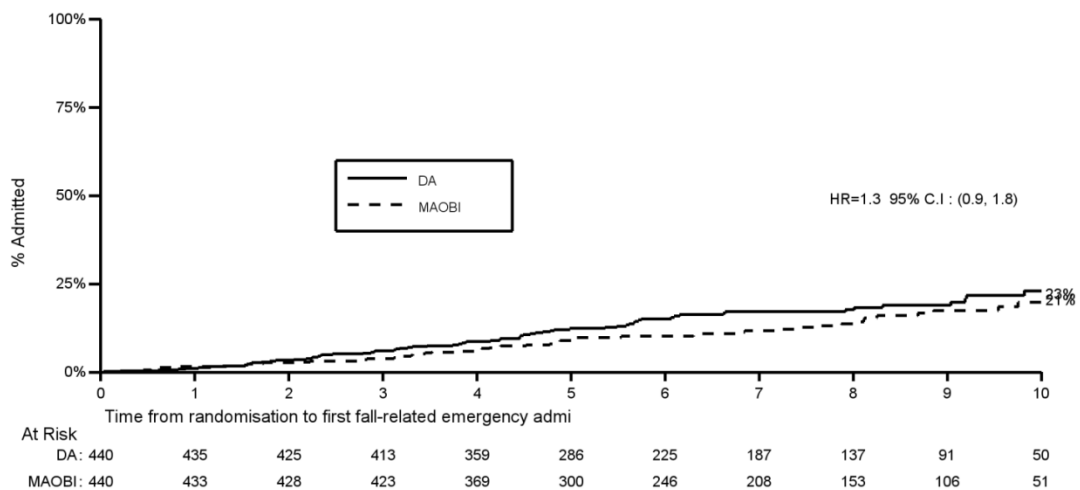
Figure 14: Time to first PD-related admission (dopamine agonists versus MAOBI) for PD MED EARLY patients who had non-elective admissions



There were 129 (15%) of patients who had a fall/fracture/injury related admission.

There was no difference in the time to fall-related admission between the arms (see figure 15).

Figure 15: Time to first fall/fracture/injury related admission (dopamine agonists vs MAOBI) for PD MED EARLY patients who had non-elective admissions



PD MED LATER

Patient Demographics

486 of patients from the PD MED LATER were included from England and Wales: mean age 73 ± 8.2 years mean disease duration 5.6 ± 3.9 years. 341 (70%) of these patients: mean age 73 ± 8.1 years, 63% male and mean disease duration 5.7 ± 4.1 years, were admitted during the study period. 42% of the patients who were admitted had H&Y stage 3 or more (table 10).

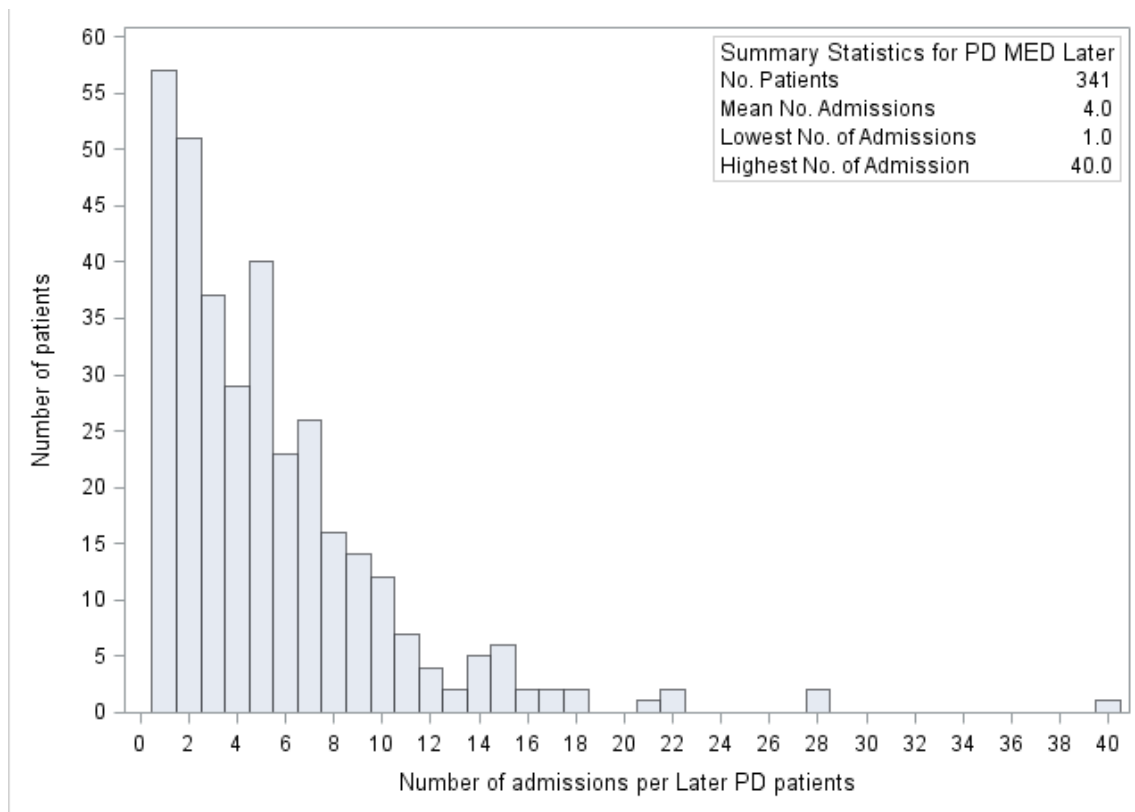
Table 10: Baseline demographic data for all PD MED LATER patients from England and Wales and the subset who had at least 1 admission

Variable	All PD MED patients included in dataset	Patients who had an admission
	N=486	N=341
Mean Age (SD)	73 (8.2)	73 (8.1)
Age (%) <50	5 (1)	3 (1)
50-59	31 (7)	19 (6)
60-69	103 (21)	63 (18)
70-79	254 (52)	179 (52)
≥ 80	93 (19)	77 (23)
Sex (%) Male	306 (63)	215 (63)
Duration of PD in years(SD)	5.6 (3.9)	5.7 (4.1)
Hoehn & Yahr N (%) 1	19 (4)	12 (3)
1.5	36 (7)	18 (5)
2	142 (29)	102 (30)
2.5	98 (20)	67 (20)
3	153 (32)	112 (33)
4-5	38 (8)	30 (9)

Hospital admissions

2 591 inpatient episodes making up 1 854 spells/admissions were obtained for the 341 patients. Of these patients, only two patients had taken part in early disease trial randomisation. The number of admissions per patient ranged from 1-40, median (IQR) 4(2-7) (figure 16). 38% (n=700) of these admissions had zero days duration, and the range was 0-434 days. Majority of the admissions were non-elective: 59% (n=1 017) and 41% (n=764) were elective. There were 73 transfers between hospitals.

Figure 16: Number of admissions per patient for the PD MED LATER patients who were hospitalised



Elective admissions

There were 764 elective admissions from 229 patients. Median number of admissions per patient was 2 (1-4) and range 1 - 39 admissions. 549 (72%) were zero bed days, median admission duration 0 (0-1), range 0- 320 days. Of the 764 elective admissions, 9% (n=68) had a primary diagnosis coded as PD-related of which 81% were coded as PD (table 11). Of the patients who had PD-related elective admissions, 6 (14%) were on dopamine agonists, 17 (41%) MAOBI, and 19 (45%) on COMTI.

Table 11: PD-related primary diagnoses (reasons for admission) for PD MED LATER who had elective admissions

Primary reason for admission	Number of admissions	%
Parkinson's disease	55	81
PD non motor symptoms	2	2.9
Neuropsychiatric disorders	2	2.9
Injuries	1	1.5
Infections	6	8.8
Fractures	2	2.9

Non-elective admissions

There were 1 017 non-elective admissions from 304 patients: 62 (27%) were on dopamine agonists; 69 (31%) MAOBI, and 96 (42%) on COMTI. Median number of admission per patient was 3(1-4), mean 3.3 ± 2.7 (figure 17), range 1 - 15 admissions. 91 (30%) of the 304 patients had one non-elective admission and the rest had more than 1 non-elective admission. 150 (15%) of the admissions were zero bed days, median admission duration 6 (1-17) days and range 0 - 131 days. Of all 1 017 non-elective spells, 532 (53%) had the primary diagnosis coded as a PD-related diagnosis (table 12)

Figure 17: Number of admissions per patient for the PD MED LATER patients who had non-elective admissions

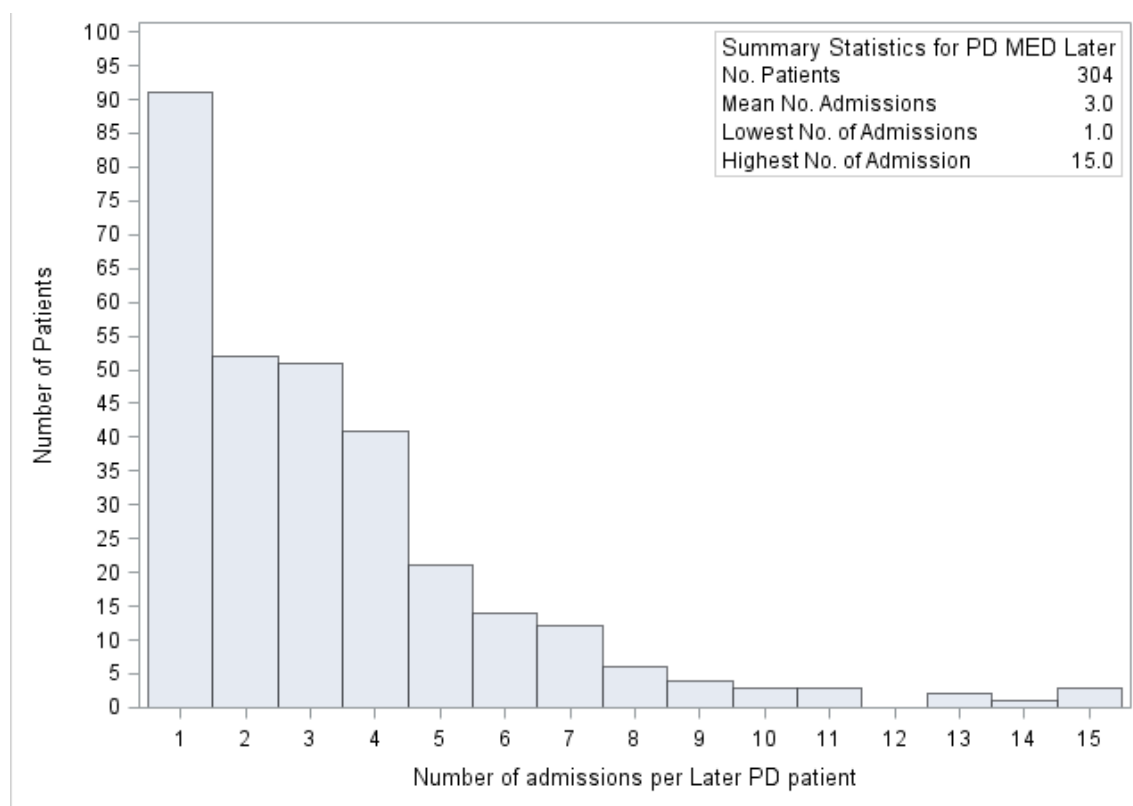


Table 12: PD-related primary diagnoses (reasons for admission) for PD MED LATER patients who had non-elective admissions

Primary PD-related reason for admission	Number of admissions	%
Fall/Fracture/Injuries	141	26.5
Parkinson's disease	120	22.5
Infections	176	33.1
Problems related to blood pressure	53	10.0
Neuropsychiatric disorders	35	6.6
PD non-motor symptoms	6	1.1
Other	1	0.2

Hospital admissions by treatment arm.

Non-elective admissions

Dopamine agonist versus MAOBI and COMTI

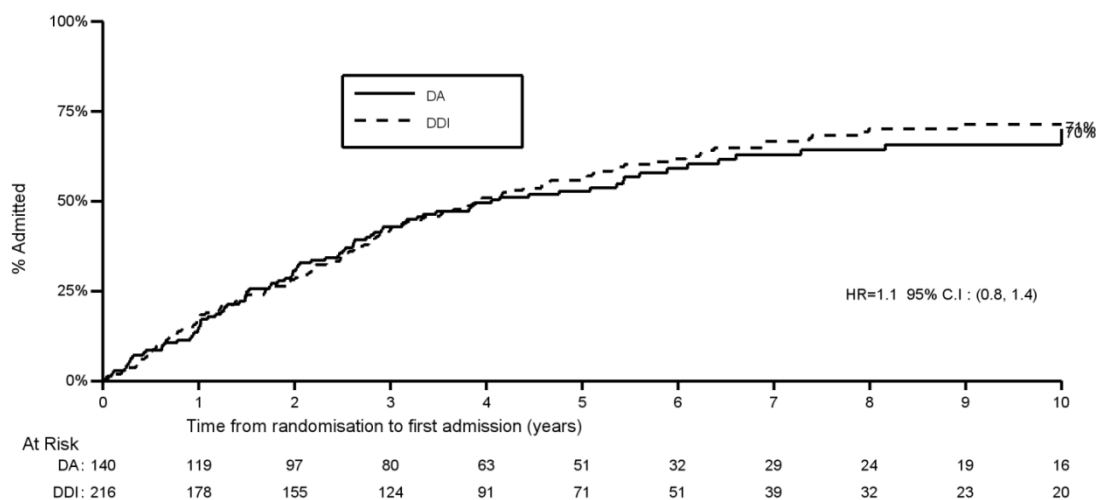
When dopamine agonists, and combined MAOBI and COMTI were compared there was no difference in the rate of admissions (RR 1.0; 95% CI 0.78, 1.38), admission duration (RR 1.0 95% CI; 0.81, 1.20), or time to first non-elective admissions (HR 1.1, 95% CI 0.8, 1.4): table 13, figure 18.

Table 13: Time to first admission, admission rates and duration for PD MED LATER trial patients who had non-elective admissions (levodopa versus levodopa sparing arms)

		DA	MAOBI and COMTI
N		140	216
Number of admissions	Mean (SD)	2.2 (2.96)	2.2 (2.69)
	Median (IQR)	1.0 (0.0-3.0)	1.0 (0.0-4.0)
N*		86	138
Time to first admission (years)	Mean (SD)	2.6 (2.24)	2.6 (1.99)
	Range	0.01 – 11.40	0.02 – 8.91
Total Admission Duration (per patient)	Mean (SD)	50.0 (55.2)	49.9 (57.0)
	Median (IQR)	32.5 (8.0-73.0)	33.0 (9.0-74.0)
N		302	483
Admission Duration (days)	Mean (SD)	14.2 (20.93)	14.3 (21.20)
	Median (IQR)	6.0 (1.0-17.0)	6.0 (1.0-17.0)

*Only including those patients who had an admission. The comparisons in this table exclude individuals who were randomised between the MAOBI vs COMTI arms. Total admission duration was calculated as the sum of the duration of admissions for all patient divided by the number of patients. Admission duration was average admission duration for all the admissions in each group.

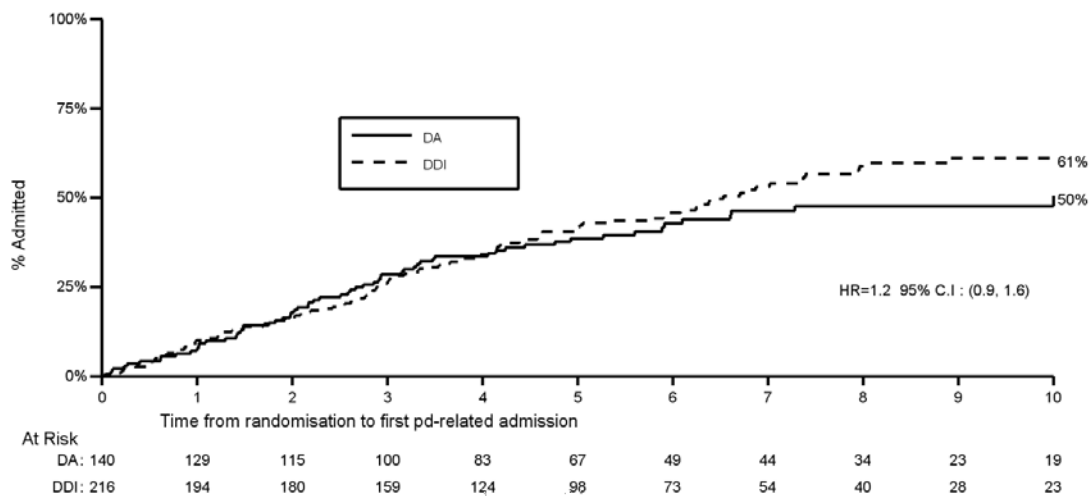
Figure 18: Time to first admission (dopamine agonists versus COMTI and MAOBI) for PD MED LATER patients who had non-elective admissions



DDI: dopamine degradation inhibitor (MAOBI and COMTI)

When comparing the number of PD-related non-elective admissions, no difference in rate of admissions was found between the two groups (RR 1.1; 95% CI 0.79, 1.53). After including H&Y stage, disease duration, gender and institutionalisation into a negative binomial model, there was no difference between dopamine agonists versus COMTI and MAOBI groups (RR 1.2, 95% CI 0.84, 1.60). For PD-related admission duration, a negative binomial regression model without covariates found no difference between the dopamine agonists and combined COMTI and MAOBI arms (RR 0.9 95% CI 0.73, 1.22). PD duration was the only significant covariate and when included, this did not change the inferences. Similarly, including patient admission duration as a random effect did not alter the results (RR 0.9, 95% CI 0.65, 1.17). There was no difference in the time to the first PD-related admission (figure 19).

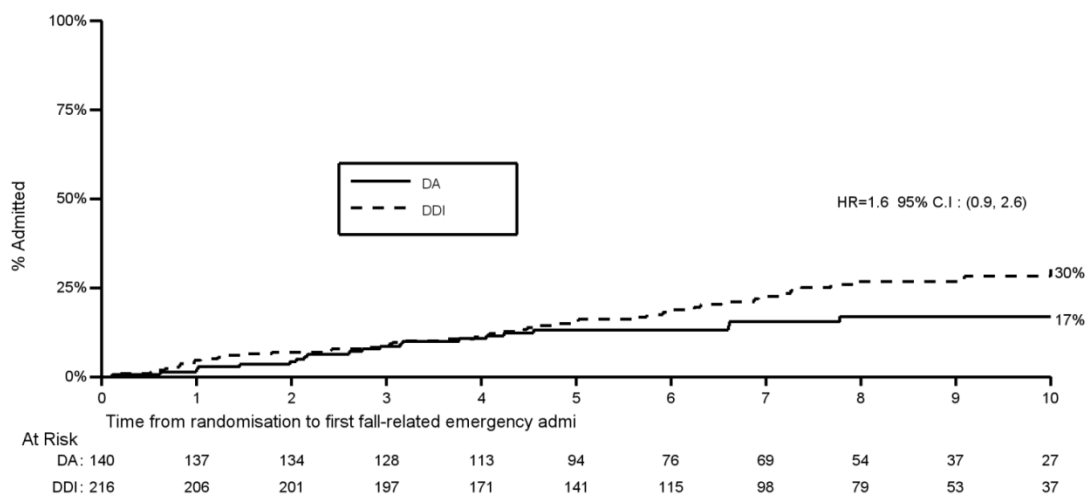
Figure 19: Time to first PD-related admission (dopamine agonists versus COMTI and MAOBI) for PD MED LATER patients who had non-elective admissions



DDI: dopamine degradation inhibitor (MAOBI and COMTI)

There were 70 (20%) patients who had a fall/fracture/injury related admission. There was no difference in the time to first fall related admission between the dopamine agonist compared COMTI and MAOBI groups (figure 20).

Figure 20: Time to first fall/fracture/injury related admission (dopamine agonists versus COMTI and MAOBI) for PD MED LATER patients who had non-elective admissions



DDI: dopamine degradation inhibitor (MAOBI and COMTI)

MAOBI versus COMTI

There was no difference in rate of admissions (RR 1.1; 95% CI 0.85, 1.52; table 14) and the time to first admission: HR 1.1, 95% CI 0.8, 1.4 (figure 21) between the MAOBI and COMTI groups.

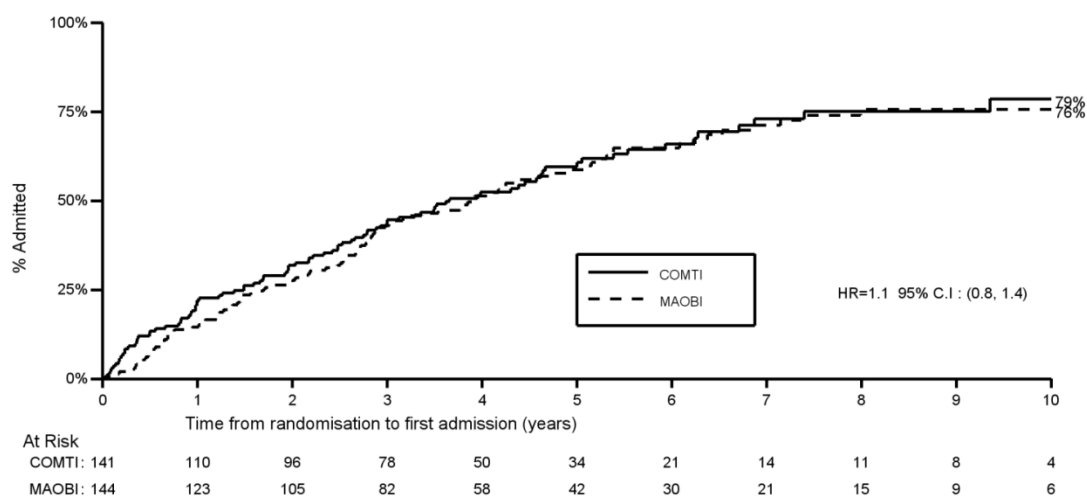
Table 14: Time to first admission, admission rate and duration for PD MED LATER patients who had non-elective admissions (MAOBI versus COMTI)

		MAOBI	COMTI
N		144	141
Number of admissions	Mean (SD)	2.0 (2.38)	2.3 (2.78)
	Median (IQR)	1.0 (0.0-3.0)	1.0 (0.0-3.0)
N*		95	91
Time to first admission (years)	Mean (SD)	2.7 (1.92)	2.4 (2.42)
	Range	0 – 10	0 – 9
Total Admission Duration (per patient)	Mean (SD)	36.6 (44.31)	51.3 (59.41)
	Median(IQR)	18.0 (6.0-54.0)	36.0 (9.0-72.0)
N		285	318
Admission Duration (days)	Mean (SD)	12.2 (18.56)	14.7 (22.01)
	Median (IQR)	6.0 (1.0-15.0)	6.0 (1.0-19.0)

*Only including those patients who had an admission. The comparisons in this table exclude individuals who were randomised between the DA versus MAOBI and COMTI arms. Total admission duration was calculated as the sum of the duration of admissions for all patient divided by the number of patients. Admission duration was average admission duration for all the admissions in each group.

When the admission duration was assessed using a negative binomial regression model adjusting for age, gender and institutionalisation, there was some evidence that patients in the COMTI arm had longer admissions (RR 1.2; 95% CI 0.99, 1.58). After including an offset for years in the trial, however, strong evidence of a difference between the arms in admission duration (RR 1.5; 95% CI 1.15, 1.97) was obtained. When similar analysis as that performed when comparing dopamine agonist versus COMTI and MAOBI was used, and adjusting for significant covariates, there was no evidence of a difference in admission duration (RR 1.2; 95% CI 0.92,1.63) between the COMTI and MAOBI treatment arms.

Figure 21: Time to first admission (MAOBI vs COMTI) for Later PD MED patients who had non-elective admissions



When comparing PD-related non-elective admission rate without adjusting for covariates, the admission rate ratio was 1.2 (95% CI 0.87, 1.78). Including the only significant predictors in the model (institutionalisation), did not change the inferences on admission rates (RR 1.2; 95% CI 0.84, 1.70). In addition, no difference between the arms in time to first PD-related admission was found (figure 22). For PD-related admission duration, no difference between the MAOBI versus COMTI arm (RR 1.2; 95% CI 0.92, 1.67) was found. There were no significant predictors for admission duration. Fitting a model with total admission duration per patient showed some evidence that the patients in the COMTI arm spent longer time in hospital (RR 1.5; 95% CI 1.0, 2.3). 58 (20%) of patients who had a fall/fracture/injury related emergency admission. There was no difference in the time to the first fall/fracture/injury-related admission, between the arms (figure 23).

Figure 22: Time to first PD-related admission (MAOBI vs COMTI) for PD MED LATER patients who had non-elective admissions

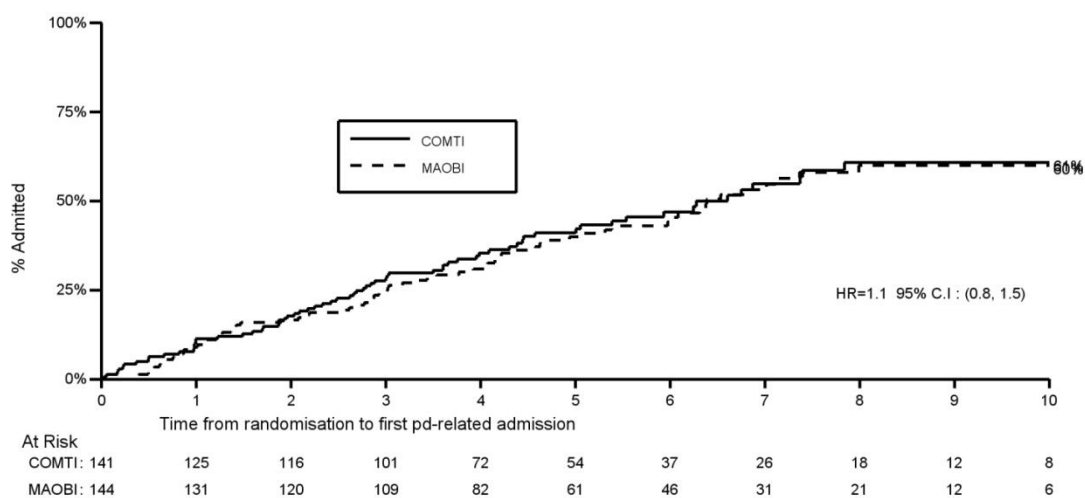
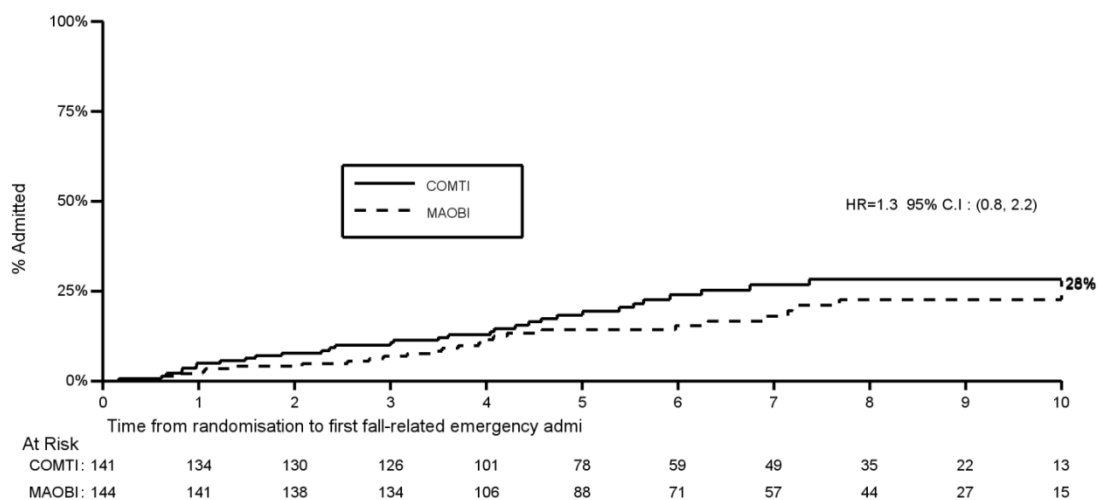


Figure 23: Time to first fall/fracture/injury related admission (MAOBI vs COMTI) for PD MED LATER patients who had non-elective admissions



3.4. Discussion

This study assessed hospitalisation patterns in two large cohorts of PD patients with early and later stage disease. 73% and 70% from the PD MED EARLY and LATER study, respectively, were admitted to hospital at least once during the study period. Prospective hospitalisation data from HES was used in conjunction with PD MED data, which allowed comparisons of hospital admission rates by randomised treatment arm. To our

knowledge, this is the first study to assess PD hospitalisation by treatment class in a prospective and randomised fashion. PD MED was a large study which included a real world population and therefore results of this study can be generalised to the PD population seen in routine clinical practice. Another strength of this study is its large sample size and availability of treatment, disease duration, and disease severity data from the PD MED database, which allowed comparisons to be made. These data were also used to control for potential confounders. Furthermore, all included patients had a PD diagnosis confirmed by a movement disorders specialist and based on the UK PD Brain Bank Criteria. Evaluating hospitalisation by treatment option may also provide insight into strategies that may be useful for preventing hospitalisation in PD

The high rate of hospitalisation demonstrated here is comparable to that reported in a study in Ontario which compared hospitalisation between 15 306 patients with Parkinsonism and 30 612 age and gender-matched counterparts without Parkinsonism (Guttman et al., 2004). Similar to the current study, Guttman et al. (2004) assessed both elective and non-elective admissions and showed that 68.4% of the Parkinsonism patients were admitted over the 6 year study period (Guttman et al., 2004). Nevertheless, their study included all patients with Parkinsonism as study authors could not validate the diagnosis of included patients (Guttman et al., 2004). The current study only included patients with a diagnosis of idiopathic PD. The admission rate reported in our study is therefore more likely to reflect the correct PD hospitalisation frequency.

There were no differences in hospitalisation rates, length of stay, and time to first admission, between levodopa and levodopa sparing arms. No difference was found when dopamine agonist and MAOBI arms were compared. In PD MED LATER trial cohort, no differences were found between the different adjunctive therapies. Since poor control

of motor symptoms and motor complications of treatment in later disease may lead to problems such as falls and fractures, the time to admission for these complications was also compared. But no difference in the time to admissions for falls and fractures was observed, when the treatment arms compared. These findings contrast with previous retrospective reports which demonstrated fewer emergency admissions and shorter length of stay in PD patients treated with rasagiline compared to dopamine agonists (Grubb et al., 2012).

There are a two plausible explanations for the lack of a difference in hospitalisation rates and duration between the treatment groups. Firstly, the current study showed that patient age, disease duration, and severity were significant predictors of hospitalisation. These factors were shown to be associated with an increased risk of falls (Wood et al., 2002, Allcock et al., 2009, Wielinski et al., 2005), low mineral density (Di Monaco et al., 2006), and dysphagia (D'Amelio et al., 2006), all of which increase the risk of hospitalisation in PD. Since age, disease duration and severity are not amenable to symptomatic treatments, therapy is unlikely to influence hospitalisation rates in PD. Secondly, addressing motor aspects of PD alone without addressing critical non-motor factors such as dementia, anxiety, and depression is unlikely to be effective in reducing admissions in PD. A more comprehensive approach which covers the management of both motor and non-motor aspects of the disease may be required.

Repeat hospital admissions were common among the PD MED patients who were included in this study. For example, from the group of patients who had non-elective admissions, 63% and 70% (PD MED EARLY and LATER, respectively) had repeat hospital admissions during the study period. Hassan et al. (2013) also reported high rates of repeat hospital encounters in 51% of PD patients (Hassan et al., 2013). These results

demonstrate substantial utilisation of health resources by PD patients across all disease stages.

As expected, the majority of the elective or planned admissions were of short duration: 80% and 72% of elective admissions in EARLY and LATER PD MED groups, respectively, were zero bed days.

Considering non-elective admissions, there was no difference in the median duration of stay between the PD MED EARLY and the LATER cohort. This observation was unexpected since patients with more advanced disease are more likely to develop complications during hospital admissions which can prolong length of stay. In addition in advanced disease, treatment regimens are complicated, therefore treatment errors are more likely to occur resulting in longer duration of hospital stay. The commonest reasons for non-elective admission in both groups were the same: falls fractures, infections and motor symptoms (primary diagnosis: PD). This provides an explanation for the similar lengths of stay.

The admission duration reported in this study is similar to what has been previously reported in other UK studies which assessed emergency admissions only. A recent study in England used HES data to evaluate the cost of PD hospitalisation and also assessed other aspects of hospitalisation including length of stay, reasons for admissions and mortality (Low et al., 2015). The study reported mean lengths of stay which ranged from 5-17 days among PD patients of different age groups (Low et al., 2015). Another retrospective study assessed the reasons for PD emergency admissions to a district general hospital in England and reported a mean length of stay of 21 days among the 120 PD patients (Woodford and Walker, 2005). Furthermore, a prospective study reported a mean duration of stay of 12 days during emergency admissions to a general hospital in northern

Italy over a 1 year period (Martignoni et al., 2004). In Turkey, a small retrospective study including 66 PD patients admitted to emergency department reported a mean duration of 12 ± 7 days. A number of factors may influence length of stay during. Low et al. (2015) showed an increasing length of stay with patient age in PD patients (Low et al., 2015). Differences in healthcare systems, hospital reimbursements, hospital bed availability, and social care structures may also influence length of stay, therefore direct comparisons of duration of admissions between countries may not be accurate.

Although the current study did not have a control group of people without PD, there is clear evidence that PD patients stay in hospital longer than non-PD controls (Lubomski et al., 2015, Low et al., 2015, Guttman et al., 2004). Low et al. (2015) showed that PD patients stayed in hospital 7 days longer than controls (Low et al., 2015). A large retrospective Australian study compared demographic details and hospitalisation aspects of PD and non-PD patients and reported a median length of stay 7 (3-13) in PD patients and 1 (1-7) days in those without PD ($p < 0.001$) (Lubomski et al., 2015). Similarly, Guttman et al. (2003) reported 1.19 times longer length of stay in PD patients compared to controls (Guttman et al., 2003). Several reasons including deterioration in PD motor symptoms, account for the prolonged admissions during PD hospitalisation. Gerlach et al. (2012) showed that 20% of PD patients who were admitted to hospital reported a deterioration in motor symptoms and a further third develop complications such as confusion and infections (Gerlach et al., 2012). The risk of motor deterioration was associated with poor PD medication management (Gerlach et al., 2012, Gerlach et al., 2013). Magdalinou et al. (2007) performed a case note review of 35 patients admitted to hospital and reported prescription errors and a lack of awareness by junior doctors of the available formulations of PD medication, which resulted in complications (Magdalinou et al., 2007). An association between PD medication errors and prolonged length of stay

was also demonstrated in a recent cross-sectional study (Martinez-Ramirez et al., 2015). The authors reviewed records for 212 PD patients who had 339 admissions and showed that those who had missed/delayed doses and had dopamine blockers prescribed stayed in hospital for longer periods than those who did not have similar prescription errors (Martinez-Ramirez et al., 2015).

Infections, falls, fractures, and injuries were the commonest reasons for non-elective admissions in both PD MED EARLY and LATER groups, which is consistent with previous reports. There were more PD-related non-elective admissions in the later than the early disease group, which is expected: 59% and 45%, respectively. Disease and medication related complications such as postural instability, hypotension, falls, fractures, and neuropsychiatric symptoms occur more frequently in later stages of the disease leading to frequent hospital admissions for these complications. Nevertheless, patients with early disease were also admitted to hospital for these complications. Falls, infections, and fractures are also an early disease phenomenon, albeit less frequent. Walker et al. (2013) assessed the incidence of hip fractures in people with PD compared with non-PD controls. 72% of the PD patients who had hip fractures were reported to have mild to moderate PD (H&Y stages 1-3) (Walker et al., 2013). Low mineral bone density, a known significant risk factor for fractures, (Schneider et al., 2008) has been demonstrated in patients with early PD (Van den Bos et al., 2013b). Another study reported falls in 23% of patients with early PD (Voss et al., 2012). Dysphagia, silent aspiration, and impaired cough efficacy are thought to contribute to the increased risk of respiratory tract infection in PD and these abnormalities have been demonstrated in both early and advanced disease (Potulska et al., 2003, Ebihara et al., 2003) which may explain the high number of infections leading to admissions in the current study. Occurrence of these complications in early disease suggests that similar strategies may be employed

across all disease stages to reduce hospitalisation in PD. Targeting patients with early disease may prevent future hospitalisation. Such interventions may include osteoporosis prevention for fractures, falls management, and screening for dysphagia to reduce the risk of aspiration pneumonia. The effectiveness of these preventative strategies should be examined in further studies.

Some patients were admitted to hospital for management of motor symptoms (primary diagnosis code PD), these only contributed to a small proportion of non-elective admissions: 7% and 12% in early and later PD MED, respectively. Since the majority of the admissions were for reasons other than management of motor symptoms, this observation may also explain the lack of a difference in hospitalisation between patients on the different oral treatments for PD motor symptoms.

There are a number of limitations to this study. Firstly, there was a possible underestimation of PD admissions. Incomplete episodes at the beginning or end of the study period were excluded which probably resulted in an under estimation of the number and duration of admissions. Evaluation of coding of PD admissions at a tertiary hospital in England showed significant underreporting of PD admissions on the Secondary Uses Services database which is linked to HES (chapter 4). This suggests that the extent of PD hospitalisation may be even higher than reported in the current study. Finally, hospital admissions to private hospitals are not included in HES which may have resulted in underestimation of PD hospitalisation, but this only contributes to a small proportion of hospital admissions in the UK.

In conclusion, this study demonstrated a high rate of hospitalisation in patients with early and advanced PD. Although available symptomatic treatments are effective in controlling motor symptoms, they had no differential effect in reducing hospitalisation rates and

length of stay in PD. Addressing motor aspects of the disease alone may not be sufficient to reduce the burden of hospitalisation in PD. Disease related complications which are frequently encountered in advanced disease, contributed to a large proportion of non-elective admissions in both groups of PD patients. Therefore, screening and early management of these complications in addition to addressing motor and non-motor aspects of the disease may prevent unplanned admissions in PD and reduce the associated costs. These prevention strategies should be introduced earlier in the course of the disease. Future studies should evaluate the effectiveness of preventative interventions that address both motor and non-motor aspect of the disease.

**CHAPTER 4: UNDER-REPORTING OF PARKINSON'S DISEASE HOSPITAL
ADMISSIONS**

4.2. Background

Hospitalisation in PD is associated with a substantial economic burden (Low et al., 2015). It is crucial that metrics relating to hospital episodes are accurate. These data form part of the Secondary Uses Services (SUS) database in England which is used for hospital reimbursements and health care planning (Payne, 2013). In this study, the number and characteristics of PD admissions to a large Birmingham hospital were estimated and the coding accuracy of the admissions was also assessed.

4.3. Methods

This service evaluation was approved and registered with the University Hospital Birmingham (UHB) audit department. The hospital is a large tertiary centre in the UK's second largest city, with a capacity of 1 213 inpatient beds, and providing secondary care services to an estimated 800 000 patients per year (University Hospital Birmingham NHS foundation Trust, 2013). The hospital also has a large catchment area beyond the West Midlands and is the regional centre for functional surgery in PD (University Hospital Birmingham NHS foundation Trust, 2013). Data extraction was performed by the UHB informatics department and data were stored in a secure SQL server.

The SUS database is a database where all data pertaining to health resource use in NHS trusts in England are stored (Payne, 2013). The SUS was searched for PD admissions during the period 1st July 2009 to 30th June 2013. Admissions where PD was recorded as a primary or secondary diagnosis were identified from this database using the ICD-10, codes G20 and F023 for PD and PD dementia, respectively, based on the discharge date.

The UHB electronic inpatient Prescribing Information and Communications System (PICS) database, stores information on medications prescribed during hospital

admissions. The PICS database was searched for all admissions where PD medications were prescribed during the study period.

Quality assurance checks on the data were performed. Firstly, since PD is less prevalent in younger age groups (Pringsheim et al., 2014), all patient records for admissions identified from SUS and PICS, where the patient age was under 40 years, were reviewed, and those with diagnoses other than PD were excluded. Secondly, since dopaminergic drugs used for treating PD are also used in other conditions, a sample of 20 medical records for patient admissions identified from PICS database search were reviewed, to ascertain if they had PD. Of the 20 records, 55% (n=11) had PD and were all on levodopa, 35% (n=7) were on low dose non-ergot dopamine agonists for restless legs syndrome or cabergoline for pituitary adenomas, and the remaining 2 patients had normal pressure hydrocephalus and dystonia. Patient records for all admissions obtained from the PICS database, where bromocriptine, cabergoline and low dose non-ergot dopamine agonists were prescribed (pramipexole <1.05mg, ropinirole <6mg/day, rotigotine <6mg/day) were reviewed, and excluded admissions for patients with other diagnoses. Case notes for patients contributing to the remaining admissions identified by the PICS search were also reviewed and admissions for those who did not have PD were also excluded.

For all admissions identified in the SUS database using G20 and F023 ICD-10 codes, and the PICS database, patient hospital numbers were obtained. A supplementary search of the two databases was then undertaken using these patient hospital numbers to identify any additional hospital admissions missed by the SUS and PICS criteria, but still occurring in the evaluation period.

To estimate the number of PD patients who were admitted to hospital, data from the electronic search of SUS and PICS databases were used and capture-recapture methods

were applied to estimate the number of patients missed by both searches (Hook and Rega, 1995). This is a recognised method for estimating population sizes or disease occurrence and assumes that the available data sources do not identify the entire population being studied (Brittain and Böhning, 2008). Chao's lower bound method which provides a reliable estimation of the total population size was used (Brittain and Böhning, 2008).

The same capture-recapture analysis was also applied to the number of PD admissions that were identified after electronic searches of SUS and PICS. Admissions obtained from the supplementary search using hospital numbers were not included in the capture-recapture analysis, as this was not an independent source of admissions.

Assessing the coding error rate

The coding error rate was ascertained as the proportion of PD admissions not recorded in the SUS database. The denominator was the sum of all admissions identified by the three searches (PICS, SUS, and hospital number search) described above and the numerator was the number of PD admissions not appearing in the SUS database. Descriptive analyses of the type of patients (age, gender) and information on the admission (e.g. number of admissions per patient during the study period, length of stay, and reasons for admissions) were performed.

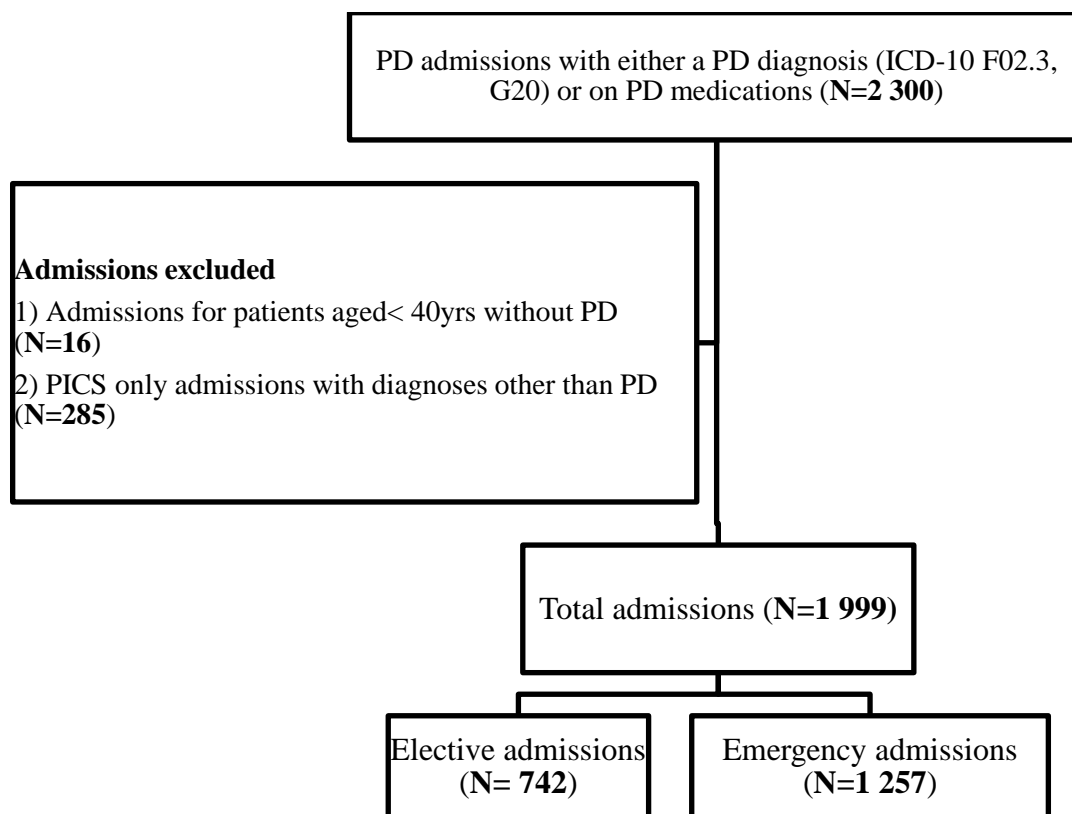
4.4. Results

Exclusions

2 300 possible PD admissions were identified from SUS and PICS databases over the 4 year period. 16 admissions for patients below the age of 40 years who had diagnoses other than PD (dopa-responsive dystonia, pituitary tumours, and drug-induced Parkinsonism) were excluded (figure 24). After reviewing case notes for patients who had

admissions identified from the PICS database, 285 admissions for patients who did not have PD were also excluded.

Figure 24: PD admissions identified from SUS, PICS databases search



Numbers of patients

997 patients were identified from SUS (656 of these were in both PICS and SUS, and 341 patients in SUS only), and 71 patients were identified from PICS only (figure 25). Therefore, in total, the PICS and SUS database identified 1 068 PD patients as having been admitted during the 4 year study period: median age (IQR) was 75 (67-82) years and 59% were male. Capture-recapture methods estimated that the number of patients missed by both databases was 59, meaning that over the 4 year period an estimated 1 127 PD patients, 95% CI 1 107 to 1 146, were admitted to UHB. Therefore, the SUS database missed an estimated 130 PD patients (range: 110-149 missed patients).

Figure 25: The number of Parkinson's disease patients by method of ascertainment

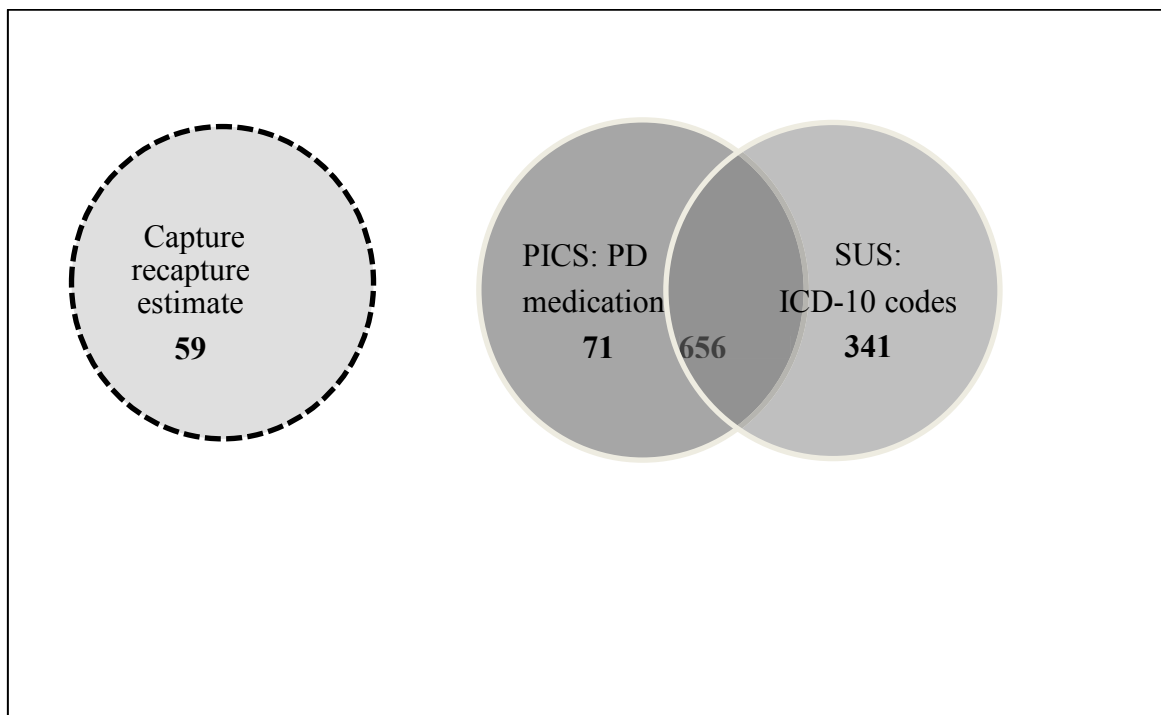


Figure 25 above shows the number of PD patients obtained by searching the PICS, SUS databases. The capture recapture method estimated that 59 patients were not recorded in these two databases.

Numbers of admissions

SUS database identified 1 811 admissions during the study period. Of these, 1 053 admissions appeared in both databases (PICS and SUS), and 758 admissions were in SUS only. 188 admissions were in PICS only. Therefore, in total, 1 999 admissions were identified from the two databases. Capture-recapture methods estimated that the number of admissions missed by the SUS and PICS searches was 212, meaning that over the 4 year period, there were an estimated 2 211 PD admissions, 95% CI 2 169 to 2 252, to UHB.

A supplementary search of both databases using the patient hospital numbers of the 1 068 patients obtained from initial SUS and PICS searches, identified an additional 479 PD

admissions during the study period. For these admissions, no PD ICD-10 code was recorded on SUS and no PD medications were prescribed on PICS (table 15). Therefore, when the three search criteria (SUS, PICS and hospital numbers) were combined, a total of 2 478 PD admissions were identified during the study period.

Table 15: Number of Parkinson’s disease admissions by data source

Data Source	Admissions		
	Total	Elective	Non-Elective
SUS	1 811	700	1 111
PICS only	188	42	146
Hospital number search	479	324	155
Total	2 478	1 066	1 412

Coding errors

The admissions coding error rate was calculated using data from the three sources: 1 811 PD admissions from SUS database, 188 from PICS database only, and 479 admissions from the hospital number supplementary search. Therefore, 27% (667 not recorded on SUS/2 478) of these admissions were not coded as having PD on discharge from hospital, and were therefore not recorded in the SUS database during the study period.

Capture-recapture methods estimated that there were 2 211 admissions of PD patients. There were 1 811 admissions recorded on SUS, which gives an estimated coding error rate of 18% (400 admissions not recorded on SUS/2 211 admissions).

Non-elective admissions

Of the 2 478 admissions identified during the study period, there were 1 412 (57%) non-elective admissions in 747 patients: median age 79 (71-85) years and 57% were male. 60% (n=451 patients) were admitted only once during the study period and 40% had repeat hospital admissions (range 2-18 admissions). Median length of hospital stay was 6 (1-18) days. 45% of the admissions were under the general medical team, 21% geriatric medicine, 7% general surgery, 5% trauma and orthopaedics, 5% accident and emergency, 4% neurosurgery, and only 1% were admitted to the neurology ward. Infections (20 %), and falls, fractures and injuries (15%) were the commonest reason for non-elective admission (Table 16).

Elective admissions

There were 1 066 elective admissions in 502 patients during the 4 year study period: median age 71 (63-79) years and 64% were male. Median length of stay was 0 (0-1) days. The majority of elective admissions were to neurosurgery (24%), nephrology (19%), neurology (9%), general surgery (9%), cardiology (6%), and ophthalmology (4%). The commonest reasons for elective admission included PD (20 %), genitourinary disorders (20%), neoplasms (13 %), musculoskeletal disorders (11 %), central nervous system and disorders of sense organs (7%), gastrointestinal disorders (7%) and PD surgery (6 %).

Table 16: Commonest primary reasons for non-elective and elective admissions

		Total		Elective		Non-Elective	
Age		Median:75 IQR:67-82		Median:71IQR:63-79		Median:79 IQR:71-85	
		Range:40-101		Range:40-94		Range:44-101	
Cause of Admission	Infections	186	11.6%	0	0	186	17.2%
	Cardiovascular and circulatory disorders	155	9.7%	48	8.6%	115	10.6%
	Falls fractures	112	7.0%	4	0.7%	109	10.0%
	Gastrointestinal disorders	102	6.4%	34	6.1%	76	7.0%
	Other injuries	75	4.7%	1	0.2%	74	6.8%
	PD	218	13.6%	164	29.4%	64	5.9%
	PD mental health symptoms	65	4.1%	1	0.2%	64	5.9%
	Problems related to blood pressure	61	3.8%	1	0.2%	61	5.6%
	Musculoskeletal and connective tissue disorders	102	6.4%	48	8.6%	56	5.1%
	Central nervous system and disorders of sense organs	86	5.4%	42	7.5%	44	4.0%
	Genitourinary tract disorders	51	3.2%	13	2.3%	41	3.8%
	Respiratory tract disorders	38	2.4%	0	0.0%	38	3.5%
	PD gastrointestinal	30	1.9%	2	0.4%	28	2.6%
	Endocrine disorders	32	2.0%	7	1.3%	25	2.3%
	Neoplasms	108	6.7%	89	15.9%	23	2.1%
	Complication of device implants or prosthesis medical and surgical procedure	30	1.9%	11	2.0%	21	1.9%
	PD surgery	71	4.4%	58	10.4%	16	1.5%
	Other infectious disease	12	0.7%	0	0.0%	12	1.1%
	PD other NMS	12	0.7%	0	0.0%	12	1.1%
	Dermatological disorders	16	1.0%	9	1.6%	7	0.6%

4.5. Discussion

This study showed a significant under-coding of PD on discharge from hospital. 8% (n=188) of all admissions were identified by the prescription database only, implying that although PD medications were prescribed, PD diagnosis was not coded on discharge so these admissions were not found in the SUS database. Another 19% of PD admissions were identified based on searching both PICS and SUS databases using hospital numbers of patients previously identified using PD and PD dementia ICD-10 codes and PD medication, suggesting further underestimation of PD admissions by SUS. In total, 27% of PD admissions were not recorded on the SUS database during the study period.

Capture-recapture methods showed slightly lower estimates for PD under-coding on SUS (18%), which indicates that the method is not always reliable when used to estimate populations. However, the method also showed that PICS and SUS searches underestimated the number of PD patients admitted during this period by 59 patients, suggesting that the capture-recapture coding error rate is higher than 18%. The methods used for ascertaining admissions in the present study were exhaustive, so our estimate for under-reporting of PD admissions on SUS is likely to be accurate.

Under-coding of other chronic conditions as secondary diagnoses has also been reported when hospital inpatient data were used (Whitston et al., 2012, Anwar et al., 2011). Anwar et al. (2011) reported 41% under-coding of diabetes admissions when Scottish inpatient hospital data were compared with the Scottish National Diabetes register (Anwar et al., 2011). In another study in England, an estimated 9% of diabetes hospital admissions were not coded when HES data were compared with data from the English National Diabetes audit (Whitston et al., 2012). Although these studies showed a wide disparity in diabetes under-coding, results suggest that improvements in diagnosis coding are required. This is crucial since resource allocation (Payne, 2013), performance metrics used for comparing NHS trusts, and hospital reimbursement (Department of Health, 2013) are derived from inpatient data. A recent audit of accident and emergency and inpatient care data of NHS trusts in England (2012/13) conducted by the Audit Commission reported a mean coding error rate of 11% for all primary diagnoses and 15% for secondary diagnoses for inpatient spells (Department of Health, 2013).

When compared to these previous figures, the present study showed a much higher coding error rate. Several reasons may account for the difference in coding error rates. Firstly, the current study was disease-specific whereas the NHS audit reported an overall coding

error rate for several hospitals and all diagnoses. Coding accuracy for PD is likely to vary across different hospitals, as occurs with other chronic conditions (Anwar et al., 2011, Whitston et al., 2012). The majority of admissions in the current study (57%) were non-elective and infections, falls, fractures, and injuries were the commonest primary reasons for admissions. Since PD was a secondary diagnosis during these admissions, it was less likely to be recorded. Lastly, 78% of non-elective admissions were recorded under teams other than neurological or geriatric teams, which may account for the high PD under-coding. Although no comparison of coding error differences between different admitting specialties was performed, it is likely that teams which are familiar with PD management are more likely to record the disease on discharge. This requires further verification in the future.

The high under-reporting of PD admissions shown in our study questions the quality and usefulness of SUS data for PD healthcare planning. The financial implications of such errors on individual hospital trusts are likely to be substantial as previously shown (Nouraei et al., 2009, Razik et al., 2013). A recent study to evaluate the reasons, rate, and cost of PD hospitalisation in England reported expenditure of £907 million on PD admissions over a 4 year period (Low et al., 2015). If 27% of PD admissions are not recorded on the SUS database as shown in the present study, then the NHS expenditure on PD admissions is underestimated by approximately £61 million annually.

The English NHS audit in 2012/13 found that coding inaccuracies for inpatient spells were mainly due to poor recording of secondary diagnosis codes by coders, (Department of Health, 2013) who are not medically trained. In addition, discharge summaries which contained inadequate and inaccurate information were used to obtain information for coding (Department of Health, 2013). Coders also relied on poorly documented medical

notes for extracting data, which led to coding errors (Department of Health, 2013). In other studies, lack of involvement and awareness of the importance of coding by medical staff were some of the key factors contributing to coding errors (Razik et al., 2013, Pillai A and Medford A.R, 2013)..

A number of recommendations aimed at tackling these potential sources of coding errors can be proposed. These include dissemination of information regarding the importance of accurate and legible handwritten medical documentation on all wards and in the doctor's mess. Coding of neurological diagnoses for patients who attend the neurology outpatients department has also been proposed. Senior clinicians who review patients in these clinics are more likely to record accurate neurological diagnoses. Our hospital uses electronic outpatient records and these are linked to the inpatient prescribing database which is also available to coders for use during coding. This may improve coding of neurological diagnoses when patients are discharged from hospital. The effect of this intervention on inpatient coding of neurological diagnoses will be assessed in the future. If results show an improvement in the coding error rate, this intervention will be trialled in other departments. Further training of coders is also required.

Limitations of this study include its reliance on retrospective data which may lead to inclusion of admissions for patients without PD. This potential problem was addressed by excluding admissions from the PICS database where small doses of dopamine agonists were prescribed for conditions such as restless legs syndrome and pituitary adenomas. Case notes of all patients who were identified by the PICS database only were reviewed and non-PD cases were excluded. Admissions identified from SUS database only but having no PD medications prescription may reflect prescription errors which are common when PD patients are hospitalised (Derry et al., 2010). It is also plausible that a proportion

of these could have been incorrectly coded as having PD. For example patients with other types of Parkinsonism not requiring dopaminergic medication may have been coded as having PD. Patients with early PD, who have mild motor symptoms, which do not require treatment could potentially account for a small proportion of these patients.

If under-reporting of PD admissions on the SUS database is repeated nationally, the NHS expenditure on PD admissions in England is underestimated by approximately £61 million annually. If such coding errors are not addressed, these figures will continue to rise with increasing life expectancy, and thus PD prevalence, in coming decades. The NHS should take urgent action to implement measures to improve coding accuracy to improve healthcare planning and hospital reimbursement.

**CHAPTER 5: DEVELOPMENT OF A MAGNETIC RESONANCE IMAGING
(MRI) SENSITIVE PROBE FOR IMAGING IN PARKINSON'S DISEASE**

5.1. Background

Dopamine metabolism

The monoamine dopamine (3,4hydroxyphenylethyl-amine) was discovered by Arvid Carlsson in the 1950s (Carlsson, 2003). The neurotransmitter plays a crucial role in many processes including movement, cognition, learning, appetite, mood, and also in peripheral sympathetic nervous system (Beaulieu and Gainetdinov, 2011). A large proportion of dopaminergic neurons in the brain are found in the substantia nigra and ventral tegmentum area of the midbrain. These neurons have three major projections to the striatum, limbic and prefrontal cortical regions (Bjorklund and Dunnett, 2007). Degeneration of the substantia nigra dopaminergic neurons is the main mechanism by which the motor syndrome of PD develops. Understanding the processes involved in dopamine synthesis, release, storage and uptake at the presynaptic terminal is crucial for a number of reasons. Firstly, it may provide insights into processes that lead to the degeneration of dopaminergic neurons. For example, impaired dopamine metabolism may lead to production of toxic oxidative species which have been implicated in the pathogenesis of the dopaminergic neuron degeneration (Meiser et al., 2013). Secondly, current PD treatments exert their action by either binding to the dopamine receptors or by inhibiting various enzymes that are involved in dopamine degradation. Further, ligands that are currently used in imaging the dopaminergic system bind to various targets involved in dopamine metabolism (figure 2).

Dopamine synthesis and degradation

The initial step in dopamine biosynthesis involves conversion of tyrosine to dihydroxyphenylalanine (levodopa) by the enzyme tyrosine hydroxylase (TH), a process regulated by the cofactor tetrahydropterin (Elsworth and Roth, 1997, Meiser et al., 2013). Levodopa is then decarboxylated by aromatic amino acid decarboxylase (AADC) to form

dopamine which is in turn transported actively from the cytoplasm into storage vesicles, (Elsworth and Roth, 1997) by the vesicular monoamine transporter 2 (VMAT2) (Meiser et al., 2013). Dopamine is pumped via a proton gradient into the vesicles and can reach very high vesicular concentrations (estimate 0.1M) compared to the cytoplasmic concentration (Elsworth and Roth, 1997). This prevents formation of oxygen reactive products from dopamine oxidation in the cytoplasm (Meiser et al., 2013). Dopamine then exerts its functions by binding to and activating its receptors (D1-5) on the post synaptic terminal (Beaulieu and Gainetdinov, 2011). Dopaminergic neurotransmission is regulated by dopamine auto-receptors (D2-like), which are present on presynaptic terminal, and these receptors inhibit dopamine synthesis and release, and also slows down the firing rate (Elsworth and Roth, 1997). These D2 auto-receptors also potentially regulate dopamine transporter (DAT) by increasing its surface expression thus increasing dopamine uptake (Eriksen et al., 2010). DAT and other monoamine transported such serotonin transporter (SERT) and noradrenaline transporter (NET) belong to a family of transporters called solute carriers 6 (SLC6) which transport monoamines using a sodium transmembrane gradient and chloride co- transport (Eriksen et al., 2010). Once in the cytoplasm, dopamine is either actively transported back into storage vesicles for further release or metabolised by the enzyme monoamine oxidase (MAO) to form 3,4 dihydroxyphenylacetic acid (DOPAC) (Elsworth and Roth, 1997, Meiser et al., 2013). The remaining dopamine in the cytoplasm is degraded by monoamine oxidase and catechol-O-methyl transferase (COMT) to form homovallinic acid (HVA) by glial cells (Meiser et al., 2013). Figure 26 below illustrates the dopamine metabolism discussed above.

Figure 26: Dopamine synthesis and degradation

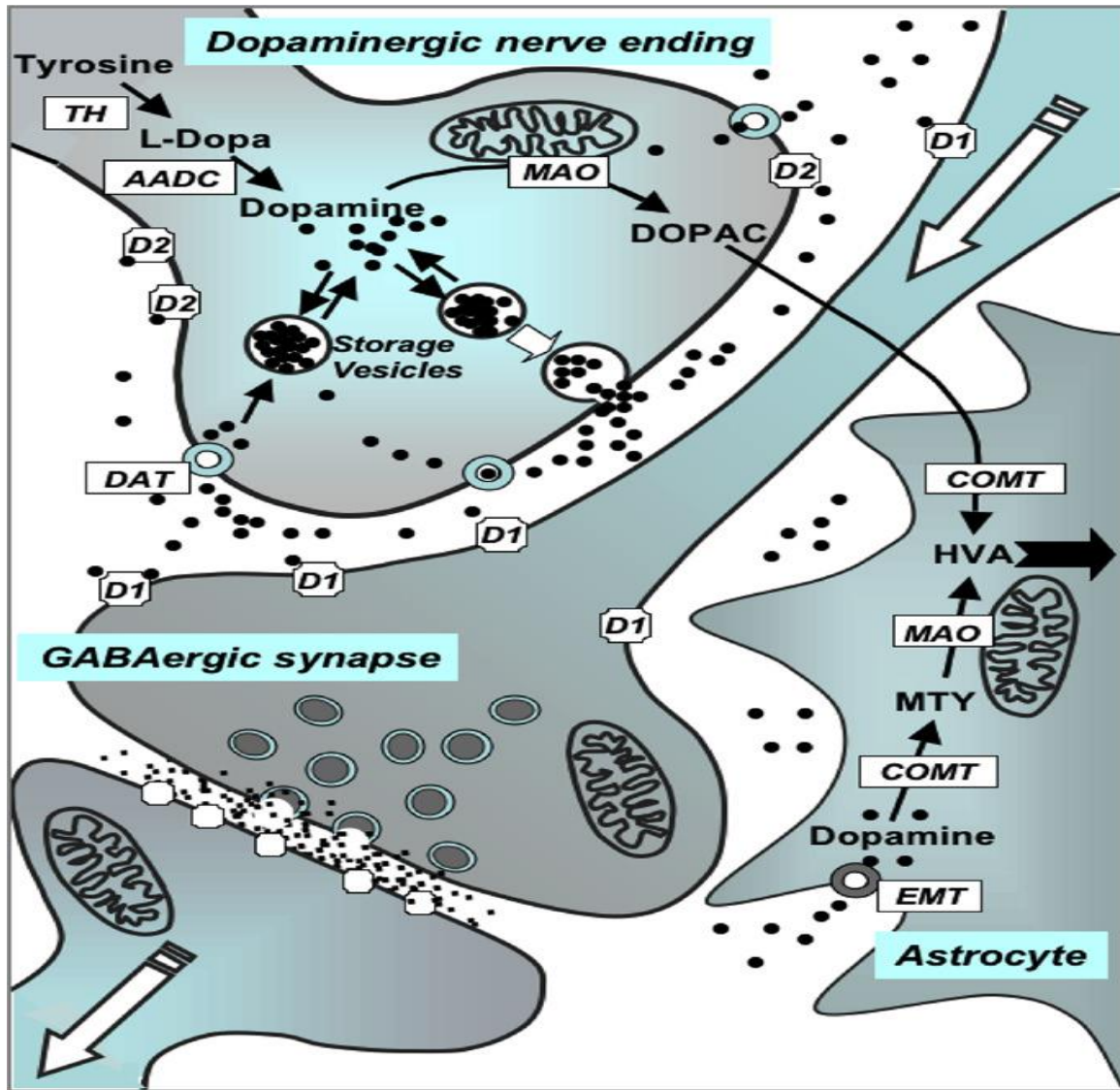


Figure obtained from Eisenhofer and Reichmann 2012: Dopamine synthesis, storage, release, uptake, and degradation. Enzymes involved in dopamine synthesis: TH, tyrosine hydroxylase and AADC, aromatic acid decarboxylase, Transporter for dopamine storage: VMAT2 (not shown) is found the storage vesicles. Reuptake transporters: DAT, dopamine transporter; EMT, extracellular monoamine transporter. Receptors: D1, dopamine D1 receptor; D2 dopamine D2 receptor. Degradation enzymes: MAO, monoamine oxidase; COMT, catechol-O-methyltransferase. Degradation products: DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; MTY, 3-methoxytyramine.

Figure obtained from Eisenhofer G, Reichmann H (2012) Dopaminergic Neurotransmission in Primer on the Autonomic Nervous System (Third Edition), Paton D, Robertson I, Biaggioni G, Burnstock PA, Low JFR, eds. Academic Press, San Diego, pp. 63-65, with permission from Elsevier

5.2 Aims

MRI has several advantages over radionuclide imaging in PD, including availability, lower costs, and no exposure to radiation. But its use as a diagnostic tool in PD is limited by low sensitivity (Brooks, 2010). Nevertheless, as highlighted in Chapter 1, small research studies have shown that when various MRI modalities such as diffusion tensor imaging, susceptibility imaging, and high field strength are combined, MRI gives high sensitivity and specificity for differentiating brains of individuals with PD from healthy control brains,(Peran et al., 2010) atypical forms of the disease, (Prodoehl et al., 2013) and may also have a role in assessing disease severity and progression (Zhang et al., 2010, Ulla et al., 2013). This suggests that with further developments, MRI has potential utility as a diagnostic and prognostic biomarker.

The main objective of this study was to determine the feasibility of using two types of MRI sensitive probes (lanthanide and ^{19}F based) for imaging dopaminergic cells in PD. These probes may be used in the future in conjunction with other MRI techniques such as DTI, high field strength and SWI to increase the sensitivity of the tool and this project forms the initial stage in its development.

5.3. Materials and methods

Lanthanide probes

Lanthanides are a group of rare earth metals, used as molecular probes and contrast agents by utilising their fluorescent and magnetic properties (Heffern et al., 2014). MRI contrast agents used in clinical practice are based on the lanthanide, gadolinium which has magnetic properties. Europium has fluorescent properties and its long stokes shift (difference between excitation and emission wavelengths) is utilised in fluorescence microscopy studies to abrogate the influence of cellular auto-fluorescence by use of

interference filters (Diamandis and Morton, 1988, Murphy and Davidson, 2012). For purposes of this study europium based compounds were used for fluorescence microscopy. Since europium and gadolinium are isoelectric, when a lead compound is obtained, europium will be replaced with gadolinium to form a probe which will then be tested in small animals using magnetic resonance imaging.

DAT ligands used in clinical practice are cocaine analogues which bind to surface DAT with very high affinities. The hypothesis for this study was that the lanthanide based probe would be taken up by dopaminergic terminals via DAT or neutral amino acid transporters in a similar fashion as PET ligands. This approach potentially allows intracellular accumulation of the substrates in sufficiently high amounts to allow visualisation by fluorescence microscopy and also by MRI in subsequent studies. Hence, compounds that are DAT or neutral amino acid transporter substrates, instead of those that only bind to surface DAT, were synthesised.

Given that dopamine and levodopa are actively pumped into the dopaminergic terminals via DAT and neutral amino acid transporter respectively,(Camargo et al., 2014) probes which are potential DAT or ADCC substrates were synthesised by conjugating europium to either dopamine or levodopa. Feasibility of using the europium probes was demonstrated in a previous pilot study in our laboratory (unpublished). In the current study cellular uptake of europium compounds by PC12 cells was assessed using fluorescence microscopy.

Europium-dopamine (MT172, MT630) and Europium-L dopa (MT519) conjugates, were synthesised at the Oxford Chemistry Research Laboratory courtesy of Dr Manuel Tropiano and Professor Stephen Faulkner. The chemical structures and fluorescence spectra of the compounds MT172, MT630, and MT519 are shown in figure 27 below. As

preluded above, the europium compounds characteristically have long stoke shifts: excitation wavelength is in the ultraviolet to blue range and emission within the visible (red) range of the electromagnetic spectra. Each compound was diluted with physiological saline (PSS) to make a stock solution of concentration 10mM and stored in 100 μ L aliquots at -20⁰ C. On the day of the experiments the compounds were thawed and diluted further with PSS to make 30 μ M, 100 μ M, and 300 μ M solutions for the experiments and warmed to 37⁰C.

PC12 Cells

PC12 cells were obtained as a gift from Professor Ann Logan's laboratory at the University of Birmingham. The cells were used for these experiments for several reasons. Firstly, the cell line has been used broadly in PD. Greene and Tischler (1976) demonstrated that the large quantities of cells could be produced and maintained easily (Greene L.A and Tischler A.S, 1976), which is a useful property for screening studies. Furthermore, PC12 cells possess the machinery required to produce dopamine and also contain energy dependent transporter mechanisms for uptake of monoamines into the cells (Greene and Rein, 1977, Kadota et al., 1996). These properties are essential for cellular uptake of potential DAT or ADCC substrates, which were used in this project.

Maintaining undifferentiated PC12 cells

Undifferentiated PC12 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 (+glutamax) culture media (Thermofisher: Life technologies), using established protocols (Elobi, 2014). RPMI culture media was supplemented with horse serum 10%, foetal calf serum 5% and 1% penicillin/streptomycin (50 μ g/ml) to make the complete growth media and stored at 4⁰C. Cells were grown in 25cm² flasks, in an incubator with 5% CO₂ and 95% humidified air at 37⁰C. Cell culture media change was performed every

two days. All media preparation and change was performed in a sterile fume cupboard, cleaned with 70% ethanol prior to use. Cells were transferred from the tissue culture flask to a falcon tube and gently pipetted to separate the cells. 10 μ L of the cell suspension was mixed with an equal volume of trypan blue and cells were counted using a haemocytometer. The cell suspension was centrifuged at 800 revolutions per minute (RPM) for 5 minutes. The supernatant was discarded and the cell pellet dislodged by adding fresh growth media. The cell density was adjusted by adding the required volume of fresh media to maintain a maximum cell density of 500 000 cells per ml and transferred to the tissue culture flask which was stored in the incubator.

Culture media for differentiating PC12 cells

Dulbecco's Modified Eagle Media (DMEM) (1X) (+Glutamax) (Gibco® life technologies) was used for differentiating PC12 cells. This was supplemented with 1% horse serum, 1% penicillin/streptomycin and 100ng/ml nerve growth factor (NGF) 2.5S (Sigma-Aldrich®, UK). The differentiation media was changed daily for 7 days using the protocol described by Greene and Tischler (Greene L.A and Tischler A.S, 1976). Cell differentiation was monitored daily using an inverted microscope.

Physiological Salt solution (PSS)

One litre of PSS was made up by adding 0.368g EDTA(1mM), 4.766g HEPES (20mM), 0.373g KCl (5mM), 7.592g NaCl (130mM), 0.901g glucose(5mM), 1.3mls MgCl (from 1mM stock solution yielding 1.3mM), and 1.8mls CaCl (1.8mM) to 500mL distilled water, then and made up to 1000mls. The PH was adjusted to 7.4 using 1M NaOH (Elobi, 2014).

Cover slips for plating PC12 cells

13mm² cover slips were used for plating cells. The cover slips were sterilised with 70% ethanol and left to dry in a sterile fume cupboard. Poly-L-lysine* sigma cat number P4707 (0.01%) was diluted 1:1 with sterile filtered water. Cover slips were soaked in the diluted poly-l-lysine for 5 minutes and then allowed to air dry for 2 hours. Prior to an experiment, cells were seeded onto coverslips at density of 50 000cells per coverslip and placed in an incubator for one hour to allow the cells to attach before adding fresh culture media: RPMI or DMEM based culture media for undifferentiated and differentiated PC12 cells respectively. For undifferentiated cells, coverslips with cells were kept at 37⁰C in the incubator overnight and used for cellular uptake experiments the following day. For differentiated PC12 cells, differentiation media was changed daily for 7 days. Experiments for differentiated PC12 cells were performed from day 7 onwards.

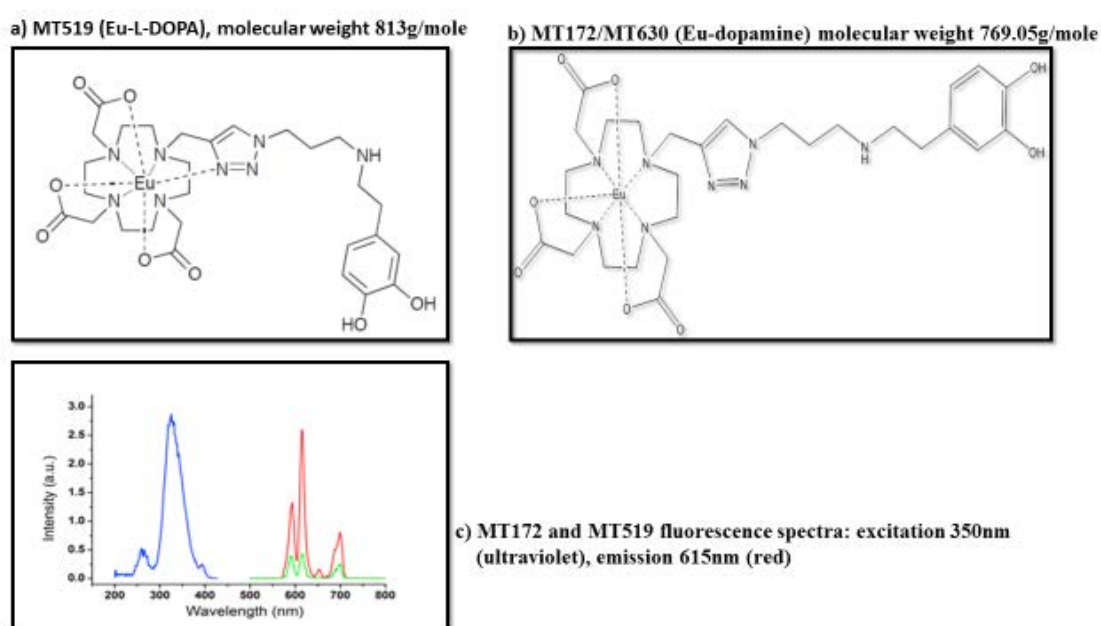
Neurotransmitter Transporter Uptake Assay (NTUA): positive control

The NTUA assay kit contains a fluorescent monoamine transporter substrate for the dopamine transporter DAT, Noradrenaline transporter (NET) and serotonin transporter (SERT) (Molecular devices, UK part number R8173). The substrate is pumped into the cells via any of these three monoamine transporters.(Jorgensen et al., 2008) The kit also contains a dye which quenches extracellular fluorescence and therefore aids in reducing any background signal.(Jorgensen et al., 2008) NTUA was diluted with 10mls of physiological saline (PSS) and aliquots were stored at -20⁰C. On the day of experiments NTUA was thawed, diluted further with PSS (1:10), and pre-warmed to 37⁰C.

Cellular uptake of lanthanide compounds by PC12 using fluorescence microscopy

PC12 cells were plated onto coverslips pre-coated with poly-L-lysine as described above. For the cellular uptake experiments, a coverslip with cells was placed in a heated organ bath attached to an epifluorescence microscope (Olympus BX51W1 purchased from Olympus, Southend, UK). The cells were perfused with pre-warmed PSS (37⁰C) for 20 minutes at flow rate of 0.6ml/min. After the PSS perfusion, cells were continuously exposed to either NTUA (positive control) or europium based compounds (MT172, MT630, or MT519) for 60 minutes. This was followed by a further drug washout with PSS for 20 minutes. For the NTUA cellular uptake experiments, PC12 cells and NTUA were excited with a 405nm light emitting diode (LED) source and a fluorescein Isothiocyanate (FITC) emission filter (519nm) was used.

Figure 27: Chemical structure and molecular weight of Europium complexes.



For cellular uptake of europium conjugates, excitation wavelengths, 405nm and 365nm from an LED light source (Cairn imaging, Kent, UK) and Qdot 605nm emission filters (Chroma Technology Corp, USA) were used. In subsequent experiments a mercury arc lamp light source was used with a 390nm excitation filter. Bright field and fluorescent Images were taken at time 0 then at 10minute intervals throughout the experiment using

an Orca-ER Hamamatsu digital camera (Hamamatsu photonics, UK), attached to the epifluorescence microscope. The following camera settings were used, bright field: exposure time 150ms, gain 0, offset=0 and fluorescent images: exposure time 5sec, gain 255, offset 0. Organ bath temperature was maintained between 29 and 34 °C.

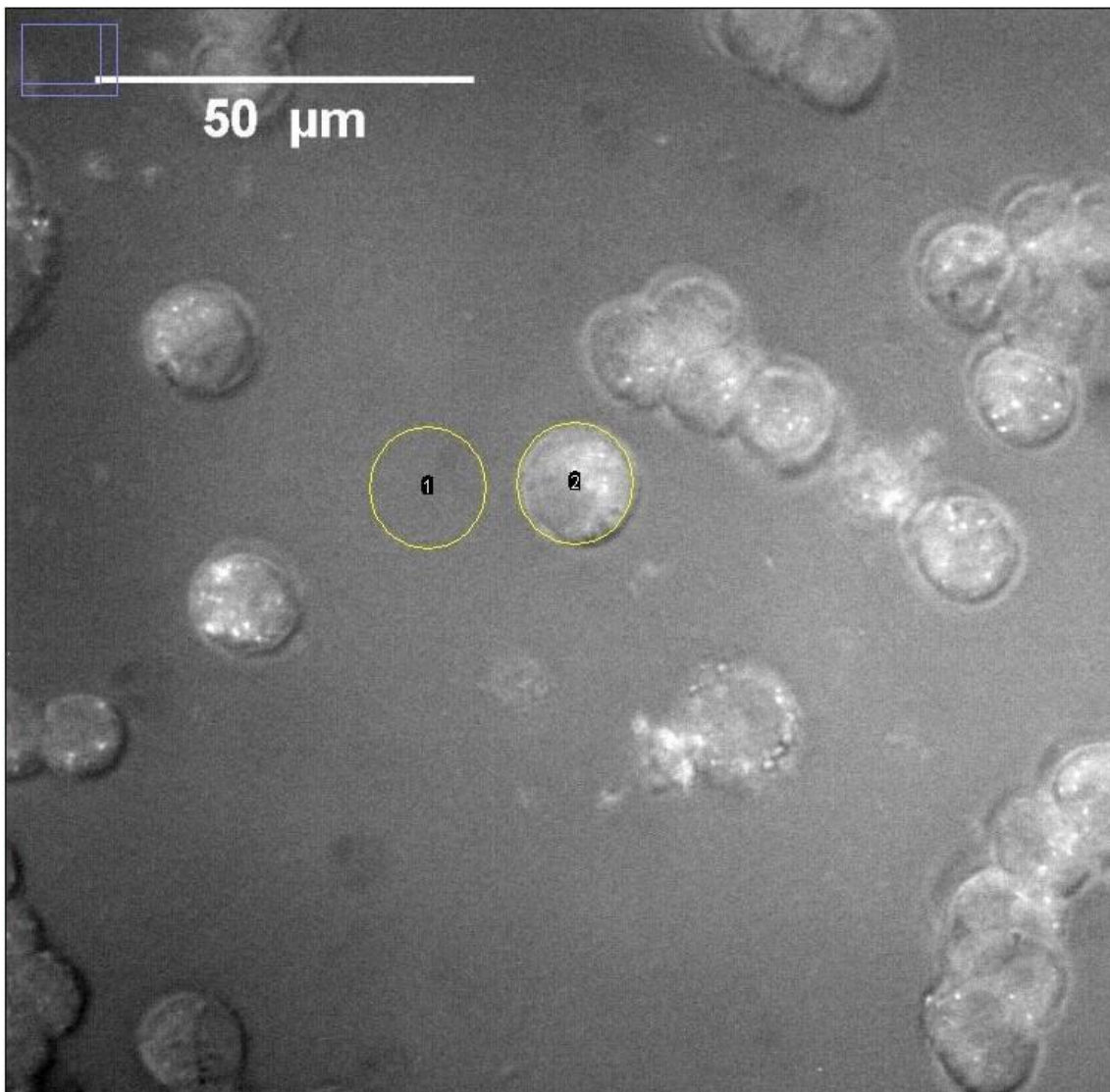
The change in PC12 cell fluorescence (negative control) over time was also assessed using methods similar to those for cellular uptake experiments.

Statistical analysis

Image analysis was performed using image J software version (1.38e/Java1.5). A region of interest (ROI) was created around an individual PC12 cell (figure 28). The ROI was automatically fitted onto subsequent images of each cell on the image stack. Since the distribution of the fluorescent vesicles was uneven, with some regions of the cells having significantly higher pixel intensity than others (figure 28), the median, instead of mean cell fluorescence was used, since the median would be more representative of such data. The same principle was applied when measuring the background fluorescence. Median fluorescence (median pixel intensity) was measured in each ROI. Another ROI of exactly the same size as the cell, was drawn adjacent to the cell and the median fluorescence of this region was considered as the background fluorescence. Fluorescence was expressed in arbitrary units (au). Corrected median fluorescence for each cell was calculated by subtracting the median background fluorescence from the median cell fluorescence (figure 28). The mean fluorescence of all cells in each experiment was calculated for all time points: 0, 10, 20, 30, 40, 50 and 60 minutes. Data were analysed using GraphPad Prism 6. Ink. The mean fluorescence was plotted against time and the rate of uptake (slope of the graph) in $\text{au}\cdot\text{min}^{-1}$ was calculated. Data were represented as mean \pm SEM.

Comparisons between groups were performed using Student t-test or Mann-Whitney U test accordingly.

Figure 28: Regions of interest for cell and background fluorescence



The areas marked in yellow represent the regions of interest. Pixel intensity in region of Interest (1) represents background fluorescence and (2): cell fluorescence, scale bar 50μm.

5.4. Results

PC12 cells developed branchlike extensions (neurites), which increased in number and length with time, when cells were maintained in DMEM based media supplemented with NGF (figure 29).

Cell Auto-fluorescence

When PC12 cells were washed with PSS over 60minutes a decline in cellular fluorescence over time was observed: rate of decline $-0.09 \pm 0.005 \text{ au}\cdot\text{min}^{-1}$ (figure 30).

5.4.2.1 Cellular uptake of neurotransmitter transporter uptake assay (NTUA) by PC12 cells using fluorescence microscopy

There was a time dependent NTUA cellular accumulation (increase in cellular fluorescence) in both differentiated (figure 31) and undifferentiated PC12 cells (figure 32), when 405nm excitation wavelength and 519nm emission filter were used. Rate of NTUA uptake was $0.27 \pm 0.02 \text{ au}\cdot\text{min}^{-1}$ and $0.33 \pm 0.01 \text{ au}\cdot\text{min}^{-1}$, in differentiated and undifferentiated cells respectively.

Figure 29: PC12 differentiation from day 1 to day 7 in NGF enriched DMEM culture media.

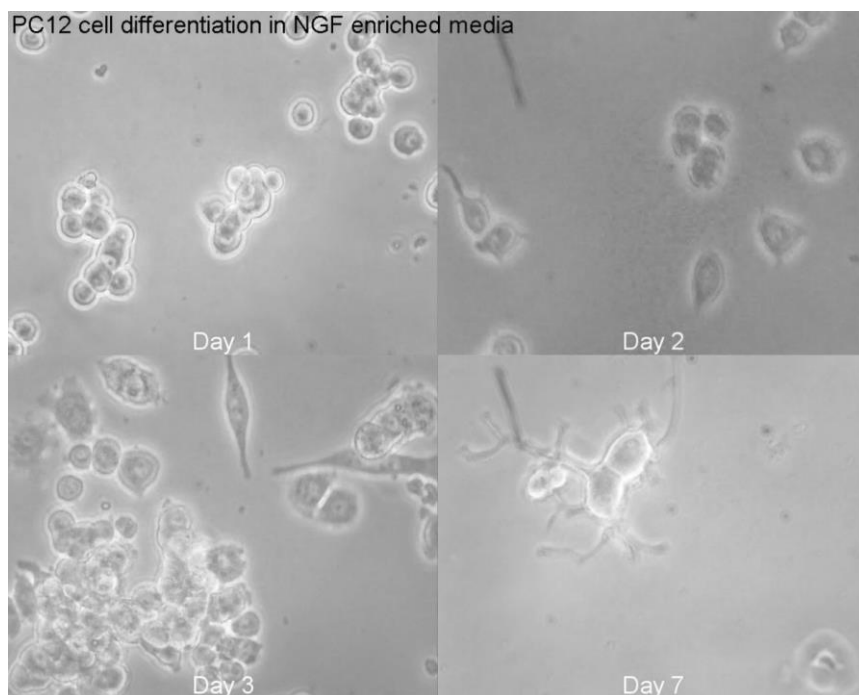


Figure 29 above shows PC12 cells grown in NGF-enriched media. Neurite number and length increased from day 2 onwards.

Figure 30: Change in undifferentiated PC12 cell auto fluorescence over time

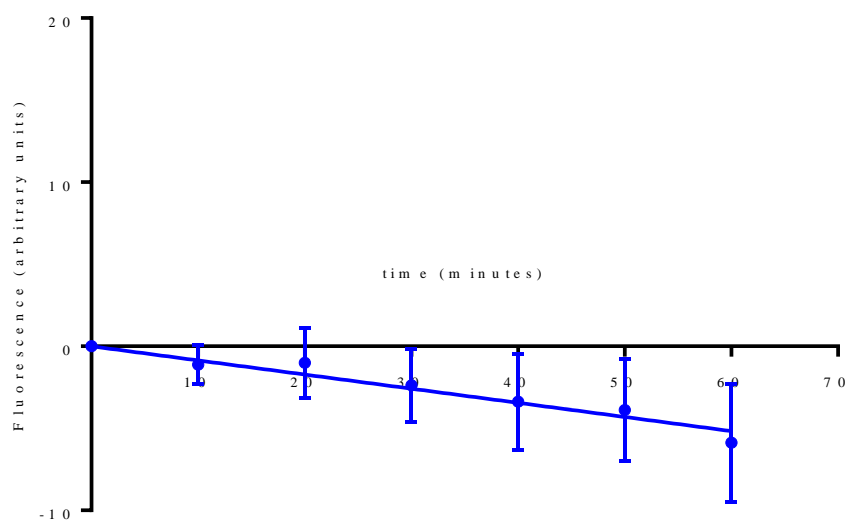
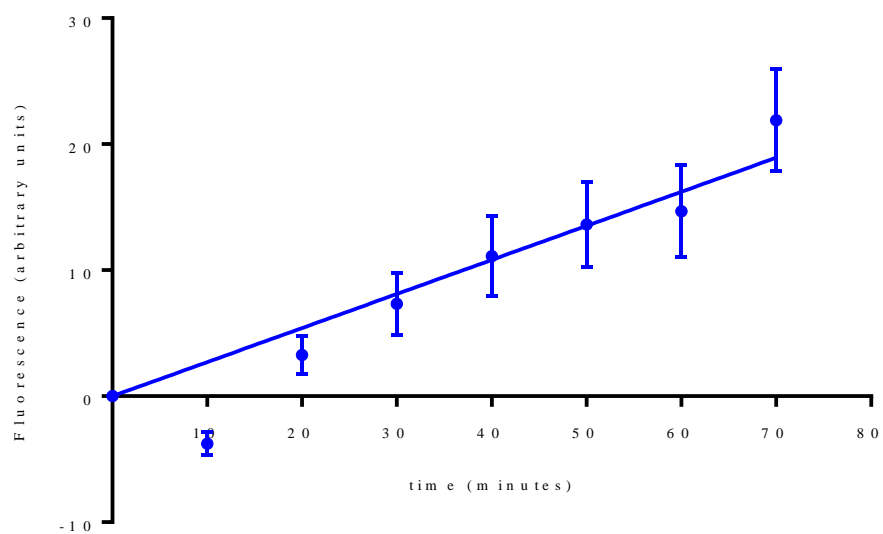


Figure 30 above, data points represent mean change in PC12 cell fluorescence \pm SEM, when cells were washed with PSS over 60minutes

Figure 31: NTUA cellular uptake by differentiated PC12 cells



b)

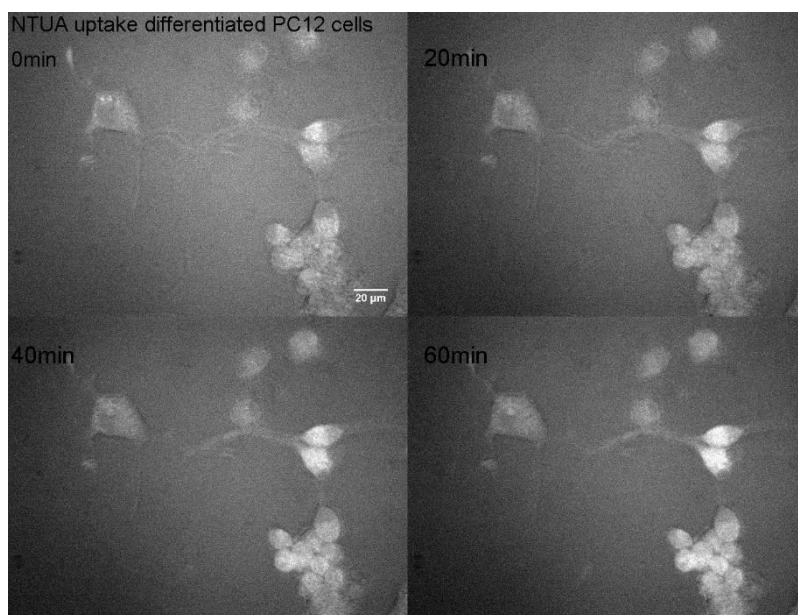


Figure 31a) Data points represent mean change in PC12 cellular fluorescence \pm SEM when exposed to NTUA over 70minutes. Results shown are for 3 experiments. b) Images showing increase in cell fluorescence with time when cells were exposed to NTUA: images shown for time 0, 20, 40, and 60 minutes. Scale bar 20 μ m.

Figure 32: NTUA cellular uptake by undifferentiated PC12 cells

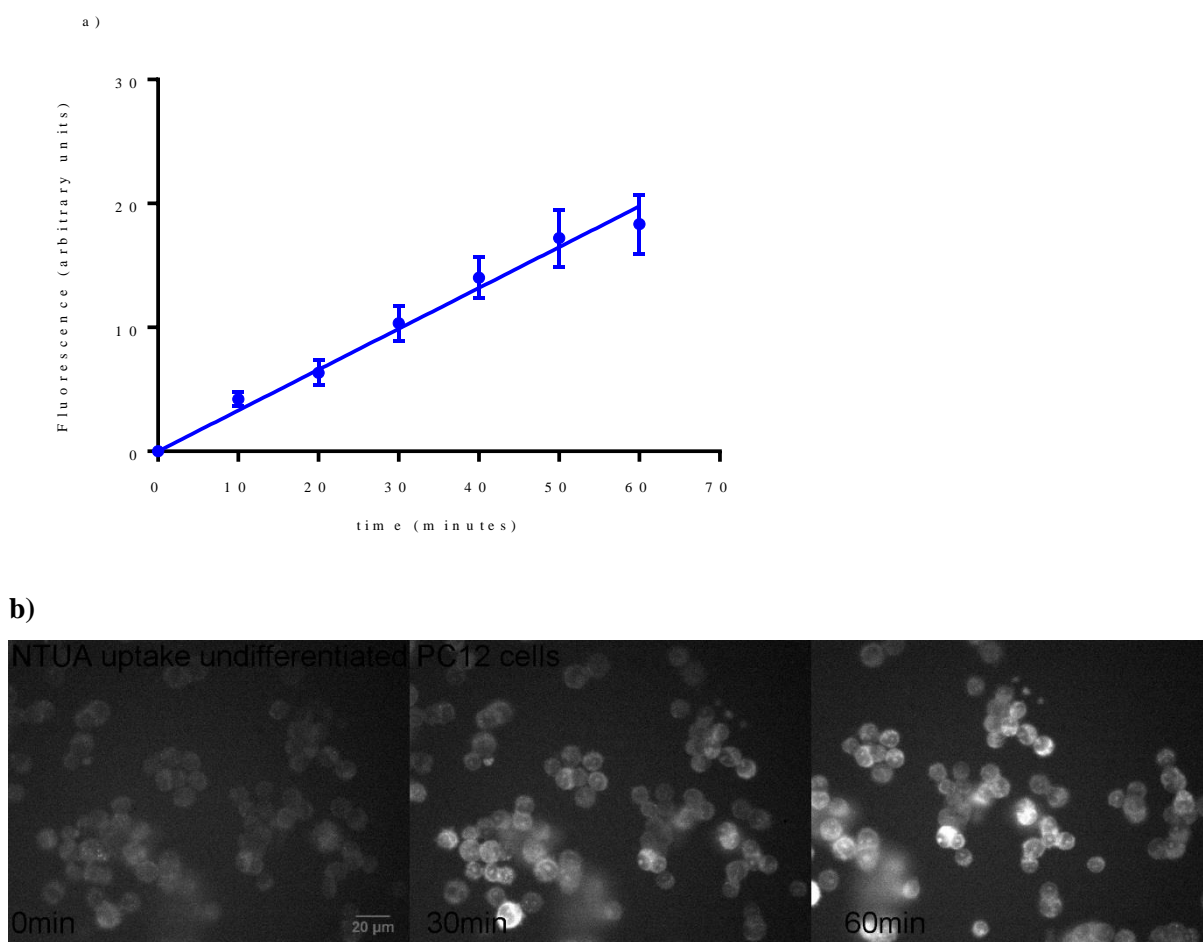
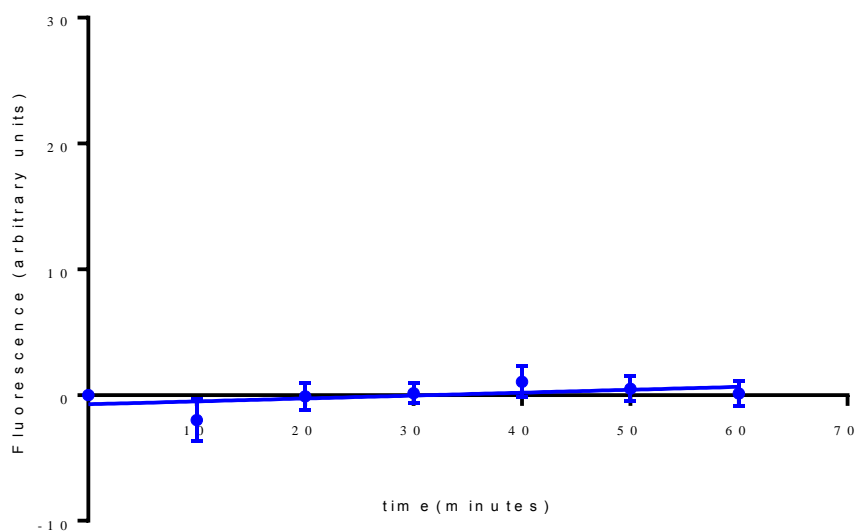


Figure 32 (a) showing a time dependent increase in mean PC12 cellular fluorescence \pm SEM when cells were exposed to NTUA over 60 minutes. Results shown are for 1 experiment. b) Images showing increase in PC12 cell fluorescence, images taken at time 0, 30 and 60 minutes. Scale bar 20 μ m.

5.4.2.2 Cellular uptake of europium conjugates by PC12 cells

Cellular fluorescence gradually decreased with time when both differentiated and undifferentiated PC12 cells were exposed to 30 μ M MT519, MT630 and MT172: figure 33-35.

Figure 33: MT519 (30 μ M) cellular uptake by differentiated PC12 cells



Data points represent mean change in PC12 cell fluorescence \pm SEM when cells were exposed to MT519, results for 5 experiments shown.

The rate of change in fluorescence when differentiated PC12 cells were exposed to MT519 was $0.01 \pm 0.01 \text{ au}\cdot\text{min}^{-1}$ (95% CI -0.013 to 0.003).

Figure 34: MT630 (30 μ M) uptake by differentiated and undifferentiated PC12 cells

MT630 uptake (30 μ M) by PC12 cells (n=4 experiments)

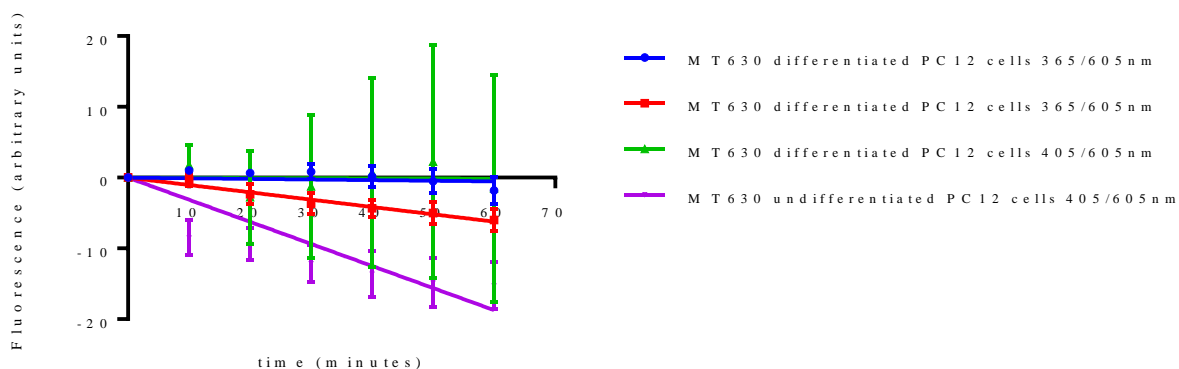


Figure 34 above: data points represent mean change in cell fluorescence \pm SEM, when cells were exposed to MT630. Negative values indicate a fall in fluorescence over time. Results for 4 experiments shown, each represented by a different colour.

When differentiated and undifferentiated PC12 cells were exposed to MT630 (30 μ M), cellular fluorescence declined with time (figure 34). Rates of change in fluorescence for each of the 4 experiments are shown in table 17.

Table 17: MT630 (30 μ M) uptake by differentiated and undifferentiated PC12 cells

MT630 uptake by PC12 cells	Experiment 1 Differentiated cells	Experiment 2 Differentiated cells	Experiment 3 Differentiated cells	Experiment 4 Undifferentiated cells
Excitation/emission λ (nm)	365/605	365/605	405/605	405/605
Rate of change in cell fluorescence au.min ⁻¹ \pm SEM	-0.01 \pm 0.01	-0.10 \pm 0.004	-0.003 \pm 0.02	-0.31 \pm 0.03
95% CI	-0.032 to 0.015	-0.11 to -0.09	-0.05 to 0.04	-0.39 to -0.23

Table 17 shows rate of change in cell fluorescence when cells were exposed to MT630. Negative values indicate a fall in fluorescence over time. λ (nm) represents the excitation and emission wavelengths used for each experiment.

Figure 35: MT172 uptake by undifferentiated and differentiated PC12 cells

MT172 (30 μ M and 100 μ M) uptake by differentiated & undifferentiated PC12 cells
(n = 4 experiments)

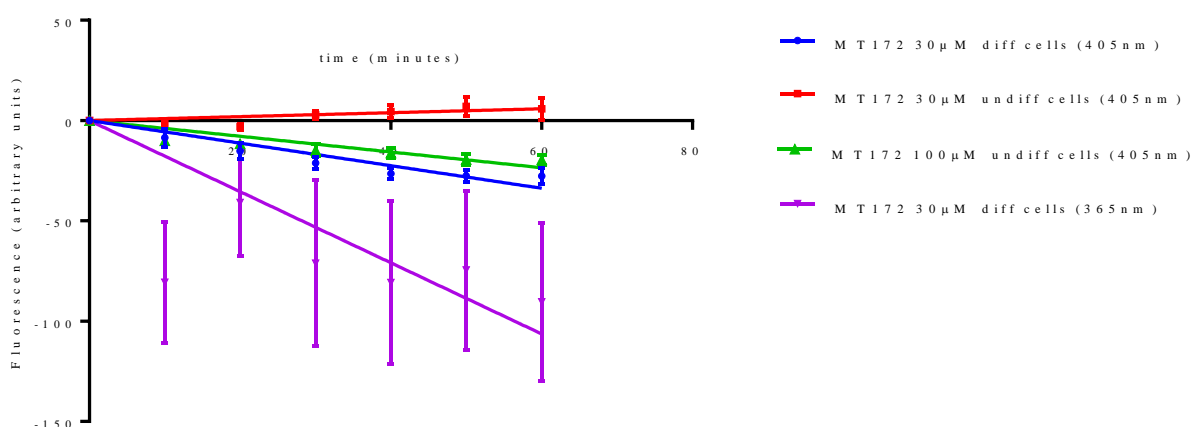


Figure 35 shows change in fluorescence during MT172 exposure only. Data points represent mean \pm SEM for cells from 4 experiments. Negative values indicate a fall in fluorescence over time

Similarly, with MT172 30 μ M and 100 μ M exposure, the change in PC12 cell fluorescence was assessed, and a decline in cellular fluorescence with time was observed (figure 35). The rate of change in cellular fluorescence for these experiments is shown in the table 18.

Table 18: MT172 (30 μ M and 100 μ M) uptake by differentiated and undifferentiated PC12 cells

MT172 uptake by PC12 cells	Experiment 1 Differentiated cells	Experiment 2 Differentiated	Experiment 3 undifferentiated cells	Experiment 4 Undifferentiated cells
MT172 concentration	30 μ M	30 μ M	30 μ M	100 μ M
Excitation/ emission λ (nm)	405/605	365/605	405/605	405/605
Rate of uptake au.min ⁻¹ \pm SEM	-0.56 \pm 0.042	-1.8 \pm 0.30	0.098 \pm 0.026	-0.39 \pm 0.037
95% CI au.min ⁻¹	-0.66 to -0.46	-2.5 to -1.0	0.035 to 0.16	-0.48 to -0.30

Table 18 shows rate of change in cell fluorescence when PC12 cells were exposed to MT172. Negative values indicate a fall in fluorescence over time. λ (nm) represents the excitation and emission wavelengths used for each experiment.

5.4.2.3 Verifying MT172 and MT630 fluorescence signal

Results of the experiments described above demonstrated a decline in cellular fluorescence over time when the cells were exposed 30 μ M and 100 μ M of the europium conjugates: MT159, MT630, and MT172. This finding was similar to that seen in the negative control experiments where cells were washed with PSS only. There are two possible explanations for this finding. Firstly it is possible that the method used for detecting the fluorescence signal had low sensitivity and hence could not detect minimal cellular accumulation of these compounds over time. However, the same protocol was used to assess NTUA uptake and demonstrated an increase in cellular fluorescence over time. Secondly, it is possible that the europium conjugates used in these experiments had a weak fluorescence signal which could not be detected at the concentration used in this study. To explore the latter point, further tests were performed to determine the minimum concentration at which the fluorescence signal could be detected and the optimum

conditions (concentration of the compound, excitation and emission wavelengths) at which the fluorescence signal could be detected.

Methods

To ascertain which one of the europium conjugates provided the strongest luminescence, 10mM solutions of MT172, MT630, and MT611 were each placed in empty capillary tubes. MT611 is a europium complex with a strong fluorescence signal, and was used as a positive control. All compounds were excited using available acousto-optical tuneable filters (excitation wavelengths): 365nm, 370nm and 405nm, 450nm and 650nm, on a confocal microscope. A 605nm emission filter was used. All three compounds produced a fluorescent signal when excited at the following wavelengths, 365nm, 370nm and 405nm. MT611 (positive control) had the brightest fluorescent signal at all excitation wavelengths used (figure 36, 37). Of the two europium conjugates (MT630 and MT172), MT172 had a brighter fluorescence signal and therefore was used for further tests (figure 36).

The minimum concentration at which the fluorescence signal could be detected was also assessed by placing MT172 and MT611 solutions in capillary tubes: concentration 100 μ M, 300 μ M, 500 μ M and 1mM. 390nm/605nm (emission/excitation wavelengths) were used since this produced a strong fluorescent signal from both compounds. An empty capillary tube was used as a negative control. For each compound, corrected fluorescence was calculated. Several regions of interest within the capillary tube with the compound were marked out using image J and the mean pixel intensity for these regions were calculated. The mean background fluorescence (obtained by drawing several regions of interest in an area outside the capillary tube and finding the mean pixel intensity for these regions) was also measured. Corrected fluorescence for each compound equated to the

mean compound fluorescence minus the mean background fluorescence. The minimum concentration above which the fluorescence could be detected (x intercept at which the fluorescence was zero) was also determined using linear regression. Both MT172 and MT611 were fluorescent as demonstrated before and the fluorescent signal increased with increasing concentration (figure 37).

The MT172 concentration above which a fluorescence signal could be detected was greater than 190 μM (x intercept where $y=0$) for MT172, this suggested that the fluorescence detection threshold in capillary had to be greater than 190 μM . Since the MT172 fluorescence signal was visible in the capillary tube at a concentration 300 μM (figure 37), further MT172 cellular uptake studies were therefore carried out using this 300 μM .

Figure 36: Images for 10mM MT172, MT611, and MT630 fluorescence signal in capillary tubes

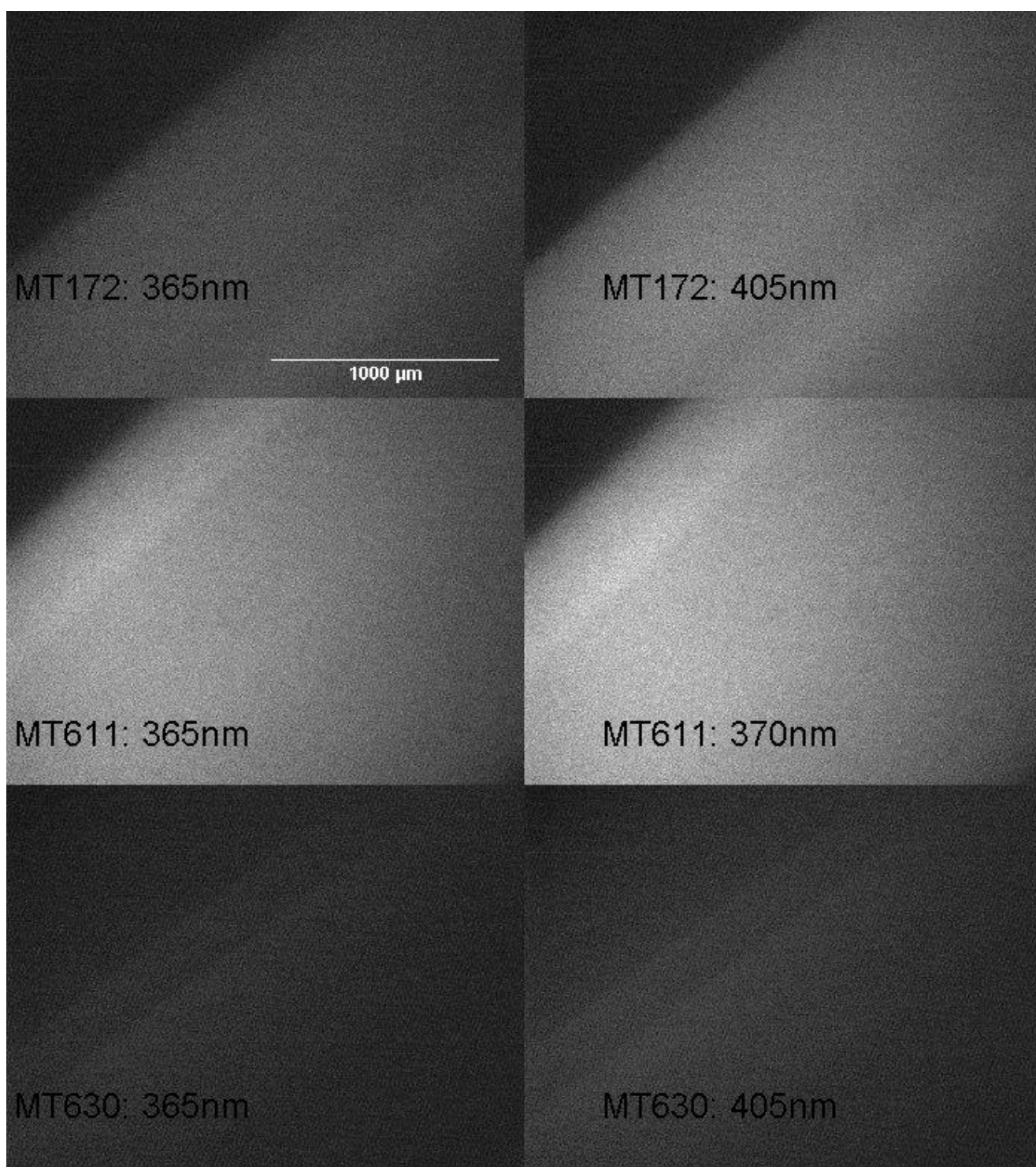
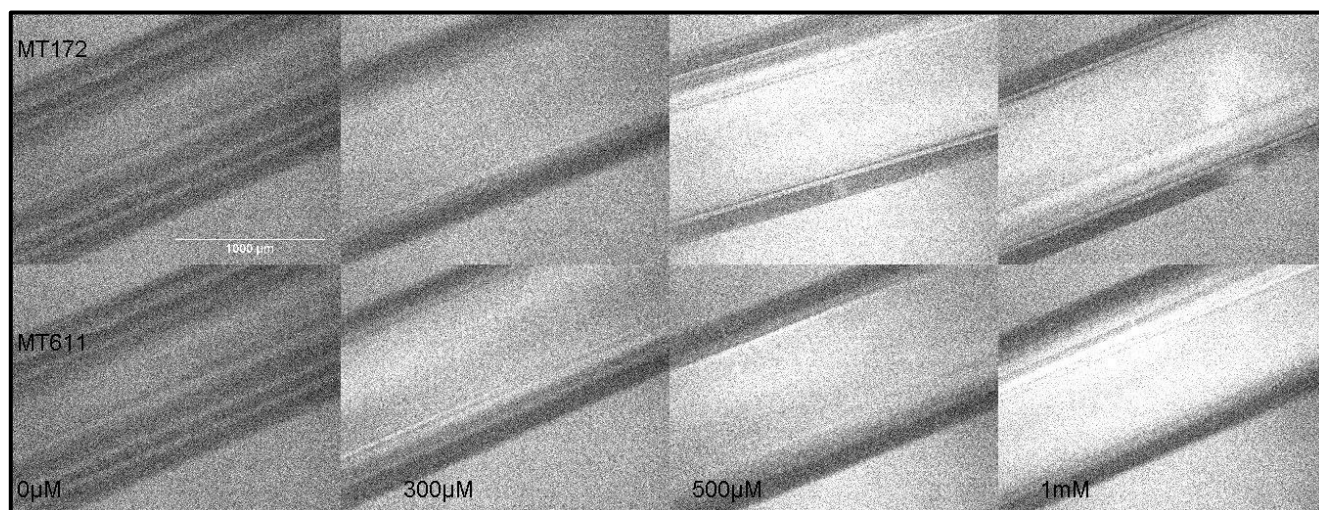


Figure 36 above shows fluorescence signal for MT611 and MT630 placed in capillary tubes and excited at various wavelengths: 365nm, 370nm, 405nm. Emission wavelength used was 605nm. Scale bar 1 000μm

Figure 37: MT172 and MT611 fluorescence in capillary tubes at different concentrations

a)



b)

MT172 and MT611 fluorescence signal at various concentrations

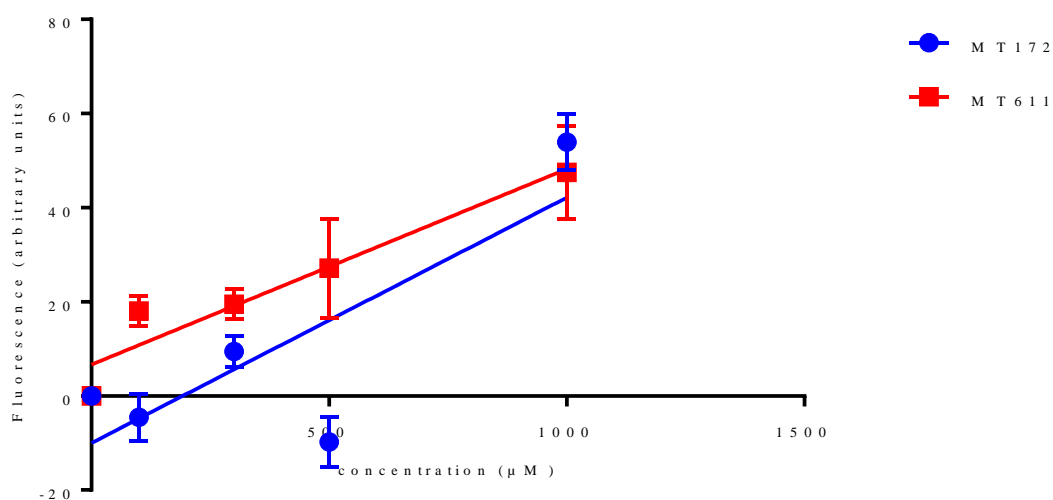


Figure 37 a) Top row shows MT172 fluorescence signal in capillary tubes, bottom row MT611 in capillary tubes at different concentrations: Columns from left to right: 0 μM, 300 μM, 500 μM, and 1000 μM. Capillary tubes with compounds were excited at 390nm and a 605nm emission filter was used. Scale bar 1000 μm. (b) Fluorescence signal (y axis) in the capillary tubes plotted against concentration (x axis), for MT172 and MT611

5.4.2.4 MT172 (300 μM) cellular uptake by PC12 cells.

When MT172 (300 μ M) cellular uptake was assessed: excitation/emission (390/605nm) a time dependent increase in cellular fluorescence in undifferentiated cells was seen (figure 38a, b). The cellular fluorescence peaked at 40 minutes. There was minimal decrease in fluorescence during drug washout with PSS (time 60-80 minutes) (figure 38b). Mean rate of increase in fluorescence during MT172 exposure to undifferentiated cells (3 experiments) was 0.73 ± 0.24 au.min⁻¹ (95% CI 0.11 to 1.35). For subsequent experiments cells were therefore exposed to MT172 for a maximum time of 60 minutes. The rates of change in cell fluorescence for the three MT172 cellular uptake experiments are shown in table 19. When 300 μ M MT172 cellular uptake was assessed in differentiated PC12 cells (n=3 experiments) (figure 39a), the mean rate of uptake for all cells in the experiments was 0.78 ± 0.15 au.min⁻¹ (95% CI 0.41 to 1.42). Table 20 shows the rates of 300 μ M MT172 uptake by differentiated PC12 cells in the 3 experiments. Cellular fluorescence continued to increase during drug washout, shown in figure 39b.

Table 19: Rate of MT172 300 μ M uptake by undifferentiated PC12 cells

Undifferentiated PC12 cells	Experiment 1	Experiment 2	Experiment 3
Rate of uptake au.min ⁻¹ \pm SEM	0.47 \pm 0.07	1.39 \pm 0.45	1.30 \pm 0.17
95% Confidence Interval au.min ⁻¹	0.30 to 0.63	0.29 to 2.49	0.89 to 1.71

Figure 38: MT172 (300 μ M) uptake by undifferentiated PC12 cells

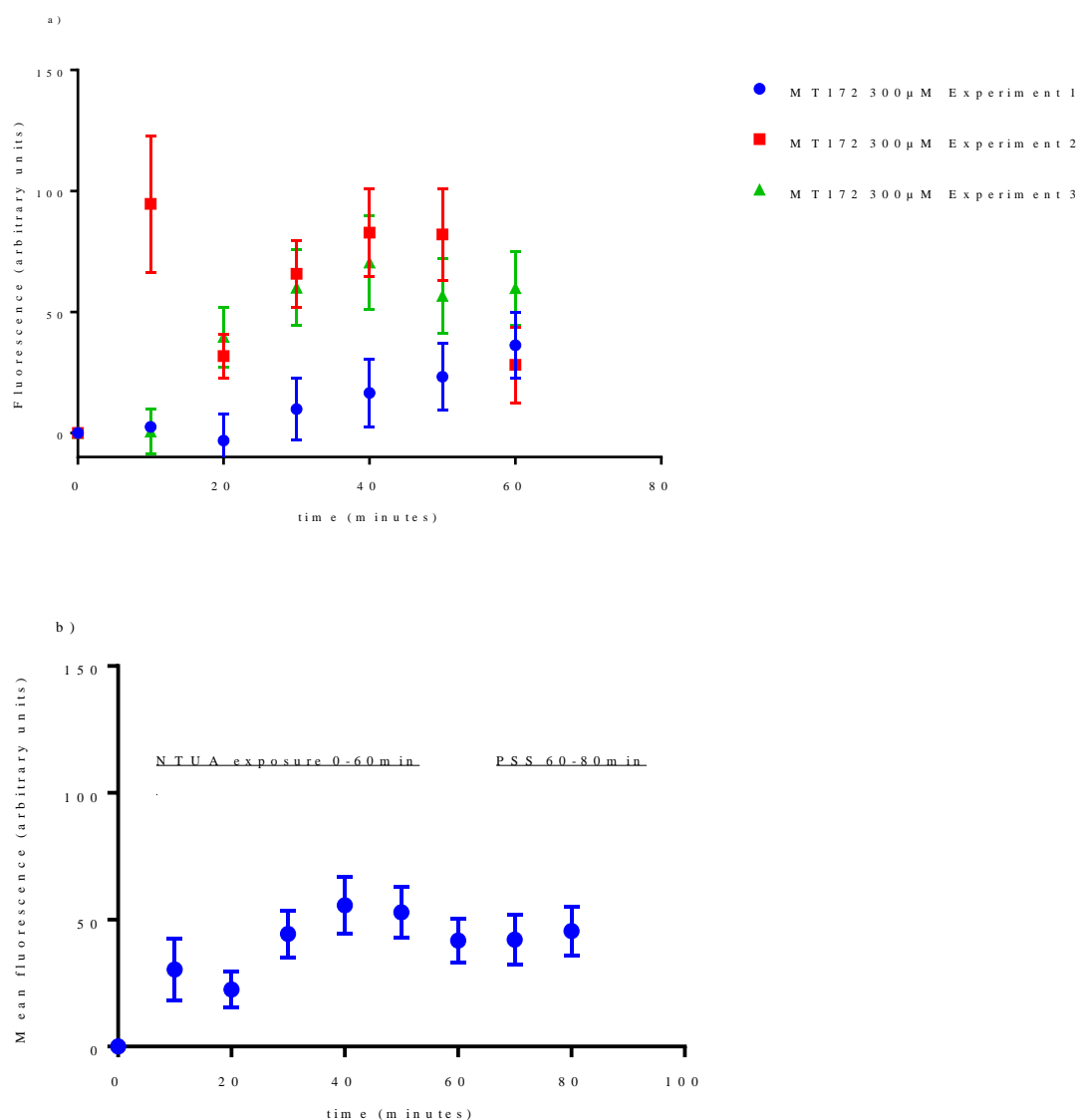


Figure 38 above. Data points represent mean change in cell fluorescence +SEM, when cells were exposed to MT172 300 μ M and excited at 390nm, emission 605nm, a) results for individual experiments b) Results for 3 experiments combined.

Figure 39: MT172 (300 μ M) uptake by differentiated PC12 cells

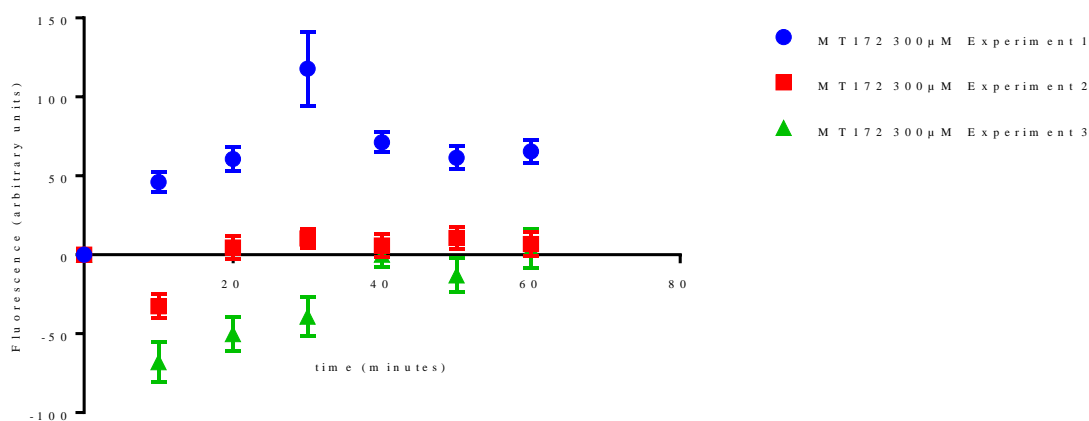


Figure 39 above .Data points represent mean change in PC12 cell fluorescence \pm SEM, when cells were exposed to MT172 300 μ M over 60minutes.Results for 3 experiments shown

Table 20: Rate of MT172 300 μ M uptake by differentiated PC12 cells

Differentiated PC12 cells	Experiment 1	Experiment 2	Experiment 3
Rate of uptake $\text{au}\cdot\text{min}^{-1}\pm$ SEM	1.66 ± 0.38	0.14 ± 0.15	-0.36 ± 0.38
95% Confidence Intervals $\text{au}\cdot\text{min}^{-1}$	0.72 to 2.59	-0.23 to 0.50	-1.28 to 0.56

Table 20 shows the rate of change of cell fluorescence for differentiated PC12 cells. Cells were exposed to MT172 300 μ M during each experiment. Negative values indicate a fall in fluorescence over time.

MT172 cellular uptake by undifferentiated (n=3) and differentiated (n=3) PC12 cells were compared using unpaired t-test and showed no significant difference in the rate of increase in cellular fluorescence between the two groups (mean difference $-0.5 \pm 0.67 \text{ au}\cdot\text{min}^{-1}$, p=0.38; figure 40).

Figure 40: Mean rate of MT172 300 μ M uptake, comparison between differentiated and undifferentiated PC12 cells

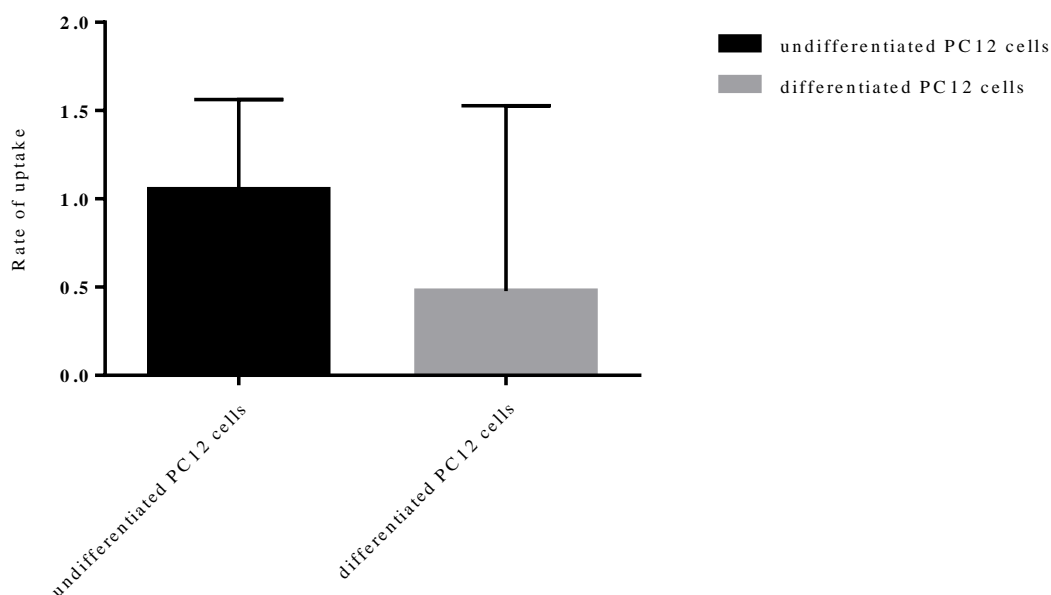


Figure 40. Compares the mean rate of change in cell fluorescence for the undifferentiated PC12 cell experiments (n=3) and differentiated PC12 cell experiments (n=3).

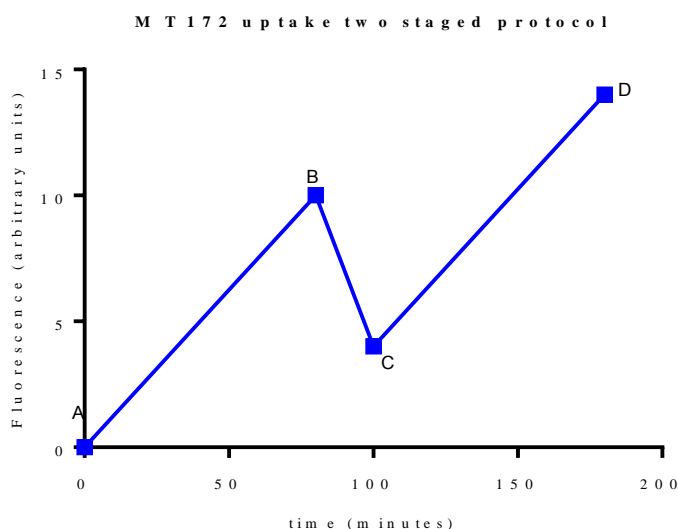
5.4.2.5 Modified cellular uptake protocol: to determine if MT172 cellular uptake by PC12 cells is mediated by dopamine transporter (DAT) or noradrenaline transporter (NET)

Methods

Further tests were performed to determine if the observed MT172 cellular uptake was mediated by the monoamine transporters DAT or NET. Given the variability in MT172 cellular uptake between experiments (figure 38 and 39), the method used for cellular uptake was modified in an attempt to improve consistency of results between experiments. A two stage protocol was devised. Cells were washed with PSS prior to starting the experiment as before in order to reduce auto-fluorescence. Stage 1: PC12 cells were exposed to 300 μ M MT172 for 60 minutes followed by a 40 minute MT172 washout using PSS. Stage 2: second MT172 exposure for a further 60 minutes (figure 41). Fewer images were taken during MT172 exposure to reduce photo-bleaching. Images were taken

at times 0 minutes (A: start of MT172 perfusion). Since previous experiments showed a minimal fall in fluorescence after drug washout with PSS for 20 minutes (figure 38b), which suggested an equilibrium was reached, for this modified protocol, further images were taken at time 80 minutes (time point B: 60 minutes MT172 exposure then 20 minutes PSS washout), and 180 minutes (time point D). Another image was taken at time 100 minutes (C: after a further 20 minutes PSS perfusion, this was considered as the baseline image for the second MT172 exposure). 390/605nm filters were used. If the protocol is robust then rate of MT172 cellular uptake in stages 1 and 2 would be equal (slope AB = slope CD), and the protocol would be used to assess for MT172 uptake in the absence and presence of DAT or NET blockers. GBR 12909, a DAT blocker was purchased from Tocris Biosciences, Bristol UK. GBR stock solution was stored at 20 °C in 1mM aliquots. This was diluted with PSS to make 1µM solution for the experiments. Desipramine, a NET inhibitor which also inhibits SERT, was stored in 10mM aliquots and diluted with PSS to 1µM solution for the experiments. A previous study in our laboratory showed that GBR 1µM and desipramine 1µM could inhibit NTUA uptake by PC12 cells (Elobi, 2014). For experiments to assess if MT172 cellular uptake was mediated by DAT or NET, cells were perfused with 300µM MT172 during stage 1 (Stage 1: AB), followed by PSS for 20 minutes. GBR 1µM and desipramine 1µM perfusion was started from 80 minutes onwards. Cells were then exposed to a second MT172 perfusion for a further 60 minutes (stage 2) Fluorescence images were taken at time 0, 80, 100, and 180 minutes as described above. NTUA was used as a positive control. Corrected median fluorescence for each cell was calculated as described before. The rates of NTUA/MT172 uptake slope (AB) and (CD) were compared for each cell using Wilcoxon matched-pairs signed rank test. Mann Whitney U test was used to compare the rate of MT172 cellular uptake before and after exposure to GBR and desipramine.

Figure 41: Modified cellular uptake protocol: 2 stages



The sketch above illustrates the two staged protocol (NB, this is not ‘real data’)

- AB: MT172 first exposure, baseline images taken at time (point A) = 0 minute, peak fluorescence images taken after 20 minute PSS wash time (point B) = 80 minute
- BC: Further MT172 washout with PSS for 20 minute
- CD: Second MT172 exposure, baseline fluorescence image (point C), time = 100 minute and peak fluorescence image taken after 20 minute washout with PSS (point D) time = 180 minute

Results

NTUA uptake by PC12 cells using a modified (two stage) protocol.

NTUA uptake by undifferentiated PC12 cells was assessed using the two staged protocol described above. Results for 20 cells were analysed (figure 42a). There was a linear increase in fluorescence during NTUA exposure: AB, CD on figure 42a. Median (IQR) rate of uptake for first NTUA exposure (slope AB) was 2.2 (0.99-3.59) au.min⁻¹, and 2.4 (0.06-3.92) au.min⁻¹ for the second exposure (slope CD). NTUA washout (BC) was associated with a reduction in fluorescence. When the rates of NTUA cellular uptake for each of the 20 cells were compared, there was no significant difference between the two stages: median difference was -0.09, p=0.48 (figure 42b).

Figure 42: NTUA uptake by undifferentiated PC12 cells, using the modified 2 staged cellular uptake protocol

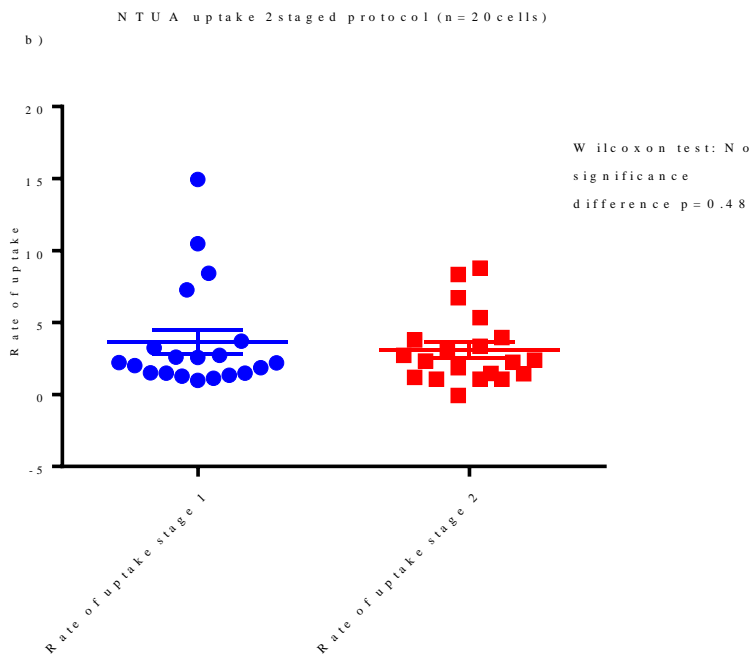
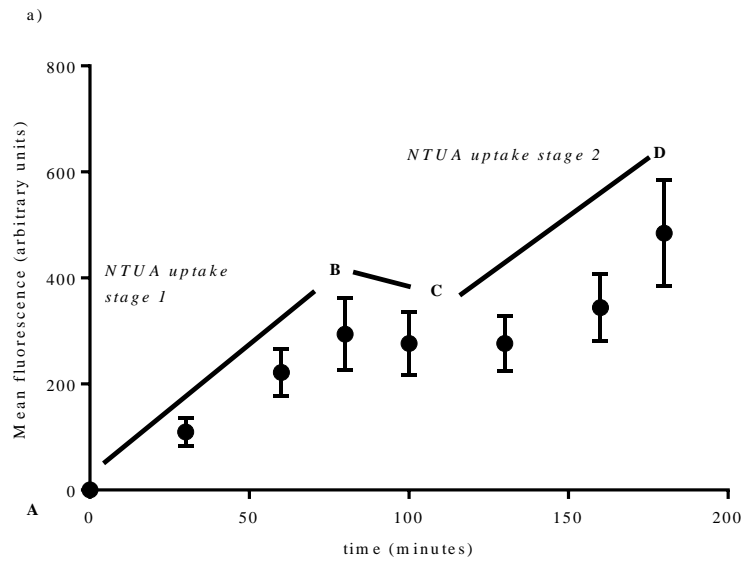


Figure 42(a) above data points represent mean change in cell fluorescence \pm SEM when cells were exposed to NTUA during stages 1 and 2. BC represents a drop in cell fluorescence when cells were washed with PSS. Results shown are for 20 cells. (b) Comparing rate of increase in cell fluorescence (NTUA uptake) for stage 1 versus stage 2. Results shown are for 20 cells.

5.4.2.6 300 μ M MT172 cellular uptake by undifferentiated PC12 cells using the modified two stage protocol

Four experiments were performed and data for 40 cells were analysed. The rate of MT172 300 μ M uptake was weak in stage 1 and 2, median (IQR) rate of uptake 0.08 (-0.11-0.14) au.min⁻¹ and 0.05 (-0.45-0.092) au.min⁻¹ respectively. There was no difference in the rate of MT172 cellular uptake between the 2 stages, median difference -0.02 au.min⁻¹, p= 0.28 (figure 43).

Figure 43: Rate of MT172 300 μ M cellular uptake by undifferentiated PC12 cells using a modified protocol stages 1 versus stage 2

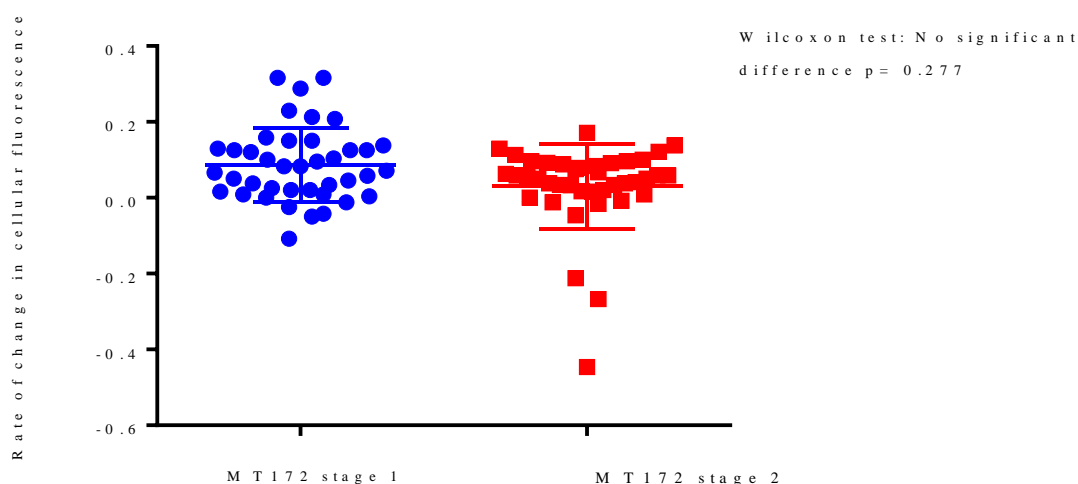


Figure 43 compares the rate of increase in cell fluorescence for each cell during stage 1 and stage 2 MT172 exposure: results for 40 cells shown. There was no significant difference between the 2 stages.

MT172 (300 μ M) cellular uptake by undifferentiated PC12 cells in the absence and presence of GBR and desipramine using 2 staged uptake protocol

In total five experiments were performed, data for 50 cells were analysed. Median (IQR) MT172 rate of uptake for stage 1 was and 2 were 0.06 (-0.07-0.15) au.min⁻¹ and 0.04 (-0.28-0.09) au.min⁻¹ respectively. There was no difference in MT172 cellular uptake

before and after adding GBR and desipramine, median difference $-0.01 \text{ au}\cdot\text{min}^{-1}$, $p=0.06$ (figure 44).

Figure 44: Comparing MT172 ($300\mu\text{M}$) cellular uptake rate by undifferentiated PC12 cells before and after GBR and desipramine ($1\mu\text{M}$) exposure, results for 50 cells.

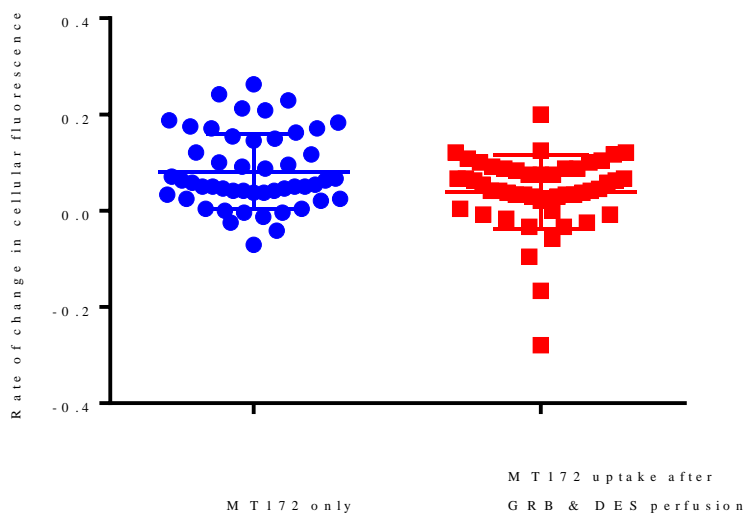


Figure 44 shows rate of change in cell fluorescence when exposed to MT172 in the absence and presence of GBR and Desipramine. Results for 50 cells shown. There was no significant difference between the 2 stages.

5.5. Discussion

This novel study sought to assess the feasibility of labelling dopaminergic cells using lanthanide based probes with the ultimate aim of using the compounds as MRI based contrast agents for labelling dopaminergic neurons in PD in the future. The project also optimised a protocol for use in screening other potential probes in the future.

PC12 cells were grown and differentiated using Green and Tischler's protocol (Greene L.A and Tischler A.S, 1976). Cell differentiation resulted in neurite growth, as previously reported (Greene L.A and Tischler A.S, 1976). Background cell fluorescence, can

interfere with the signal from potential probes. This a recognised problem in fluorescence microscopy, which is thought to arise from metabolites including NADH and flavins (Aubin, 1979). Culture media used for maintaining cells also contain vitamins such as riboflavin which may also contribute to the auto-fluorescence. In the current study, cells were washed with PSS in order to remove excess culture media before exposing the cells to the probes. A reduction in cell auto-fluorescence over time was observed (figure 30).

Use of NTUA as a fluorescent substrate for assessing monoamine transporter function has been validated before in HEK-293 and CHO-KI cell lines transfected with human DAT (Jorgensen et al., 2008). In this study NTUA was used to validate the cellular uptake protocol that will be used for screening future lanthanide probes using PC12 cells. The current study showed a time dependent NTUA accumulation in PC12 cells. A previous study in our laboratory reported similar findings and also demonstrated NTUA uptake inhibition by desipramine and GBR, (Elobi, 2014) confirming DAT and NET expression by PC12 cells.

The current study showed no evidence of cellular uptake of the europium based compounds at low concentrations. Another pilot study in our group (unpublished) demonstrated 30 μ M MT172 uptake by PC12 cells. The current study could not replicate these results even when MT172 100 μ M was assessed using the same FITC filter set used in the pilot study. In fact, a decrease in fluorescence with time was seen when cells were exposed to MT172 100 μ M. Excitation and emission wavelengths of compounds that contribute to auto-fluorescence was reported to be in the range 365-440nm and 520nm respectively (Aubin, 1979). FITC band-pass filters with an emission wavelength of 519nm may allow transmission of short wavelengths including auto-fluorescence. It is therefore plausible that the apparent europium conjugate accumulation reported in the

pilot study may have been due to auto-fluorescence. There is one potential explanation for the discordant results. The methodology of the current and pilot studies differed slightly. Cells were washed with PSS for 20 minutes prior to perfusing them with europium conjugates. This removed any excess culture media which could have contributed to auto-fluorescence, and hence observed reduction in fluorescence with time. Given that FITC emission is short (green: 519nm) and can block the europium emission (red: 615nm), the FITC filters were substituted with 390nm/605nm filter set, but no cellular uptake of the europium conjugates was demonstrated at concentrations less than 100 μ M. This observation implied that the lanthanide conjugates used in this study had a weak fluorescence signal which could not be detected at these low concentrations.

When experiments were repeated using a higher concentration (MT172 300 μ M), a time dependent increase in MT172 uptake by both differentiated and undifferentiated PC12 cells was seen. Furthermore, there was no difference in the rate of MT172 cellular uptake, when differentiated and undifferentiated PC12 cells were compared. The latter finding was unexpected. Differentiated (NGF treated) PC12 cells were shown to have a higher DAT protein expression than undifferentiated cells (Kadota et al., 1996). The reverse was true for the noradrenaline transporter. DAT expression was also demonstrated on the neurite extensions of differentiated PC12 cells in one study (Kadota et al., 1996). In the same study, Kadota et al. (1996) also compared, the rate of dopamine uptake in NGF treated and untreated cells and reported a higher rate of dopamine uptake in the earlier group (Kadota et al., 1996). If MT172 uptake was mediated via the dopamine transporter, then a higher rate of uptake should have been observed in differentiated PC12 cells. This finding implies that MT172 transport may be mediated by mechanisms other than DAT. Study results also showed variability in rate of MT172 cellular uptake between experiments (table 19 and 20). This may have arisen from a non-uniform field of

illumination from a failing mercury arc lamp light source. This light source was used for the initial set of experiments because of time constraints, but was replaced with a new arc lamp for later studies. This is not ideal as definitive conclusions cannot be drawn from the results of these experiments. . Another plausible explanation is the use of cells at different passages which may result in variable responses. It is also possible that as the number of passages increases, the behaviour of the tumour cell line becomes unpredictable, which may account for the variable MT172 uptake.

The cellular uptake protocol was therefore modified in order to improve the consistency of results between experiments. But when the modified protocol was used, the rate of NTUA uptake was more than twice and MT172 cellular uptake was significantly less than that observed in earlier experiments. This again demonstrated the unpredictable behaviour of the cells. There was no difference in the rate of NTUA uptake between stage 1 and 2, implying that that the cellular uptake protocol was robust and could be used to compare the rate of MT172 cellular uptake in the presence of the absence of transporter blockers, within the same experiment. Nevertheless, when MT172 cellular uptake was assessed before and after GBR and desipramine exposure, there was no difference in the rate of uptake. These data suggested that MT172 cellular uptake shown in this study was not mediated via DAT or NET. MT172 uptake observed in this study was by a non-specific mechanism, probably diffusion due to a concentration gradient. Support for this conclusion comes from results of earlier experiments which showed similar rates of uptake between undifferentiated and differentiated PC12 cells. Lanthanide complexes are reported to be taken up across the cell membranes by a method called macropinocytosis (New et al., 2010). The present study showed that tagging europium complexes with either dopamine or levodopa did not result in specific uptake via monoamine transporters.

The modified cellular uptake method has several limitations. It was difficult to maintain the organ bath temperature above 29 °C during the long experiments. Previous reports have demonstrated that fluorescent substrate uptake via monoamine transporters is temperature dependent (Jorgensen et al., 2008). Suboptimal temperatures in this study could have impeded any energy dependent MT172 cellular uptake. Nevertheless, NTUA uptake was still demonstrated when the modified protocol was used, which confirms the notion that MT172 uptake was nonspecific and not monoamine transporter mediated. NTUA results also confirms the robustness of the modified protocol when used for screening probes with greater luminescence. Another drawback was the long duration of the experiments, which showed that this protocol is not suitable for high throughput screening. Future experiments should address these problems. Increasing the perfusion rate of compounds during experiments and use of controlled temperature chambers may reduce the heat loss and therefore help optimise the temperature during the experiments. Use of automated methods such as FLIPR tetra was considered but plate readers with suitable excitation and emission wavelengths appropriate for the long stoke shifts were not available. Future studies should also consider using methods other than fluorescence microscopy to assess cellular uptake of these compounds. This may include inductively coupled plasma spectrometry (ICP-MS). This sensitive technique has been used in other studies to quantify cellular uptake of compounds at single cell level (Zheng et al., 2015). This may be useful for future cell based work before proceeding to testing these compounds in animals. Rational drug design using methods such as methods such as 3D x-ray crystallography, (Mandal et al., 2009) should also be considered in the synthesis of other europium complexes to improve specificity for DAT.

A human cell line will be used in future studies, to counteract the problems of unpredictable behaviour which can arise from using a tumour cell line.

**CHAPTER 6: HUMAN DOPAMINERGIC CELL LINE (LUHMES) IN THE
DEVELOPMENTS OF AN MRI SENSITIVE PROBE**

6.1. Background

LUHMES cell line

Lund Human Mesencephalon Cells (LUHMES) were developed at Lund University by immortalising human mesencephalic cells with a v-myc retroviral vector (Lotharius et al., 2002). Cell proliferation can be halted by exposing cells to tetracycline which blocks v-myc and cause the cells to exit the cell cycle (Lotharius et al., 2002). The cells can then differentiate into mature dopaminergic neurons after addition of glial derived neurotrophic factor (GDNF) and dibutyl cyclic AMP to the culture media (Scholz et al., 2011). Differentiated LUHMES cells were shown to express mature dopaminergic neuron markers including DAT, VMAT2, D₂, and tyrosine hydroxylase at varying levels depending on the stage of differentiation (Scholz et al., 2011). This cell line was considered to be ideal for the current project for several reasons. Firstly, LUHMES are a human cell line, therefore behavior is likely to be closer to that of primary dopaminergic neurons, when compared to a tumor cell line such as PC12 cells. Secondly, Scholz et al. (2011) demonstrated the ease of maintaining the cells, differentiation into mature dopaminergic neurons (Scholz et al., 2011). The same authors also confirmed the ability of these cells to take up dopamine (Scholz et al., 2011), which is an essential requirement for this project. The cell line has also been used in other PD studies to demonstrate cytotoxicity after MPP⁺ exposure and alpha-synuclein overexpression (Zhang et al., 2014).

The current study sought to validate dopamine transporter activity in LUHMES cells by assessing NTUA cellular uptake using fluorescence microscopy. The second objective was to optimise an assay method using the LUHMES cell line which can then be used for screening fluorescent DAT substrates in the future.

6.2. Materials

LUHMES cells

Cells were purchased from LGC standards, UK, and maintained using the protocol provided by LGC standards. Cells were grown in DMEM: F12 media, LGC standards, UK (stored at 4⁰C) supplemented with 1% N2 supplement (Gibco-Invitrogen, 100X stock stored at -20⁰C) and basic recombinant human fibroblast growth factor (bFGF) 40ng/ml, purchased from Gibco-Invitrogen, and stored at -20⁰C. Cells were grown in tissue culture flasks pre-coated with poly-L-ornithine 50µg/ml (Sigma-Aldrich, UK) followed by fibronectin 1µg/ml (Sigma-Aldrich, UK) (Scholz et al., 2011). Cells in culture media were placed in a humidified, 5% CO₂ incubator at 37⁰C and the growth media was replaced every 2-3 days. Materials for subculturing protocol (LGC standards, UK) which included Dulbecco's phosphate buffered saline (D-PBS) (University of Birmingham IBR stores), DMEM: F12 growth media, and 0.025% Trypsin-EDTA, were pre-warmed in a 37⁰C water bath. The old culture media was removed and cells were briefly washed with 10mls of D-PBS. 0.025% Trypsin- 0.1g/L-EDTA was used to detach cells for 3minutes, then 6ml of DMEM: F12 growth media supplemented with N2 supplement only, was added to the tissue culture flask. The cell suspension was transferred to a falcon tube and centrifuged at 190g for 7 minutes. The supernatant was removed, cells re-suspended in fresh growth media (including N2 supplement and bFGF), and transferred to another pre-coated tissue culture flasks.

Differentiating LUHMES cells

Differentiating media (Lotharius et al., 2002) was made up by supplementing DMEM:F12 with 1X N2 supplement, 1µg/ml tetracycline (Sigma-Aldrich, UK, diluted with sterile water to make 0.1mg/ml stock, stored at -20⁰C) 1mM, cyclic AMP (Sigma-

Aldrich, UK), diluted with sterile water to make 10mM stock solution stored at -20⁰C), and 2ng/ml recombinant human glial derived neurotrophic factor (GDNF): R&D systems cat number 212GD-010, reconstituted with 0.1% BSA to make 0.1mg/ml stock solution, and stored at -20⁰C. The differentiation protocol was adapted from that used by Scholz et al. (2011) (Scholz et al., 2011). 1×10^6 cells were cultured in complete growth media (DMEM: F12, N2 supplement and bFGF) in a 25cm² tissue culture flask for 24 hours, then the growth media was replaced with differentiation media. After 2 days, cells were detached using 0.025% Trypsin-EDTA and seeded onto poly-l-ornithine and fibronectin coated coverslips at a cell density of 50 000cells per coverslip and fresh differentiation media was added. The coverslips with plated cells were kept in a 37⁰C, 5% CO₂ humidified incubator. The culture media was replaced every 2 days. Experiments were performed from day 5 onwards. NTUA was prepared as described in chapter 5.

6.3. Methods

LUHMES cells were differentiated to mature dopaminergic neurons as described above. On the day of experiments, coverslips with plated cells were placed in an organ bath attached to an epifluorescence microscope. The previous study with PC12 cells showed that auto fluorescence can potentially interfere with detection of probe fluorescence. The results of the two stage NTUA PC12 cellular uptake protocol also showed a time dependent increase in fluorescence in both stage 1 and 2. The same two stage protocol was applied for the LUHMES experiments. Differentiated LUHMES were washed with pre-warmed PBS for 20 minutes prior to the NTUA uptake experiments to remove excess culture media. NTUA perfusion was run for 60 minutes. After exposing the differentiated LUHMES cells to NTUA for 60 minutes, DAT and NET blockers GBR and desipramine (1 μ M) were applied for 20 minutes, followed by a second NTUA exposure for a further 60 minutes. NTUA and the cells were excited at 405nm and a 519nm emission filter was

used. Results of the two stage protocol (figure 49a) showed a fall in fluorescence during the second NTUA exposure. Further experiments were therefore carried out without the transporter blockers to verify if the fall in NTUA uptake during stage 2 was secondary to inhibition of the monoamine transporters by GBR and desipramine. For all experiments, fluorescent and bright field images were taken at time 0 minutes (start of NTUA exposure) then at 10 minute intervals throughout the experiment. The following camera settings were used: bright field (exposure 150ms, gain 0, and offset 0), fluorescence (exposure 5 seconds, gain 255, offset 0). Image analysis was performed using image J version (1.38e/Java1.5). Median cell fluorescence (arbitrary units) was measured in each region of interest and mean cellular fluorescence for the cells calculated. Linear regression was used to calculate the NTUA transport rate. T-test or Mann U Whitney were used to compare transport rates before and after addition of GBR and desipramine. Graph pad prism software version 6 was used for statistical analysis.

6.4. Results

Large numbers of LUHMES cells were generated within a few days (figure 45). When LUHMES cells were grown in differentiation media, proliferation stopped and from day 2 onwards progressive neurite extension was seen. These increased in number and length to form a dense network of neurites as demonstrated in figure 46 below.

Figure 45: Images of undifferentiated LUHMES cells from day 0 to 5 in growth media.

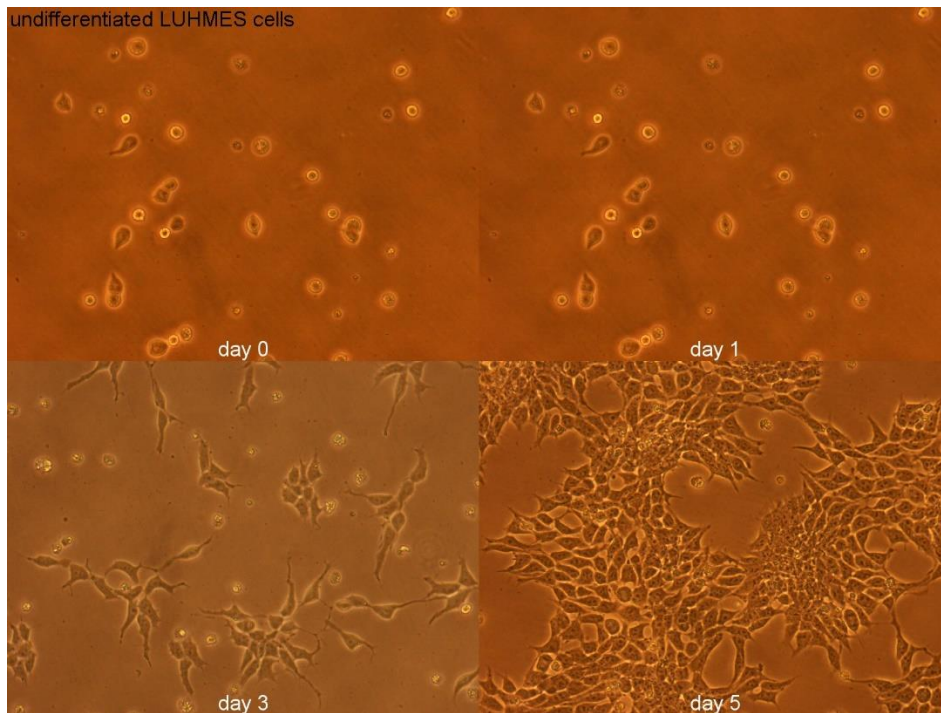


Figure 46: LUHMES cells grown in differentiation media from day 1 to day 5

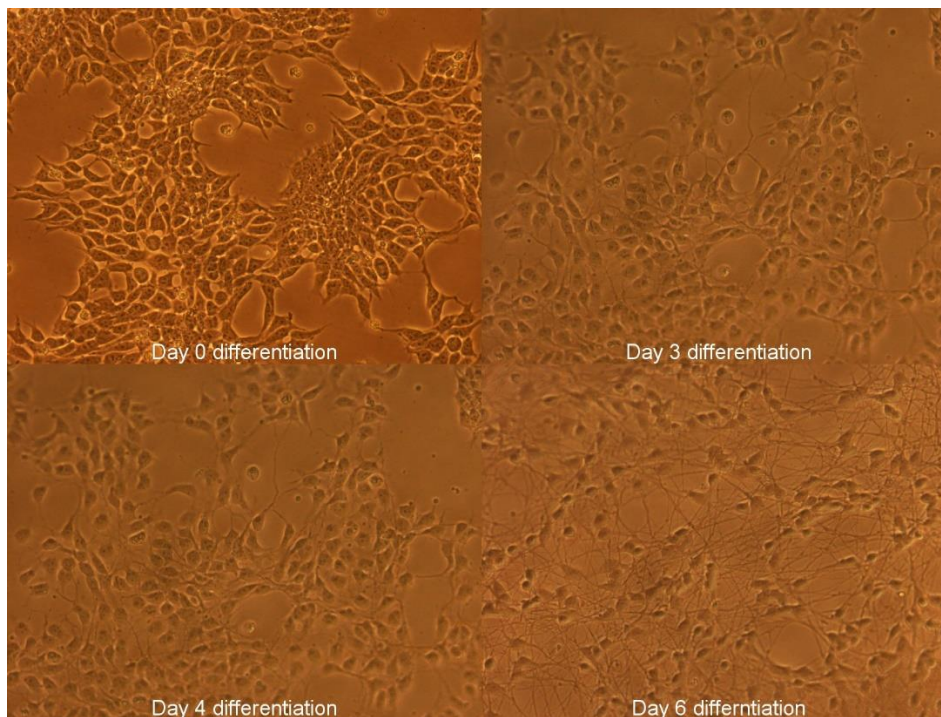


Figure 46 above: images showing an increase in number and length of LUHMES neurite network during cell differentiation from day 0 to 6,

6.4.1. NTUA cellular uptake by LUHMES cells

NTUA (1:100) cellular uptake was assessed and this showed a minimal increase in signal over time: $0.37 \pm 0.02 \text{ au.min}^{-1}$ (figure 47).

Figure 47: NTUA (1:100 dilution) cellular uptake by differentiated LUHMES

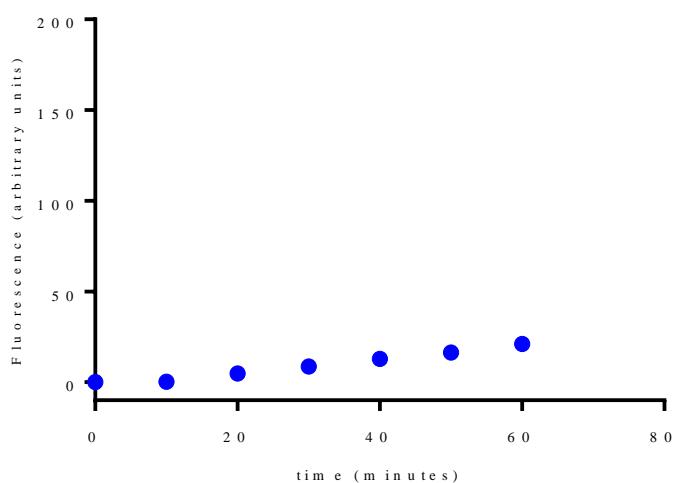


Figure 47 above, data points represent mean change in cell fluorescence +SEM, when LUHMES cells were exposed to NTUA 1:100 dilution

Experiments were therefore performed with a higher NTUA concentration (1:10 dilution). Figure 48 below shows NTUA intracellular accumulation and also in neurite extensions of differentiated LUHMES at time 40 minutes after starting NTUA perfusion. There was a time dependent increase in LUHMES cellular fluorescence upon NTUA exposure during stage 1, followed by a fall in cell fluorescence during the second NTUA exposure (figure 49 below). The rate of increase in cell fluorescence (NTUA uptake) during stage 1 was $2.5 \pm 0.2 \text{ au.min}^{-1}$ and NTUA 2 was $-0.7 \pm 0.2 \text{ au.min}^{-1}$ (figure 49a and 49b). The difference between the rate of NTUA uptake between the 2 stages was statistically significant ($p < 0.0001$).

Figure 48: NTUA accumulation by differentiated LUHMES cells using confocal microscopy (images taken using confocal microscopy)

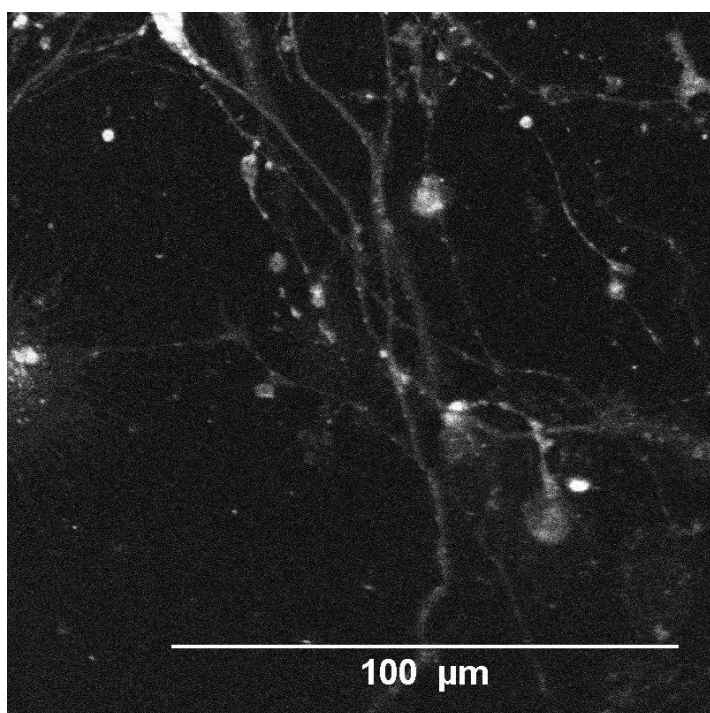
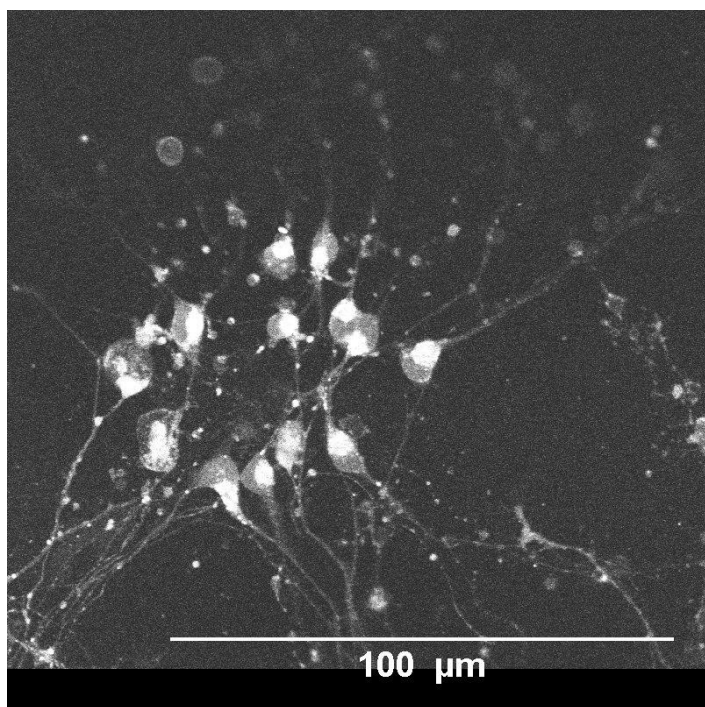


Figure 49: NTUA cellular uptake differentiated LUHMES before and after GBR/DES perfusion (a, b), results for 5 experiments

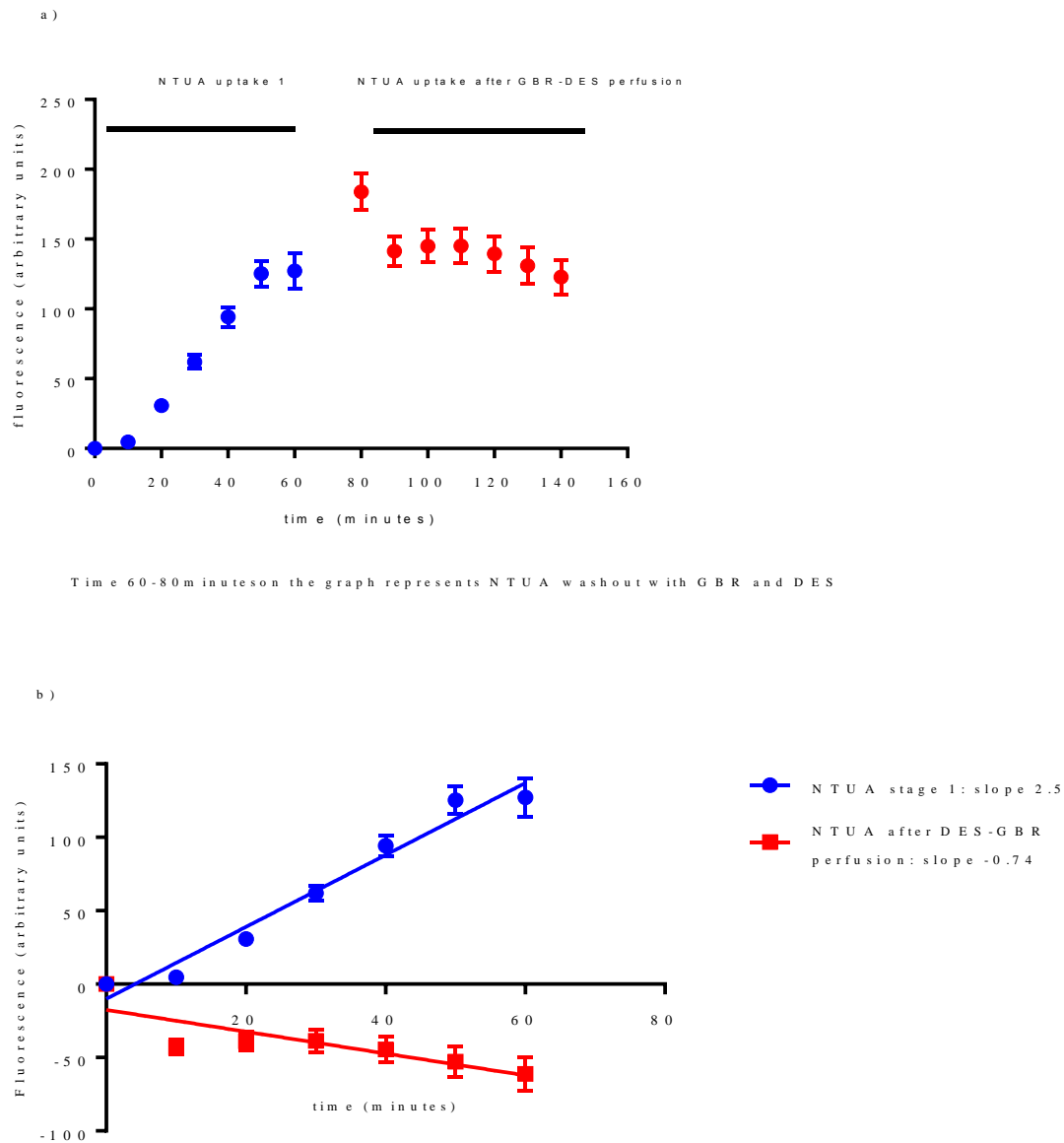


Figure 49 above: data points represent mean change in cell fluorescence +SEM, when LUHMES cells were exposed to NTUA only (blue), NTUA and blockers GBR and desipramine (red)

The two stage NTUA uptake was repeated to ascertain if the fall in fluorescence during NTUA stage 2 demonstrated above was due to transporter inhibition by GBR and DES. Cells were exposed to NTUA, then washed with PBS for 20 minutes, followed by the second NTUA exposure for a further 60 minutes.

Figure 50: NTUA cellular uptake by LUHMES cells, 2 stages: results for 4 control experiments

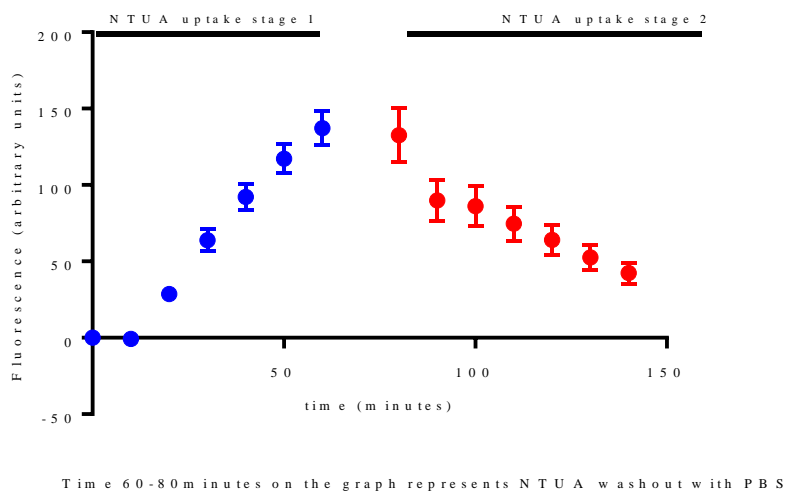


Figure 50 above: data points represent mean change in cell fluorescence +SEM, for 4 experiments when LUHMES cells were exposed to NTUA during stage 1 (blue), and NTUA stage 2 (red)

Similar to the previous results, the two stage NTUA cellular uptake control experiment showed a fall in cellular fluorescence during second stage NTUA exposure (figure 50 above). The rate of uptake in stage1 was $2.5 \pm 0.18 \text{ au}\cdot\text{min}^{-1}$ and in stage2 was $-1.3 \pm 0.19 \text{ au}\cdot\text{min}^{-1}$.

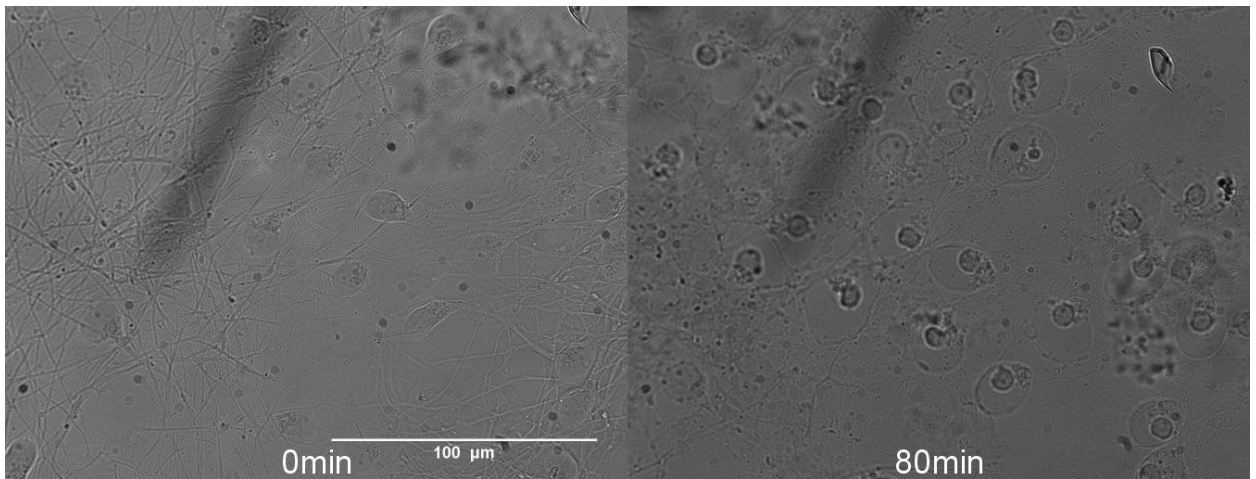
Effect of PBS supplemented with glucose, Calcium (Ca) and Magnesium (Mg)

Ca and Mg free PBS was used in the previous section. Further tests were performed to assess the effect of using different buffer constituents on stage 2 NTUA cellular uptake. Supplementing PBS with Ca (0.8mM), Mg (0.56mM) and glucose (1g/L) did not alter the rate of change in cellular fluorescence when differentiated LUHMES cells were exposed to NTUA. However, this led to loss of cell viability, as demonstrated in the images below (figure 51a). There was a fall in fluorescence (NTUA uptake) during the stage 2. The rate

of uptake for stage 1 was $2.12 \pm 0.18 \text{ au.min}^{-1}$ and for NTUA stage 2 was $-1.19 \pm 0.14 \text{ au.min}^{-1}$. Changes in cell structure were visualised during the experiments. Supplementing PBS buffer with glucose only, had a similar effect on cell viability (figure 51b).

Figure 51: Images showing effect of supplementing PBS buffer with calcium and magnesium and glucose on cell viability

a) Calcium and magnesium



b) Glucose only

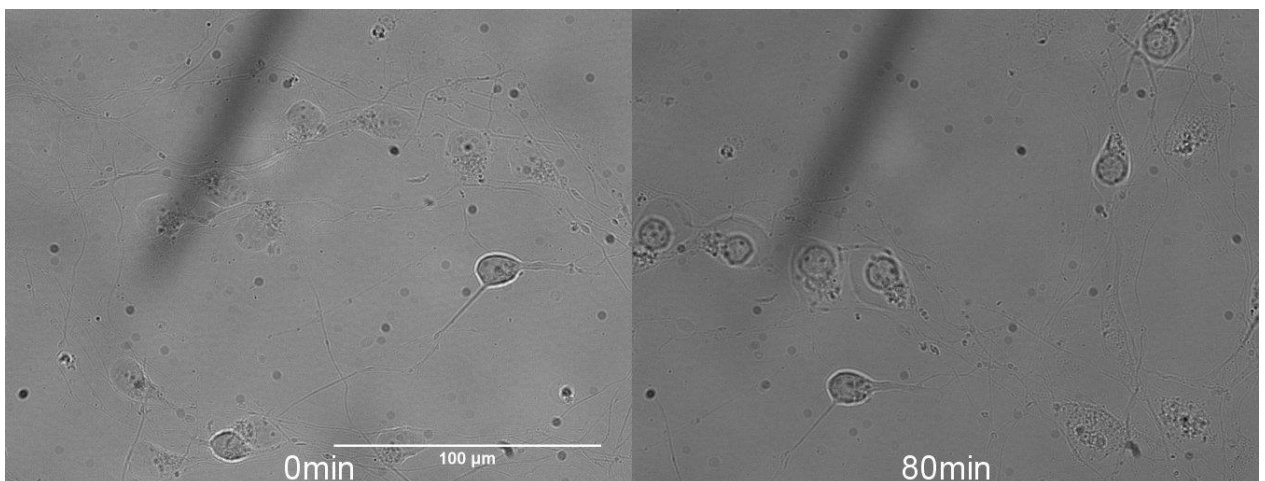


Image (a) calcium and magnesium and (b) glucose only. Scale bar 100μm.

Figure 52: NTUA uptake by differentiated LUHMES 2 stages using PBS supplemented with calcium and magnesium, results for 4 experiments

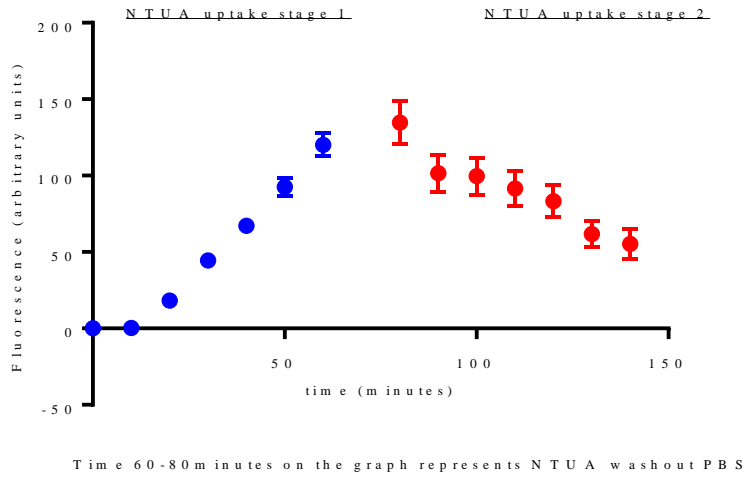
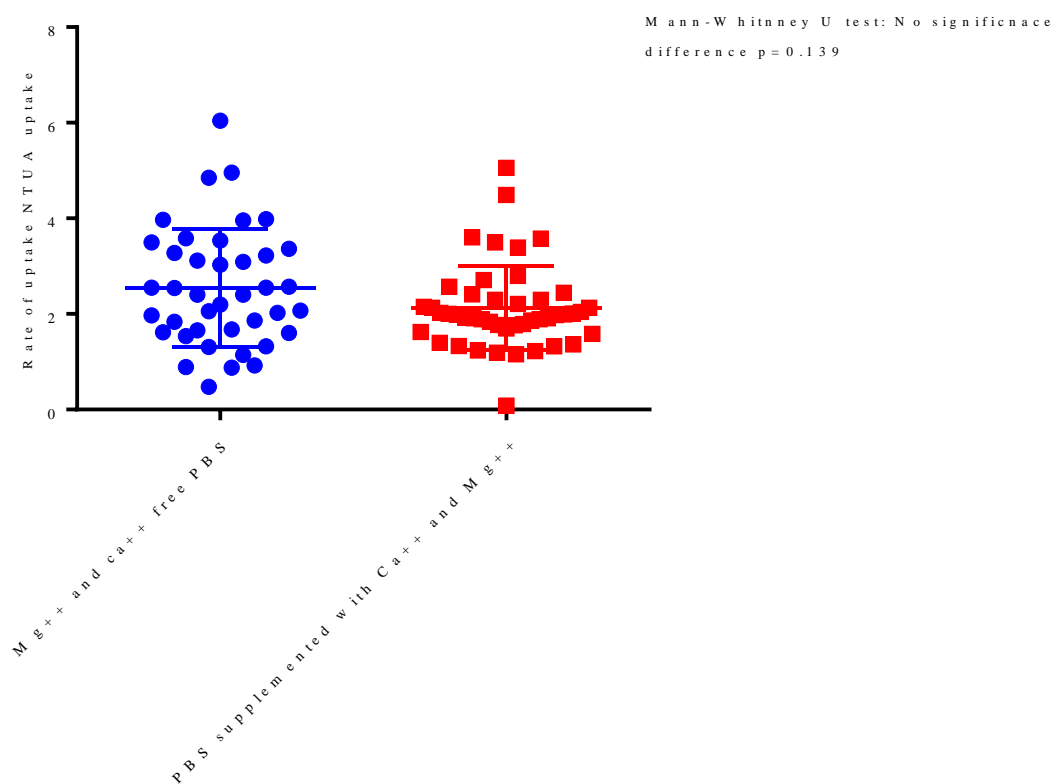


Figure 52: data points represent mean change in cell fluorescence +SEM, when LUHMES cells were exposed to NTUA during stage 1(blue) and stage 2(red) using PBS buffer supplemented with calcium and magnesium.

When results for experiments using calcium and magnesium free PBS were compared with PBS supplemented with calcium and Magnesium using Mann Whitney U test there was no significant difference in the rate of NTUA uptake during the first NTUA stage $p=0.14$ (figure 53).

Figure 53: NTUA uptake by differentiated LUHMES using PBS calcium and magnesium free compared to PBS supplemented with magnesium and calcium

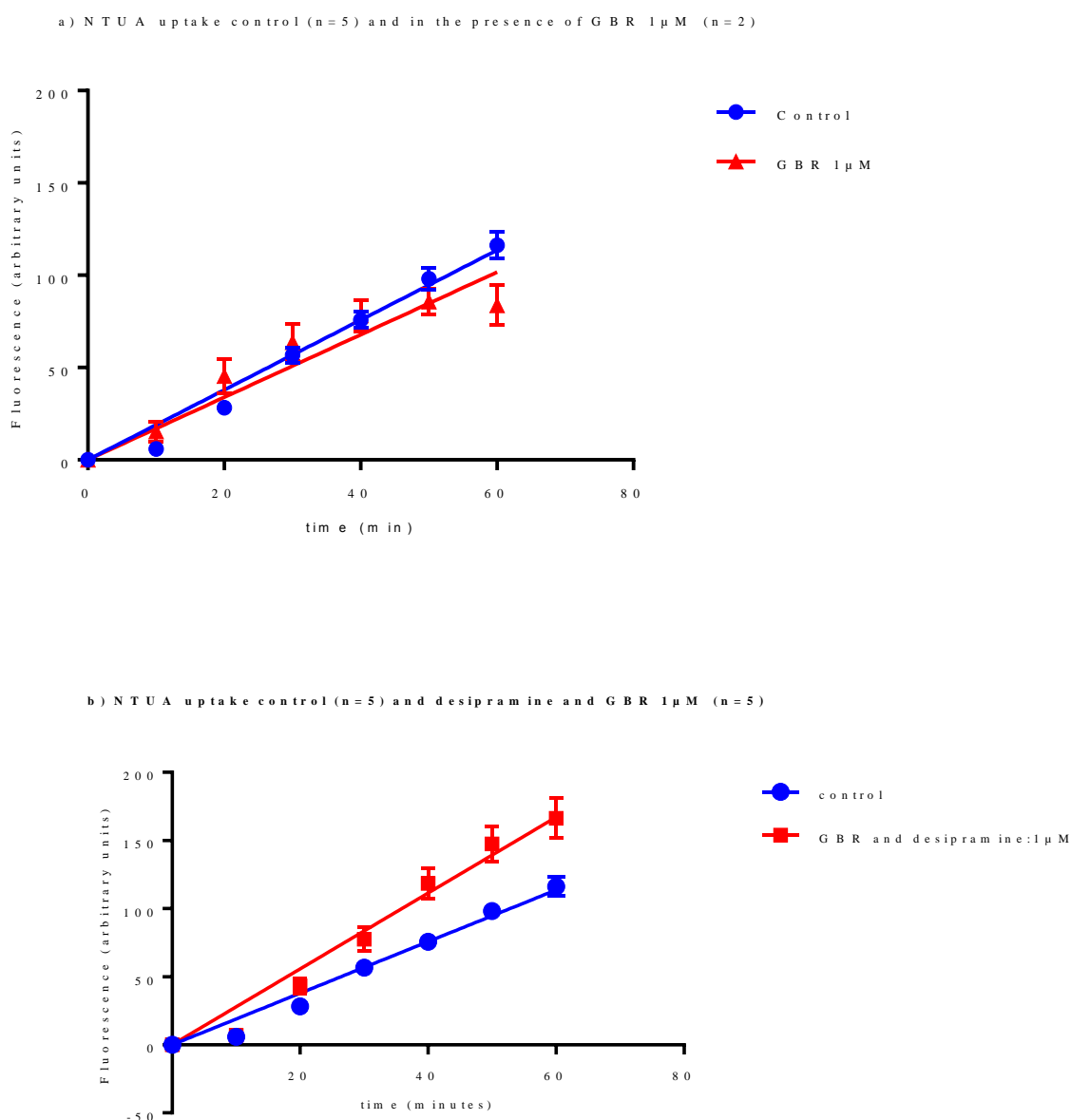


NTUA uptake (1 stage only): Is NTUA uptake mediated by monoamine transporter blockers?

Since all the experiments showed a consistent increase in cellular fluorescence for the first 60 minutes of NTUA exposure which egressed during stage 2, subsequent experiments were carried out for a maximum of 60 minutes (one stage protocol). To validate if DAT was responsible for NTUA uptake by differentiated LUHMES cells, cells were incubated with GBR 1 μ M (DAT blocker) for 15-60 minutes before the experiments then to both NTUA and GBR1 μ M for 60 minutes. Results showed an increase in cell fluorescence signal with time (rate of uptake 1.52 ± 0.21 au.min⁻¹, figure 54a). Considering that NTUA uptake could not be suppressed by GBR 1 μ M only, and that NTUA is also a NET substrate, further cellular uptake experiments were performed in the

presence of both GBR and desipramine. Results also demonstrated a time dependent increase in fluorescence, rate of uptake: $3.05 \pm 0.18 \text{ au} \cdot \text{min}^{-1}$ (figure 54b). Similarly, when NTUA uptake was assessed in the presence of NET, DAT, and SERT (fluoxetine $1 \mu\text{M}$) blockers, NTUA cellular uptake was not suppressed (rate of NTUA uptake $2.38 \pm 0.122 \text{ au} \cdot \text{min}^{-1}$; figure 54c).

Figure 54: NTUA cellular uptake by differentiated LUHMES in the presence of GBR, desipramine and fluoxetine



c) NTUA cellular uptake control (n=5) and with Desipramine, GBR, & fluoxetine (n=2)

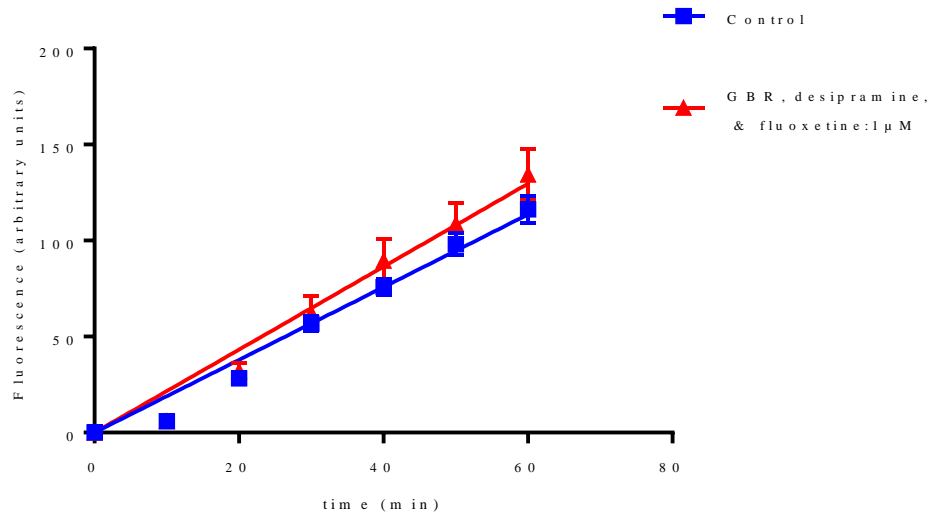


Figure 54 above: data points represent mean change in cell fluorescence +SEM, when LUHMES cells were exposed to NTUA only (blue), NTUA in the presence blockers (red) (a) GBR 1μM, (b) GBR and desipramine (1μM) (c) GBR, desipramine and fluoxetine 1μM

Effect of increasing GBR concentration

Increasing the GBR concentration to 10μM did not suppress NTUA uptake by differentiated LUHMES cells (rate of uptake $2.1 \pm 0.26 \text{ au} \cdot \text{min}^{-1}$; figure 55).

Figure 55: Effect of increasing GBR concentration on NTUA cellular uptake by LUHMES cells [DES (1 μ M) and GBR (10 μ M)]

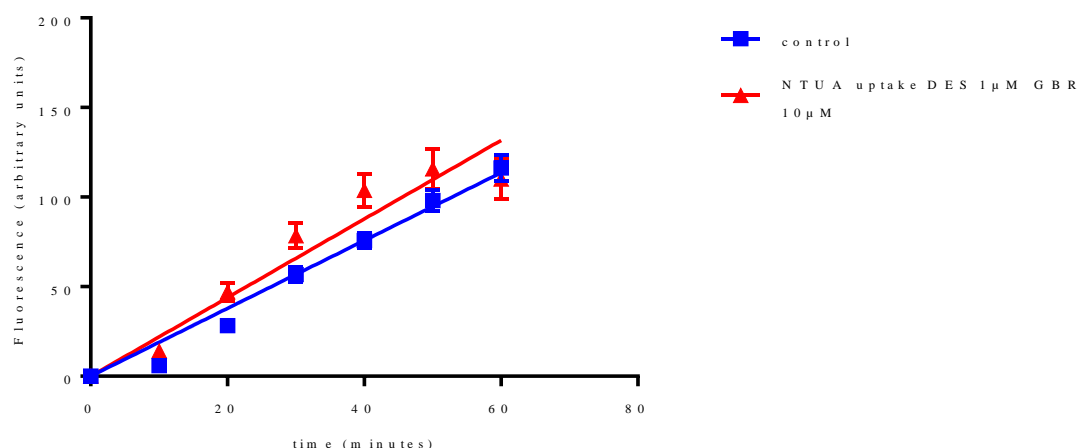


Figure 55 above: data points represent mean change in cell fluorescence +SEM, when LUHMES cells were exposed to NTUA only (blue), NTUA in the presence of GBR 10 μ M (red)

Effect of temperature on transporter rate

The effect of temperature on transporter function was also considered as a possible reason for the lack of NTUA uptake inhibition by the transporter blockers. When a lower perfusion flow rate was used, the temperature during the experiments was suboptimal (29-30 $^{\circ}$ C). Attempts were made to increase the bath temperature in order to optimise effect of the monoamine transporter blockers. This was done by increasing the flow rate of NTUA into the organ bath. Increasing the perfusion pump rate from 6RPM to 15RPM resulted in an increase in the organ bath temperature to 34 degrees which was maintained throughout the experiments. There was no change in the rate of change in fluorescence ($2.0 \pm 0.28 \text{ au}\cdot\text{min}^{-1}$). NTUA uptake in the presence of the blockers was then repeated at this temperature, but this did not alter the results.

NTUA uptake by Differentiated LUHMES cell neurite extensions.

Data analysis for NTUA uptake by LUHMES neurites was also performed. The analysis was performed for both control experiments (NTUA uptake only) and experiments with DAT and NET blockers. Results are shown in figure 56 below.

Figure 56: NTUA uptake by LUHMES neurites with and without desipramine and GBR

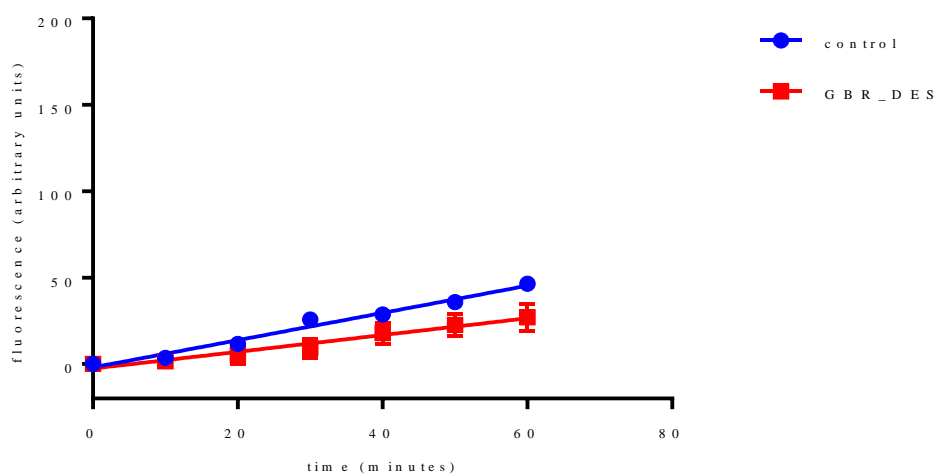
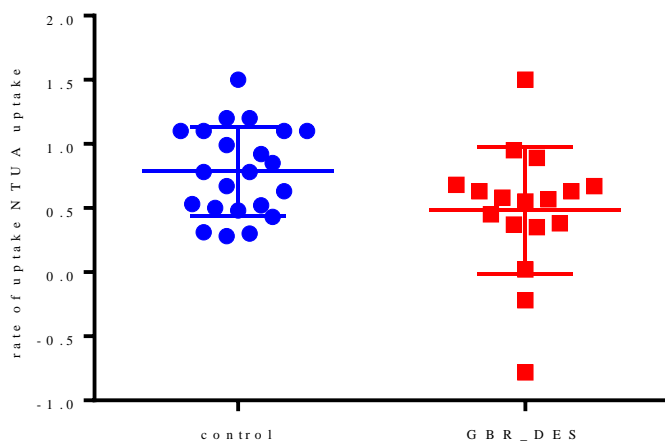


Figure 56 above: data points represent mean change in neurite fluorescence +SEM, when LUHMES cells were exposed to NTUA only (blue), NTUA in the presence of GBR 1 μ M and desipramine (red)

There was an increase in NTUA accumulation over time in both the control and experiments with blockers. But the rate of change increase in cell fluorescence in the presence of blockers was lower. The difference in NTUA uptake by the LUHMES terminals in the absence and presence of GBR and DES was statistically significant with a mean rate of uptake for control experiments without blockers 0.79 (95% CI 0.63, 0.94) au.min⁻¹ and after addition of blockers mean rate of uptake was 0.48 (95% CI 0.23, 0.74) au.min⁻¹ p=0.041 (figure 57).

Figure 57: NTUA uptake by differentiated LUHMES terminals with and without (control) GBR and desipramine



6.5 Discussion

LUHMES cells were easy to maintain and large number of cells were obtained within a short space of time as shown previously. The cells were differentiated into post mitotic neurons using methods described in previous reports (Scholz et al., 2011).

The current study demonstrated a time dependent increase in LUHMES cell fluorescence during NTUA exposure. Control experiments demonstrated similar rates of increase in cell fluorescence (NTUA accumulation) which suggests uniform differentiation of LUHMES cells. In addition to intracellular accumulation, NTUA was also seen to accumulate in varicosities along the neurites. This is consistent with previous reports which showed DAT expression on the cell bodies and also neurite extensions of dopaminergic neurons (Eriksen et al., 2009).

The two stage modified NTUA uptake protocol showed a fall in fluorescence during the second NTUA exposure. This contrasts the previous findings in PC12 cell experiments where NTUA uptake was demonstrated in both stages of the experiments. Several reasons

may explain these results. Firstly the cell lines are likely to have different saturation kinetics. Secondly, initial experiments were performed using calcium and magnesium and glucose free PBS buffer which may not have provided the necessary salts and energy requirements for neuronal function during the long experiments. This potential problem was addressed by supplementing PBS with glucose, calcium, and magnesium. However, this did not improve the rate of NTUA uptake during stage 2. Addition of glucose, Ca, and Mg to the buffer impaired cell viability, probably related to excitotoxicity as a result of sudden changes in ion influx into the cells.

Thirdly, photo-toxicity due to prolonged cell exposure to UV light during the long experiments is another possible explanation. Lastly, photo-bleaching from prolonged UV light exposure during NTUA stage 2 could have led to the fall in fluorescence, but this is, unlikely because a similar phenomenon was not seen in PC12 cells.

Only one previous study has assessed dopamine uptake and release by LUHMES cells. Scholz et al reported (^3H) dopamine uptake which was inhibited by GBR 12909 (Scholz et al., 2011). The current study sought to assess the DAT function using a fluorescence DAT substrate NTUA. In contrast to Scholz et al. (2011) study, which showed inhibition of DAT substrate uptake, incubating the cells with GBR did not inhibit NTUA cellular uptake. Given that other studies have shown that, in addition to DAT, dopamine reuptake in substantia nigra may also be via SERT and NET, (Kelly et al., 1985) and NTUA is a known substrate for the three monoamine transporters, NTUA uptake was assessed in the presence of various combinations of monoamine transport blockers: GBR, desipramine, and fluoxetine. Surprisingly, this did not alter the results, as NTUA uptake was still demonstrated. Furthermore, there was no significant change in the rate of increase in cell fluorescence in the presence of the transporter blockers. The effect of adjusting the

temperature and incubating the cells with the blockers for longer periods was also assessed. Temperature changes have been shown to correlate the DAT function (Xie et al., 2000). Xie et al. (2000) demonstrated that cellular uptake of DAT substrates including (³H)DA and (³H)MPP⁺, a rat mesencephalic cell line and a human DAT transfected neuroblastoma cell line, was greater at higher temperatures (Xie et al., 2000). In this study, the authors showed that an increase in temperature by only 3⁰C significantly increased DAT substrate uptake (Xie et al., 2000). To investigate the effect of increasing temperature on transporter rate and effect of GBR on NTUA uptake, bath temperature was increased to 34 ⁰C in the current study, but this did not alter the rate of NTUA uptake by differentiated LUHMES cells. Similarly, when the studies with blockers were repeated at a higher temperature there was no suppression of NTUA uptake. Likewise, incubating the cells with the blockers for longer periods prior to NTUA exposure and increasing the concentration of GBR did not suppress NTUA uptake. Monoamine blockers used in this study have been shown to have high potencies for inhibiting their respective transporters (GBR IC₅₀ for DAT 14nM, (Dutta et al., 1998); fluoxetine pIC₅₀ 7.8 for (³H) 5HT uptake; desipramine pIC₅₀ for (³H) noradrenaline uptake 9.1: IC₅₀ of approximately 1nM (Mantovani et al., 2009). Much higher concentration (1μM) and for GBR 10 μM were used to ensure complete blockade of the monoamine transporters. GBR and desipramine 1μM was also used in a previous study in our laboratory and this suppressed NTUA uptake in PC12 cells (Elobi, 2014).

Inability of the GBR, desipramine, and fluoxetine to suppress NTUA uptake in the current study, suggest that NTUA cellular uptake in this cell line was probably passive and not mediated by monoamine transporters. Alternatively, it is also plausible that GBR affinity for DAT in LUHMES cells and rats phaeochromocytoma (PC12 cells) DAT differ. Although speculative, another explanation for non-specific NTUA accumulation is that

the concentration (1:10) used in this study was too high. Support for this comes from a study which sought to assess whether methamphetamine induced dopaminergic neuron toxicity was DAT mediated (Xie et al., 2000). The authors tested for cellular uptake of methamphetamine at different concentrations and found that, cocaine (a DAT inhibitor) only blocked uptake when methamphetamine 10 μ M or less was used but not at higher concentration (Xie et al., 2000). The authors concluded that methamphetamine cellular uptake was DAT mediated at lower concentration and passive at higher concentration (Xie et al., 2000). To verify this hypothesis, future studies should reassess NTUA uptake inhibition by GBR using a lower concentration (1:100) or consider using higher GBR concentration than those used in this study. Lastly, it is also feasible that NTUA caused excitotoxicity to the LUHMES cells leading to formation of fluorescent substrates which was observed as a rise in cell fluorescence, this also requires verification in future studies.

Animal studies have shown different DAT densities in various brain regions (Coulter et al., 1995). It is possible that DAT density in LUHMES cell bodies and neurites is different. This was investigated by examining the rate of NTUA uptake by LUHMES varicosities and neurite extensions. Data analysis showed a time dependent uptake NTUA uptake in the LUHMES terminals. NTUA uptake was significantly lower in the presence of GBR and desipramine, probably reflecting DAT dependent NTUA uptake in the neurites. This finding is consistent with results of previous reports which demonstrated higher dopamine clearance and release in the striatum when compared to the substantia nigra, implying a higher DAT density in striatal dopaminergic terminals (Hoffman et al., 1998). Inability of GBR and desipramine to completely block NTUA uptake by the neurites suggests that the compound (NTUA), may be a substrate of other amino acid transporters present on the neurites. This requires further evaluation in future experiments.

Limitations to this study include the non-physiological conditions during the study. Suboptimal temperatures and the use of glucose and amino acid free buffer which could potentially interfere with dopamine transporter function. Increasing the bath temperature did not change the results. Use of more physiological media for future experiments, for example artificial CSF should be considered. However, some amino acids such as riboflavin can interfere with the fluorescence signal. Scholz et al. (2011) showed that electrophysiological properties of LUHMES cells lagged behind other differentiation markers and uniform properties were only demonstrated when day 10-12 differentiated cells were assessed (Scholz et al., 2011). Differentiating LUHMES cells for longer periods prior to experiments should also be considered in future studies.

In summary, this study showed that LUHMES cells can be reliably differentiated to form a uniform population of cells. NTUA uptake by the LUHMES cell bodies was non-specific in the cell bodies but GBR sensitive uptake was demonstrated in the neurites which provide further evidence for functioning dopamine uptake machinery in this cell line. Future work using a lower NTUA concentration is required to verify the utility of this cell line for screening other fluorescent DAT substrates.

**CHAPTER 7: DEVELOPMENT OF A ^{19}F MAGNETIC RESONANCE
IMAGING PROBE**

7.1. Background

In MRI, images are produced by measuring the signal from hydrogen ions/protons (^1H) of water soft tissues (Amiri et al., 2015). This is dependent on the concentration of ^1H and its surrounding environment (Armstrong and Keevil, 1991). Contrast agents are used to alter ^1H relaxation times in various tissues, which enhances the contrast of the tissue of interest from its surroundings (Amiri et al., 2015). However, increased vascular permeability or haemorrhage due to the underlying disease process may reduce specificity of a contrast agent in labelling the area of interest (Amiri et al., 2015). Use of fluorine MRI may counteract this problem.

The potential use of ^{19}F fluorine isotope (^{19}F) in MRI was first described in the 1970s (Knight et al., 2011). Fluorine naturally occurs as a stable ^{19}F isotope (100% natural abundance) and it also has a similar sensitivity and resonance frequency to that of the proton (Ruiz-Cabello et al., 2011). The later characteristic feature allows the use of ^{19}F imaging on the current ^1H MRI scanners (Ruiz-Cabello et al., 2011). In addition, fluorine is barely detectable in the body, the only small amount that is present is tightly bound to bone matrix (Chen et al., 2010, Knight et al., 2011). This provides a very high contrast to noise ratio and specificity as there is no interference from background signal on ^{19}F MRI in vivo (Ruiz-Cabello et al., 2011). Another advantage is that fluorinating a compound for use as a tracer can be achieved by replacing hydrogen atoms with fluorine, which does not interfere with the chemical or biological effects of the compound (Knight et al., 2011). But one drawback of using fluorine imaging is that high concentrations and therefore higher number of fluorine atoms per compound are required in order to obtain similar image quality as that produced with standard ^1H MRI (Ruiz-Cabello et al., 2011). Molecules with few fluorine atoms have long longitudinal relaxation times thus low signal to noise ratio (Amiri et al., 2015). Longer acquisition/scanning times are therefore

required which has practical implication when it comes to clinical application (Amiri et al., 2015). To overcome this problem, perfluorocarbon (PFC) nanoparticles have been used in ^{19}F MRI studies including for cell migration studies, vascular diseases, and to assess tissue hypoxia in tumours (Chen et al., 2010). These compounds can reach very high ^{19}F atom concentration of up to 100M which can be detected by low field strength MRI of 1.5T used in clinical practice (Chen et al., 2010). But tolerability of compounds with high density of fluorine atoms, required to achieve such concentrations is unknown.

Recent studies have also reported potential use of ^{19}F MRI in proteinopathies such as Alzheimer disease (AD). For example, Higuchi et al. (2005) used a ^{19}F amyloid tracer (E-E) - 1- fluoro-2.5-bis (3-hydroxycarbonyl-4-hydroxy) styrylbenzene(FSB) to detect brain amyloid β plaques in vivo in amyloid precursor protein transgenic mice (Higuchi et al., 2005). In another study, the authors used ^{19}F curcumin derivatives to label brain amyloid β plaques using 7.0T MRI in an AD mouse model (Yanagisawa et al., 2011, Tooyama et al., 2016). The application of ^{19}F tracers shown in these studies suggest that ^{19}F imaging has potential for use in imaging in PD.

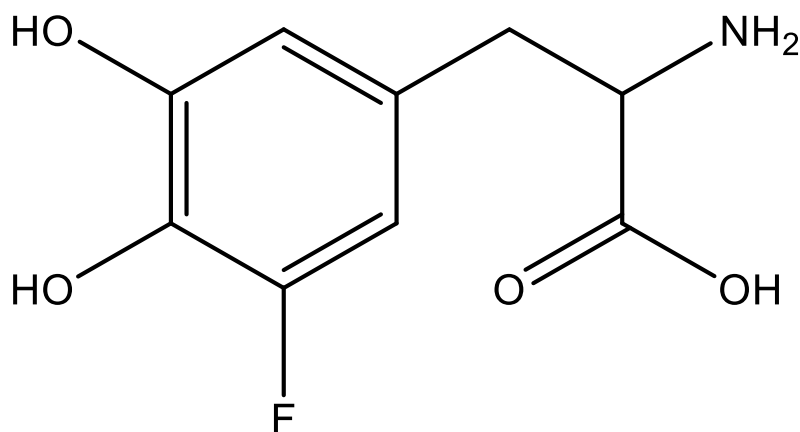
The aim of this study was to develop a ^{19}F based MRI probe for labelling nigrostriatal neurons in PD. ^{18}F -fluorodopa is already in use as a PET probe for labelling dopaminergic neurones in PD. ^{19}F 6- fluoro DL-DOPA is a non- radioactive fluorodopa ligand which is commercially available. As an initial step in the development of ^{19}F probe in PD, cellular uptake of ^{19}F 6- fluoro DL-DOPA by PC12 cells was assessed, using ^{19}F - Nuclear Magnetic Resonance Imaging (NMR) spectroscopy.

7.2. Measurement of ^{19}F 6-Fluoro DL-DOPA signal using Nuclear Magnetic Resonance (NMR) spectroscopy.

7.2.1. Materials and Methods

The study hypothesis was that ^{19}F 6- fluoro DL-DOPA (figure 58) would be taken up by PC12 cells and metabolised to ^{19}F 6- fluoro-dopamine which then accumulates in vesicles in a similar manner as dopamine. Firstly, the optimum concentration at which ^{19}F 6- fluoro DL-DOPA could be detected by ^{19}F NMR spectroscopy was determined.

Figure 58: Chemical structure of ^{19}F 6- fluoro DL-DOPA



Molecular weight 296.09

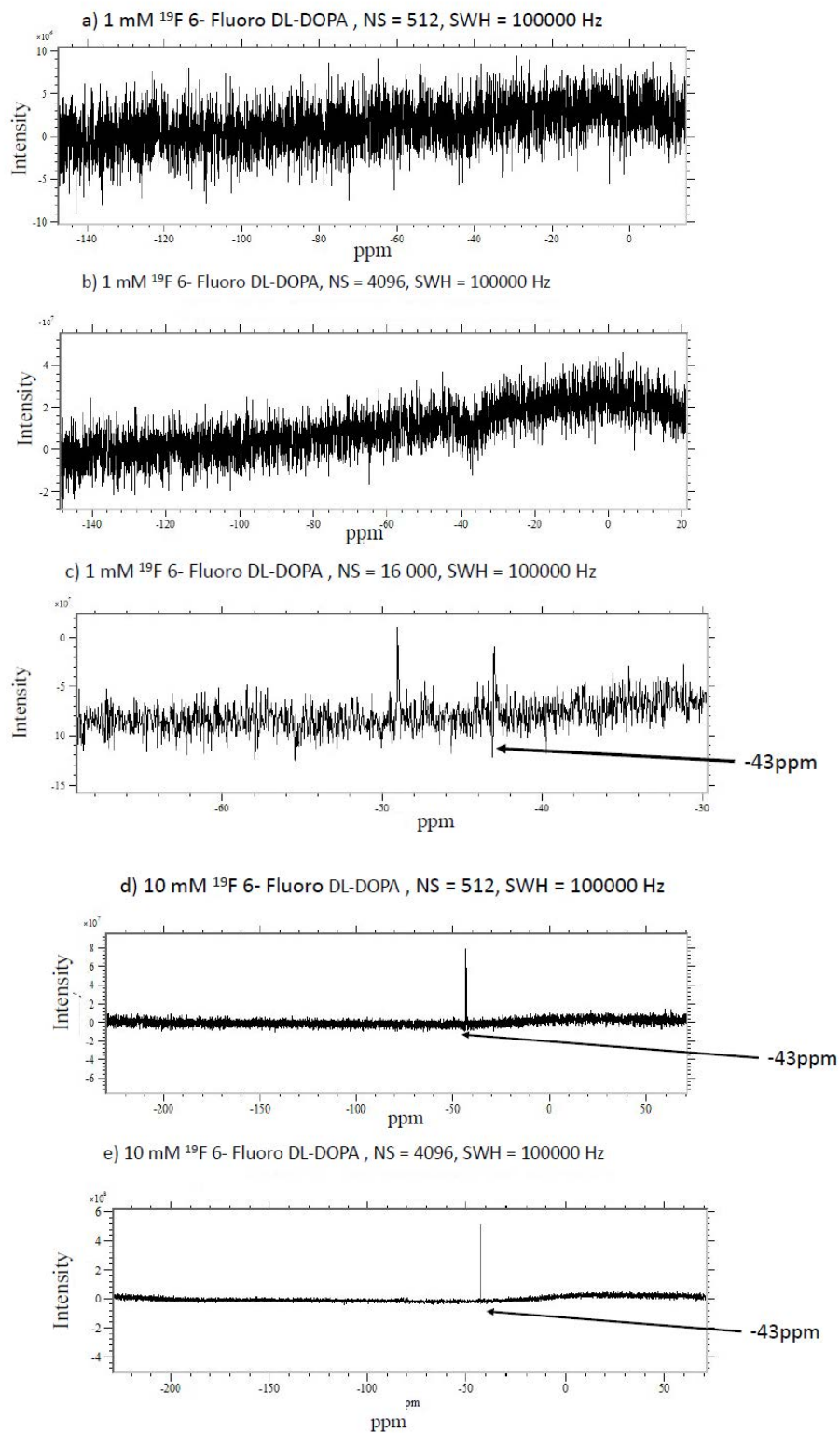
^{19}F 6- fluoro DL-DOPA was purchased from Santa Cruz Biotechnology, Germany. The compound was diluted with sterile water to make a 10mM stock solution and stored in aliquots at -20°C . For the experiments, ^{19}F 6- fluoro DL-DOPA was diluted with PSS to make 10mM, 1mM, 100 μM and 30 μM solutions which were then placed in 3mm NMR tubes (volume 200 μL), for NMR spectroscopy. Data were collected on a Bruker DMX 300 spectrometer equipped with a 7 T vertical wide-bore superconducting magnet operating at a fluorine resonance frequency of 282 MHz with a 30 mm RF bird cage coil. The following experimental conditions were applied (temperature 20°C , Spectral width

(SWH) 100 000Hz, 90^0 pulse (P_1) 10 μ s, time domain (TD) 16 384, acquisition time (AQ) 0.819s, receiver gain (RG) 8 192, Dwell time (DW) 5 μ s, nucleus (NUC)- ^{19}F , power level (PL1) 6db, field – 3 526 Hz, Pulse Program ZG, receiver gain (RG)= 8 192, DE 7.14 μ s, and frequency of ^{19}F nucleus (SF01) was 282.4Hz). The number of scans (NS) was gradually increased from 4 to 4 096 depending on the ^{19}F signal obtained.

7.2.2. Results

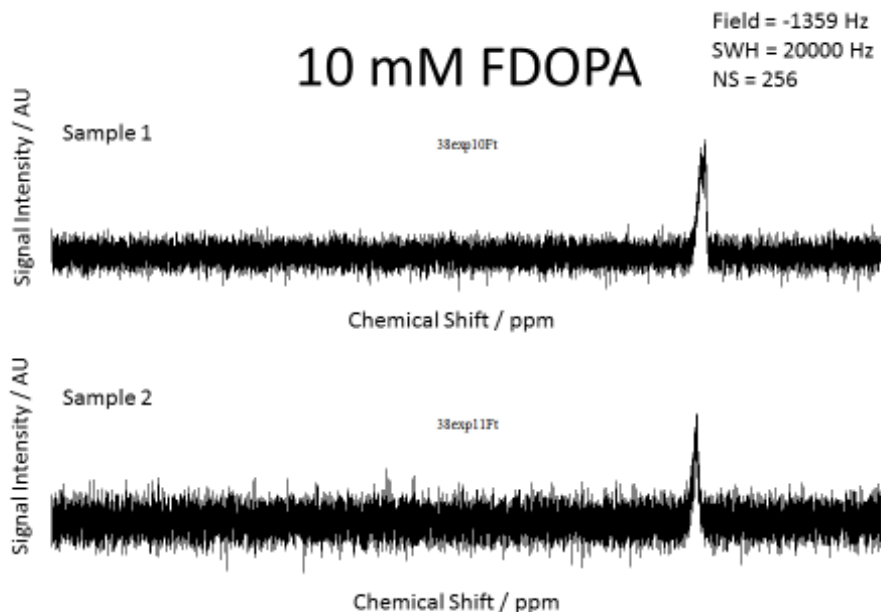
When ^{19}F 6- fluoro DL-DOPA 10mM was assessed, no signal was detected at NS=4. The number of transients was gradually increased from four to 4 096 scans. A small ^{19}F signal was detected (-43parts per million: ppm) at NS=512 (figure 59). The intensity of peak increased with increasing number of transients (NS=4 096, figure 59e). Increasing the number of scans also increased the required scanning time. ^{19}F 6- fluoro DL-DOPA 1mM, and 100 μ M solutions were also tested using similar settings but no signal was detected up to NS= 4 096 (figure 59 a and b). A small peak with marked background noise was seen with ^{19}F 6- fluoro DL-DOPA 1mM after NS=16 000 (total scanning time of over 8hours; figure 59c). Shimming the ^{19}F 6- fluoro DL-DOPA 10mM sample on tri-fluoroacetic acid (TFA) 1mM also increased the size of the peak and allowed the number of scans to be reduced to 256 (figure 60), but showed marked background noise.

Figure 59: ^{19}F 6- fluoro DL-DOPA 1mM and 10mM signal at various number of transients (NS) and spectral width (SWH) 100000Hz



(a) 1mM NS=512, (b) 1mM NS=4 095, and (c) 1mM NS=16 000, (d) 10mM, NS=512 (e) 10mM NS=4 096. X axis (ppm): parts per million, y axis represents signal intensity (arbitrary units).

Figure 60: 10mM ^{19}F 6- fluoro DL-DOPA spectra after shimming with Tri-fluoroacetic acid



7.3. ^{19}F fluoro DL-DOPA cellular uptake by PC12 cells using ^{19}F NMR spectroscopy

7.3.1. Methods

Given that no ^{19}F signal was detected at 100 μM concentration, ^{19}F 6- fluoro DL-DOPA 300 μM was therefore used for the cellular uptake experiments. It was postulated that ^{19}F 6- fluoro DL-DOPA 300 μM would accumulate in PC12 vesicles and reach a detectable concentration greater than 1mM. To assess for cytotoxicity of 300 μM ^{19}F 6- fluoro DL-DOPA, cells were incubated with the compound for 2 hours then cell viability was assessed by examining morphology of the cells using microscopy. 40×10^6 PC12 cells were incubated with 300 μM ^{19}F -fluoroDopa for 2 hours in a 5% CO_2 incubator at 37 $^\circ\text{C}$. After the 2 hour incubation, there were no obvious morphological changes to the cells when visualised using light microscopy. ^{19}F -fluoro DL-DOPA and PC12 cell suspension was transferred into a falcon tube and centrifuged at 800 RPM for 5 minutes. The

supernatant was removed and the cell pellet was gently washed with PSS to remove excess extracellular ^{19}F -fluoro DL-DOPA. The cell pellet was then re-suspended in 2mls of PSS and transferred into 10mm NMR tubes (Norrell Select Series S300 purchased from Sigma-Aldrich catalogue number NORS3007-5EA) for NMR spectroscopy. Experimental conditions were set as before and number of scans was gradually increased until a ^{19}F -fluoroDopa was detected. Tri-fluoroacetic acid (TFA) shimming sample was also used as the reference.

7.3.2. Results.

No ^{19}F -fluoro DL-DOPA signal was detected when the number of scans was increased from 512 to 4 096. In total 3 samples were assessed. Results for 1 024 and 2 048 scans are shown below shown (figure 61). Results remained negative even when the samples were shimmed on trifluoroacetic acid (TFA). Figure 62 shows the TFA signal only.

Figure 61: ^{19}F -fluoroDL-DOPA $300\mu\text{M}$ cellular uptake by undifferentiated PC12 cells

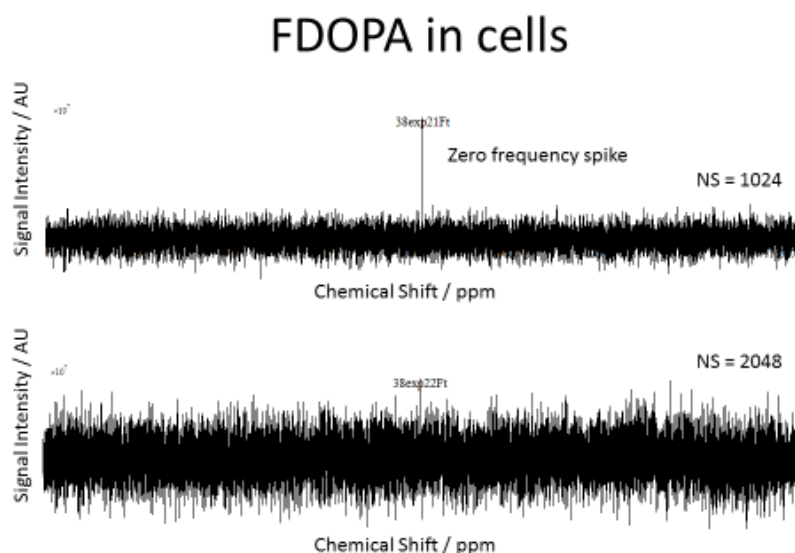
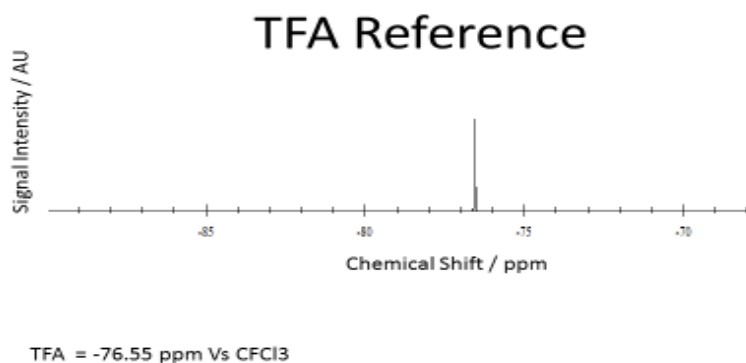


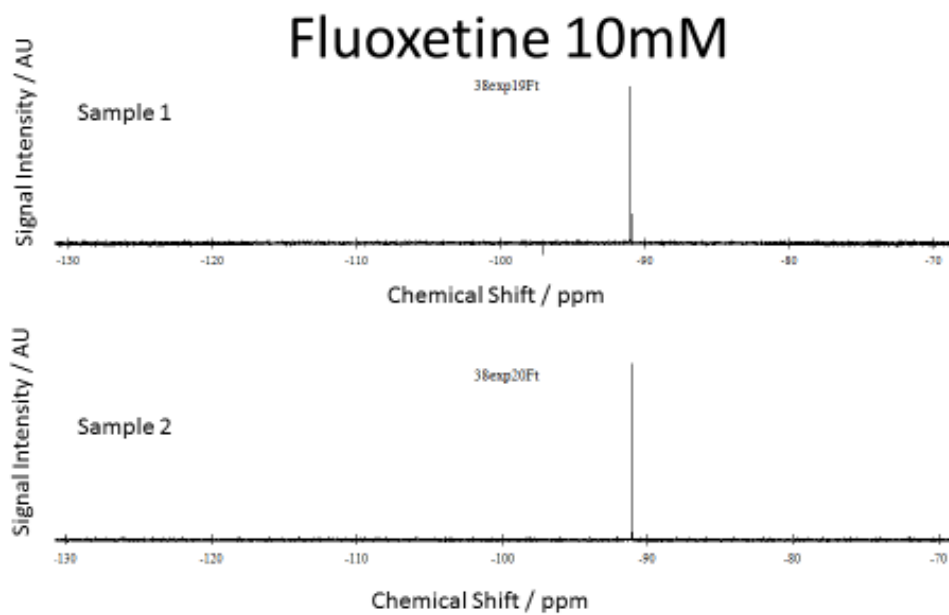
Figure 62: Trifluoroacetic acid (TFA) 1mM signal



Effect of increasing the number of fluorine atoms on the ¹⁹F NMR signal.

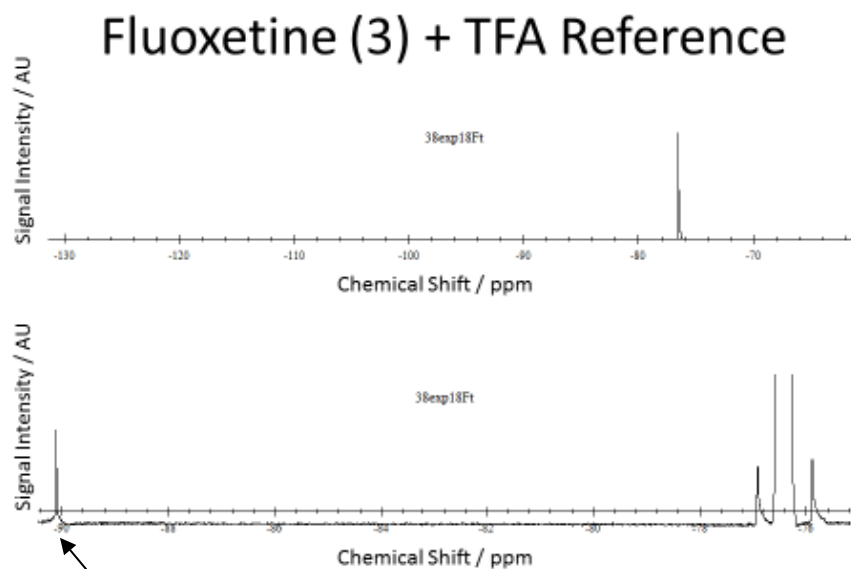
Given that increasing the number of fluorine atoms can increase the ¹⁹F NMR signal, we sought to confirm this by comparing 10mM fluoxetine HCL signal with that of 10mM ¹⁹F 6- fluoro DL-DOPA. Fluoxetine HCL contains three fluorine atoms, and was purchased from LKT laboratories Inc., UK cat No F4780. 10mM solution was made by diluting fluoxetine with PSS and placed in 3mm NMR tubes (volume 200μM). Scan settings NS=256, TD 32 768 SWH 20 000Hz, RG 256, P₁ 28 μs. PL16dB, field 1359. 3 samples were scanned, with and without TFA reference. Figures 63 and 64 show sharp fluoxetine peaks at NS= 256 with and without TFA, at -90ppm.

Figure 63: Fluoxetine 10mM NMR signal



Fluoxetine signal at -90ppm

Figure 64: Fluoxetine 10mM NMR signal with Trifluoroacetic acid (TFA) reference

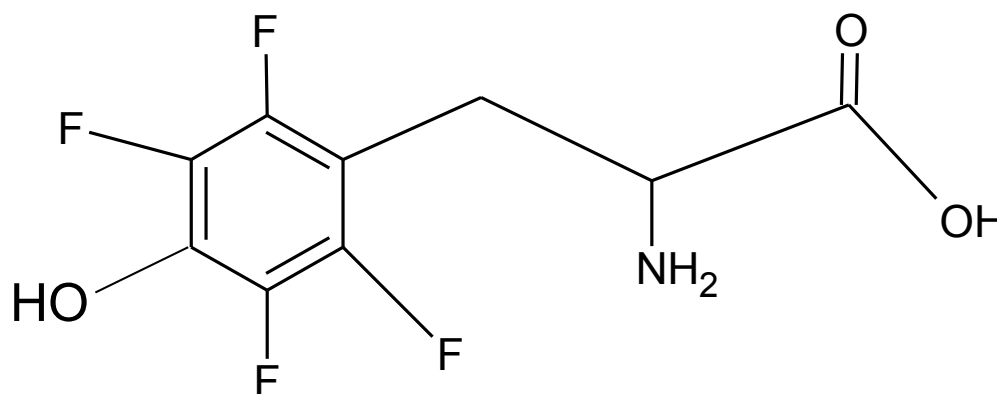


Fluoxetine signal at -90ppm

7.4. 2, 3, 5, 6-Tetrafluorotyrosine cellular uptake by LUHMES cells using NMR spectroscopy

Earlier experiments showed that increasing the number of fluorine atoms and concentration of the compound increase the ^{19}F signal intensity. Poly-fluorinated compounds have also been used in previous studies demonstrating a strong ^{19}F signal (Chen et al., 2010). Since poly-fluorinated ^{19}F fluorodopa was not available commercially, an alternative was sought for the initial study. Tyrosine is a levodopa precursor. Both amino acids are substrates of neutral amino acid transporters (Camargo et al., 2014). 6- (^{18}F) fluoro- L-meta-tyrosine analogue has been evaluated for imaging of the striatal dopaminergic neurons in animal studies and was shown to produce images with high contrast (DeJesus et al., 1997). 2, 3, 5, 6-Tetrafluorotyrosine is commercially available and was therefore purchased from Manchester organics, UK, catalogue number C15374 (chemical structure shown in figure 62 below). If 2, 3, 5, 6-tetrafluorotyrosine signal and cellular uptake of the compound can be demonstrated by NMR spectroscopy, then poly-fluorinated ^{19}F fluorodopa will be synthesised locally at the University of Birmingham with a plan to repeat the cellular uptake experiments and subsequently further testing in small animals, if results are positive.

Figure 65: 2, 3, 5, 6-Tetrafluorotyrosine chemical structure



7.4.1. Methods

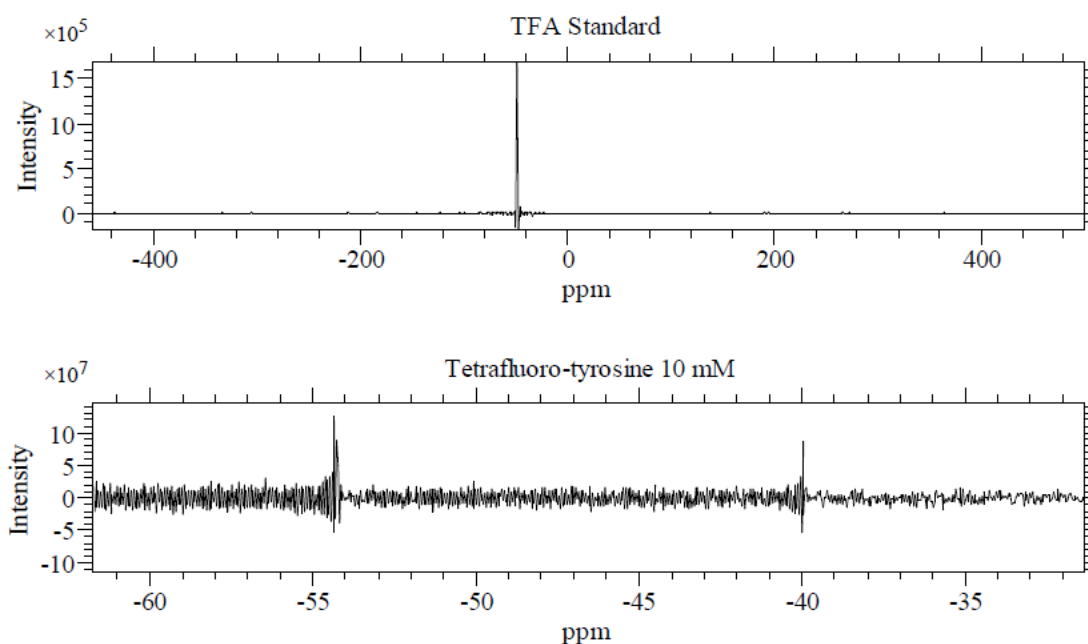
2, 3, 5, 6-Tetrafluorotyrosine was dissolved in water to form 10mM stock solution which was stored at -20°C . The compound was diluted to make 300 μM , 1mM, and 10mM solutions for NMR spectroscopy as previously described. The following settings were used for TFA: TD = 16 384, NS = 16, SWH = 100 000 Hz, RG = 16. For 2, 3, 5, 6-Tetrafluorotyrosine: SWH 100 000h=Hz, NS 2048 and RG 819216384 Number of scans = 2 048, Dummy Scans = 2, Spectral Width = 100 000 Hz, acquisition time = 0.0819 s, RG = 8192.

For cellular uptake experiments, the stock solution was diluted with D-PBS to make 300 μM solution. LUHMES cells were plated onto poly-L-ornithine and fibronectin coated multiwall plates and differentiated into mature dopaminergic cells using previously described methods. Experiments were performed from day 5 of differentiation onwards. On the day of the experiment, the differentiation media was removed from the wells and the cell layer was briefly washed with pre-warmed D-PBS. 2mls of 300 μM 2, 3, 5, 6-Tetrafluorotyrosine were added to each well with cells and incubated at 37°C for 1 hour. D-PBS only was also added to two wells and culture media only was added to an equal number of wells for control experiments. After incubation for 1 hour, cells were viewed under the microscopy to assess for viability. The 2, 3, 5, 6-Tetrafluorotyrosine, PBS, and culture media were removed from the wells and the cell layer was washed with PBS twice. 400 μM of cellLytic M (Sigma, catalogue number C2978) was added to each well and cells were incubated on a shaker for 15 minutes. A cell scraper was used to ensure that all cells were removed from the plates. The cells were transferred to Eppendorf tubes and centrifuged for 15 minutes at 15 000g. The supernatant was removed and transferred into NMR tubes for spectroscopy.

7.4.2. Results

No signal was seen when 2, 3, 5, 6-Tetrafluorotyrosine 1mM and 300 μ M were assessed. When 10mM 2, 3, 5, 6-Tetrafluorotyrosine was assessed, small peaks (figure 66) at 40ppm and -54ppm were seen.

Figure 66: NMR signal for 1mM TFA and 10mM 2, 3, 5, 6-Tetrafluorotyrosine



Due to the unavailability of time slots for using the local NMR spectrometer at the University of Birmingham, samples for cellular uptake of 10mM 2, 3, 5, 6-Tetrafluorotyrosine by LUHMES cells were sent to the University of Warwick for further experiments. Formal report showed that after two attempts, 'it was possible to detect the highest concentration (10mM), but were not overburdened by the signal'. The recommendation from the University of Warwick was that future work, would therefore need to be done with the upper end of the concentration scale.

7.5. Discussion

This study confirmed the feasibility of detecting ^{19}F fluoro DL-DOPA signal using NMR spectroscopy. At lower concentrations, greater number of transients (NS= 16 000 scans for 1mM) and thus longer scanning time (8hrs) were required to detect a signal. Such long scanning times are not practical for imaging in clinical practice. As expected, at higher concentration (10mM) fewer scans (NS=512) were required and a sharp ^{19}F fluoro DL-DOPA signal intensity was detected. Shimming is a method used in MRI and spectroscopy to increase the homogeneity of the magnetic field around sample and thus increase the signal (Bothwell and Griffin, 2011). In this experiment TFA was used as the shimming sample and this increased the ^{19}F fluoro DL-DOPA signal and also reduced the scanning time.

PC12 cells can concentrate dopamine in vesicles to 110mM (Kozminski et al., 1998), which exceeds the 10mM concentration detected by NMR spectroscopy. ^{18}F fluorodopa is already in use as a PET ligand and can be taken up by dopaminergic neurons. Substituting ^{18}F with ^{19}F is unlikely to interfere with metabolism or cellular uptake of fluoro DL-DOPA by PC12 cells. The ^{19}F fluoro DL-DOPA cellular uptake experiments, did not detect a signal, despite increasing the number of scans. ^{19}F is known to be hydrophobic and its mobility and thus ability to produce a signal is influenced by the surrounding environment (Tooyama et al., 2016). If ^{19}F fluoro DL-DOPA was taken up by the PC12 cells and converted to fluoro DL-dopamine, it is plausible that the latter was trapped in intracellular vesicles, reducing its mobility, hence reducing the NMR signal. But the most likely explanation is that the concentration used for these experiments was too low to produce a meaningful NMR signal.

A large number of PC12 cells (40×10^6) was used in-order to increase the volume fraction thus concentration of ^{19}F fluoro DL-DOPA, but this caused the cells to settle at the bottom of the NMR tube, which can also cause an indistinct NMR spectra (Rangus, 2007). To counteract this problem, cell lysis methods were used for the 2, 3, 5, 6-Tetrafluorotyrosine cellular uptake experiments. This study also showed that increasing the number of fluorine atoms by using fluoxetine increased the signal intensity and reduced the signal to noise ratio. Surprisingly 2, 3, 5, 6-Tetrafluorotyrosine which has a four fluorine atoms showed a small signal at 10mM concentration. Only one experiment was performed at this concentration. The low signal maybe due to insoluble particles in the solution which can broaden the spectra (Rangus, 2007). It is unlikely that the low signal was related to sensitivity of the NMR spectrometer because a stronger signal was obtained from ^{19}F 6-fluoro DL-DOPA which contains one fluorine atom per molecule.

Tooyama et al. (2016) showed the feasibility of labelling amyloid β plaques in AD mouse model using a probe containing only three ^{19}F fluorine atoms (Tooyama et al., 2016). In another study, the same authors also used another amyloid probe with six ^{19}F atoms. Although higher signal intensities were seen when solutions of the probe with six atoms were compared to the probe with three ^{19}F atoms, no ^{19}F signal was detected in the mice brains after intravenous injection, of the probe with six fluorine atoms (Yanagisawa et al., 2014). The study authors demonstrated that increasing the hydrophilicity of a probe by increasing the number of polyethylene glycol chains (PEG) increased the ^{19}F signal (Yanagisawa et al., 2014). Nevertheless, when number of PEG chains exceeded seven this reduced blood brain barrier permeability (Yanagisawa et al., 2014). This highlights the importance of getting the correct balance between the number of fluorine atoms and hydrophilicity of a probe for animal studies. This needs consideration when synthesising probes for future experiments.

Given that there is ample evidence to suggest that PD is a multisystem disorder with both central and peripheral alpha-synuclein deposition, (Beach et al., 2010) and there is temporal progression of Lewy body deposition with disease progression (Braak et al., 2002), future studies should focus on developing ^{19}F probes which not only label dopaminergic neurons but also alpha-synuclein deposits. Such tools may be useful in monitoring disease progression and in evaluating effects of treatments which target alpha synuclein deposition. Feasibility of imaging alpha-synuclein deposits in PD using radionuclide ligands has been assessed before. For example Bagchi et al (2013) synthesised a radiolabelled alpha-synuclein fibril ligand (SIL23) and demonstrated that the compound could bind to alpha-synuclein fibrils in a PD mouse model and also in post-mortem human PD brains, but not in controls (Bagchi et al., 2013). The ligand binding sites also correlated with the amount of alpha synuclein (Bagchi et al., 2013). This approach in imaging PD pathology, may also be useful in early disease when current clinical diagnostic criteria have low sensitivity in differentiating PD from its atypical forms (Bagchi et al., 2013). Ideally, ligands should have a higher selectivity for insoluble alpha-synuclein fibrils which are characteristically found in synucleopathies than tau and amyloid β 42 which are seen in other atypical parkinsonian syndromes (Bagchi et al., 2013). At present no studies have assessed the utility of ^{19}F probes in imaging alpha-synuclein, therefore further work in this area is warranted.

CHAPTER 8: SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

Hospitalisation in PD

This project provided further confirmation of high rates of hospitalisation in both early and advanced PD. Disease related complications contributed to a large proportion of non-elective admissions.

A systematic review of literature was performed in order to identify models of care which can be adopted in PD to reduce unplanned hospital admissions. Results of the review highlighted a significant gap in literature for effective interventions. A number of inferences were drawn from the results of available retrospective studies. These suggested that frequent specialist consultations which allow earlier detection and treatment of complications, as well timely alteration of medication for controlling PD symptoms may reduce hospital admissions. Although, this aids in identifying individuals who are at risk for hospitalisation, this intervention may not be applicable in countries where individuals pay for their health insurance. Furthermore, cost effectiveness of this intervention needs to be evaluated. Jarman et al (2002) showed that involvement of PD nurses in the management of PD patients in a community setting was cost neutral (Jarman et al., 2002).. In the UK, PD nurses already provide care in both the community and secondary care settings, but their effectiveness on reducing PD hospitalisation has not been systematically assessed. PD nurse specialists run community based clinics and also offer domiciliary/nursing visits for those with limited mobility. Availability and provision of these services vary across the country, therefore the resultant effect on PD hospitalisation, if any, may not be apparent. The majority of the non-elective PD admissions in this study were for infections, falls and fractures and other non-motor symptoms which cannot be easily prevented. Considering these complexities in PD management, assessing the role of PD nurses only, in reducing hospitalisation is unlikely to produce significant results. A multifaceted approach may be more appropriate, where the community PD nurse plays

a central role in identifying those at high risk of admission in the community and then referring to other teams for management in the community. For example physiotherapy for falls management, early referral to the primary care physicians for treatment of urinary tract infections, involving speech and language therapist for those with dysphagia and at risk of aspiration, or other PD specialists for management of other motor or non-motor symptoms. For PD patients who are discharged from hospital, early community review or home visits can potentially reduce re-admission rates. Future RCTs should therefore consider cluster randomisation, where PD patients within the catchment area of a number of hospitals, are assigned to the active arm, where there is access to frequent nurse led community clinics, domiciliary/nursing home visits, early reviews post discharge from hospital, and referral to the multidisciplinary team. The number of hospital admissions and accident and emergency visits in these clusters is then compared with the control group which continues with the current practice. Disadvantages of such a randomised trial is the difficulty in designing the study and blinding participants.

Effect of telemedicine on reducing PD hospital admissions has not been evaluated before. A recent US study demonstrated the feasibility of teleconsultations in patients with PD.(Beck et al., 2017) The study also showed similar quality of life measures in patients who had teleconsultations compared to those who had standard hospital outpatient reviews. (Beck et al., 2017) Considering the lower costs associated with teleconsultations, (Beck et al., 2017) and that previous retrospective studies have shown a trend towards reduced hospital admissions with frequent specialist consultations,(Willis et al., 2012, Ney et al., 2013) future studies should consider assessing the effect of frequent remote consultations on PD hospitalisation.

Patient and carer targeted educational programmes coupled with frequent specialist consultation were shown to be effective in other chronic conditions, (Gibson et al., 2002) and therefore should also be examined in individuals with PD.

Evaluation of the effect of dopaminergic medication on various aspects of hospitalisation was performed and showed no difference in hospital admission rates or length of stay among patients treated with different medications in both early and advanced disease. The prospective and randomised nature of the data used in the analyses allowed for definitive conclusions to be drawn from the results. Although these treatments are efficacious, they do not address the non-motor aspects of the disease, and have no effect on other disease complications such as infections, low mineral bone density, and fractures. Future interventions should involve preventive strategies for disease related complications. Since fracture prevention measures have been shown to reduce hospitalisation in other patients groups, examining the effect of bisphosphonates on hospital admissions rates for fractures in PD patients may be warranted. Considering the complexity of managing PD, it is likely that a preventive approach which addresses motor and non-motor complications in early disease, and community-based management of palliative aspects of the condition in end-stage disease may be effective in reducing hospitalisation.

The third aspect of hospitalisation assessed in the project was the accuracy of recording PD diagnosis when patients are discharged from hospital. Significant data recording errors were identified, which may have a negative impact on healthcare planning decisions and potentially leads to under-resourcing of PD services. In addition to this, hospitals may not be reimbursed correctly for the services they provide. Other chronic conditions are also likely to be under-reported during hospital admissions, where the disease may not be the primary reason for hospitalisation. Recommendations to improve

coding errors may include training coders, use of electronic records, and outpatients clinical coding of these conditions by senior clinicians. This also requires further prospective evaluation.

In terms of improving inpatient care, medication errors in PD can be reduced by involving PD experts during hospital admissions (Hou et al., 2012). The National Service Framework for long-term conditions quality requirements highlight the need for meeting patient's specific needs during hospitalisation and importance of assessment by teams with the appropriate expertise in with chronic neurological illnesses (Department of Health Long-term Conditions NSF Team, 2005). In addition, emphasis is placed on the importance of adherence to PD medication regimes and assisting patients who self-medicate in order to prevent symptom worsening and development of other complications (Department of Health Long-term Conditions NSF Team, 2005). As discussed above, current inpatient PD management falls short of these quality of care standards. Involvement of PD specialists during hospital admission is likely to improve the quality of care of patients as suggested by a previous study (Cheng et al., 2007). In the post-operative period, early consultation by a neurologist was shown to shorten hospital stay and was also shown to improve UPDRS scores (Mehta et al., 2008). Other authors have recommended computerised alert systems which trigger early referral to a PD nurse specialist when a PD patient is admitted (Derry et al., 2010). Training nurses and doctors on management of PD and incorporating important aspects of PD management into hospital guidelines may be beneficial (Magdalinou et al., 2007, Chou et al., 2011). For example, providing information on alternative formulation of PD drugs, contraindicated drugs and potential complications of medication errors. The UK NICE guidelines recommend that PD patients should be allowed to take their own medication, (NICE

guidelines [CG35], 2006) which may also improve adherence to medication doses and timing. Evaluating effects of these measures on inpatient outcome may be informative.

MRI tool for imaging in Parkinson disease

Preliminary experiments in the development of an MRI sensitive probe for use in PD were conducted. Results of these experiments provide a basis for future work. The lanthanide compounds examined so far showed minimal and non-specific cellular accumulation in PC12 cells. The current study also demonstrated poor lanthanide fluorescence signal and lack of consistency in lanthanide cellular uptake between the experiments. In addition results of a previous pilot study could not be replicated. These findings suggests that performing further studies using lanthanide compounds and similar screening methods may be futile.

Several issues which arose during the course of this study should be addressed before conducting further studies. Firstly, fluorescence microscopy may not be sensitive in detecting minimal cellular uptake. Other highly sensitive assay methods such as ICP-MS, which can measure small amounts of metals at single cell level, (Zheng et al., 2015) should also be considered in future cellular uptake experiments. Secondly, if lanthanide compounds are to be considered for future work, complexes with greater luminescence should be developed. Since the structure of a molecule determines its function and influences the selectivity of the compound for a receptor, (Deschamps, 2005) rational drug design should be used in developing complexes which are DAT specific. Methods such as X-ray crystallography can provide complete and accurate information on molecular structure (Deschamps, 2005) and should be considered in designing future lanthanide complexes. This may increase specificity of future lanthanide probes for DAT. Another limitation of using fluorescence microscopy was the long duration of

experiments, which caused difficulties in controlling for variables such as temperature, and cell viability, which can interfere with cellular uptake. This method is therefore not ideal for rapid screening of compounds. Automated methods which allow rapid screening of several compounds under a controlled environment such as FLIPR tetra should be considered. Collaboration with other groups working on similar projects will allow sharing of ideas and expertise which can provide different approaches of tackling some the problems faced in the current study and thus improve the quality of future work.

This project also investigated DAT function in a human dopaminergic cell line (LUHMES cells). Uniform and avid NTUA cellular uptake was observed across all experiments. The observed GBR and desipramine sensitive NTUA uptake in the cell neurite extensions implies that a higher DAT expression in the neurites extensions of these cells. This is consistent with previous work which demonstrated higher DAT expression in dopaminergic neuron terminals in the striatum when compared to the substantia nigra (Hoffman et al., 1998).

NTUA uptake in the cell bodies of differentiated LUHMES cells could not be suppressed by DAT/NET/SERT blockers. It is feasible that the avid NTUA accumulation may have been as a result of a high intracellular VMAT vesicle density, on which the monoamine transporter blockers had no influence. The lack of NTUA uptake inhibition by the monoamine blockers, may also be to do with the high NTUA concentration (1:10) used in this study. Further work using lower NTUA concentration (1:100) should be conducted to verify DAT function in these cells. This will also provide further evidence for utility of this cell line in screening future DAT probes. It is important to note that the initial validation studies for NTUA used cell lines transfected with human DAT and at even higher NTUA concentration (1:1),(Jorgensen et al., 2008) , suggesting different saturation

kinetics between the cell lines. It is also plausible that NTUA uptake by the LUHMES cells was via other amino acid transporters which could not be suppressed by the blockers used in the study. Further characterisation of other transporters present on LUHMES is required, in order to determine if NTUA is an appropriate control for future experiments.

Another goal of this project was to assess the feasibility of using a ^{19}F MRI probe for labelling dopaminergic neurons in PD. Although experiments performed so far showed no detectable ^{19}F NMR signal when cellular uptake of ^{19}F fluoro DL-DOPA and 2, 3, 5, 6-tetrafluorotyrosine was assessed, a ^{19}F signal at mM concentration was detected. The lack of detectable signal in the cellular uptake experiments suggests that the ^{19}F concentration was low. Probes which can accumulate in cells in sufficient amounts to allow detection by NMR spectroscopy should be developed. Synthesising tri- or tetra-fluorinated-DOPA may resolve this problem. If successful, the compounds should be tested in cells before proceeding to tests in animals. No definitive conclusions can be drawn regarding the usefulness of these compounds from the few experiments performed.

Alternatively ^{19}F probes which target other aspects of the disease pathology, for example alpha-synuclein deposition, should be developed. Such probes may be valuable as diagnostic biomarkers. Additionally, since Lewy body pathology spreads with disease progression, (Braak et al., 2002) alpha-synuclein probes may also have utility in monitoring disease progression. Ongoing animal studies have shown promising results from using immunotherapy against alpha-synuclein, (Lee and Lee, 2016) so ^{19}F alpha-synuclein probes may be a useful biomarker in examining the effects of such therapies in future clinical trials. Feasibility of using ^{19}F MRI probes in proteinopathies has already been demonstrated in animal models of Alzheimer's disease which is encouraging (Tooyama et al., 2016). Collaborative work and knowledge gained from these studies

could provide insights into how similar ^{19}F alpha-synuclein probes for imaging in PD can be developed. Evidence for alpha-synuclein imaging in PD using radionuclide probes is also emerging. For example, a recent study funded by the Michael J fox Foundation identified a panel of compounds, some of which showed specificity for Lewy body pathology when tested in human tissue sections (Neal et al., 2013). This is a significant step forward and lessons learnt in the development of these compounds can also be used in designing ^{19}F probes.

APPENDICES

APPENDIX 1: PUBLICATIONS AND POSTERS FROM MD WORK

Publications

Muzerengi S, Herd C, Rick C, Clarke CE. A systematic review of interventions to reduce hospitalisation in Parkinson's disease. *Parkinsonism and Related Disorders* 24 (2016) 3-7.

Muzerengi S, Rick C, Begaj I, Ives N, Evison F, Woolley RL, Clarke CE. Coding accuracy for Parkinson's disease hospital admissions: implications for healthcare planning in the UK. *Public Health* 146 (2017) 4 – 9.

Poster presentations

Muzerengi S, Begaj I, Rick C, Ives N, Clarke CE. Hospitalisation coding errors in Parkinson's disease in a large UK teaching hospital. ***Parkinsonism Relat. Disord*** 2016; 22 Suppl 2:e36. Presented at 1) International Parkinson and Movement disorders conference June 2014, Stockholm, Sweden. 2) Parkinson UK conference, November 2014, York, United Kingdom. 3) Midlands Neurology meeting, November 2014, Leicester, United Kingdom).

Muzerengi S, Tropiano M, Faulkner S, Brain KL. Development of a lanthanide probe for labelling the dopamine transporter. www.pa2online.org/abstracts/1vol12issue3abst225p. Presentation at the British Pharmacological Society Conference, December 2014.

Muzerengi S, Woolley RL, Rick C, Ives N, Dowling F, Gray R, Clarke CE, PD MED collaborative Group. Hospitalisation following different initial therapies in early Parkinson's disease: analysis of Hospital Episodes Statistics from the PD MED EARLY trial. Presented at the Movement Disorders Conference, June 2016, Berlin, Germany.

Muzerengi S, Woolley RL, Rick C, Ives N, Dowling F, Gray R, Clarke CE, PD MED collaborators. Frequency of hospitalisation in early Parkinson's disease using Hospital Episodes Statistics. **Parkinsonism Relat. Disord** 2016; 22 Suppl 2:e36. Presented at the World Congress of Parkinson's disease and Related disorders. December 2015, Milan, Italy.

APPENDIX 2: CONTRIBUTIONS TO MD WORK

Chapter 1: Introduction

Sharon Muzerengi

Chapter 2. A systematic review of interventions to reduce hospitalisation in Parkinson's disease

Sharon Muzerengi: Literature search, study selection, data extraction, data analysis, quality assessment, data interpretation, discussion.

Clare Herd: Search strategy, reviewed search and comments on manuscript.

Caroline Rick: Commented on manuscript.

Carl E Clarke: Project conception, reviewed and commented on manuscript

Chapter 3. Frequency of hospitalisation in early Parkinson's disease using Hospital episodes statistics

Sharon Muzerengi: Cleaning, data interpretation and wrote the chapter

Rebecca L Woolley analyzed the PD MED/HES data.

Professor Richard Gray and Natalie Ives provided statistical support and advice for the project.

Dr Caroline Rick conception of the project and advice on project

Professor Carl E Clarke conception of the project and advice on project

Francis Dowling applied and obtained approval for use of the HES data from the HSCIC.

Contributions to the PD MED trial

PD MED study was supported by funding from the UK National Institute for Health Research Health Technology Assessment Programme (project number 98/03/02). The University of Birmingham Clinical Trials Unit also received support from the UK Department of Health. Richard Gray is funded by the UK Medical Research Council, Emma McIntosh by Parkinson's UK. Alastair Gray is an NIHR Senior Investigator.

I thank the PD MED writing committee, all contributors: data monitoring, steering committees, data management, health economics, and quality of life, and PD MED investigators who contributed to the trial, the patients who agreed to enter the study, and the Dementia and Neurodegenerative Disease (DeNDRoN) Clinical Research Network for their help with recruitment. The PDQ-39 questionnaire was developed by Crispin Jenkinson, Ray Fitzpatrick, and Viv Peto in 1993, who have asserted their moral rights in it, and the copyright, which is owned by Isis Innovation Limited.

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PD MED trial Contributors

RG, CEC, AG, CJ, KW, and AW designed the trial. RG, CEC, KW, AW, NI, SP, and CR ran the trial and CEC and AW recruited patients. NI, and SP analysed the data. RG, CEC, AG, NI, CJ, EM, SP, CR, KW, and AW interpreted the data and wrote the paper. The authors assume responsibility for the accuracy and completeness of the data and for the overall content and integrity of the paper.

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Chapter 4: Under-reporting of Parkinson's disease hospital admissions

Sharon Muzerengi: study design, data analysis and data interpretation and wrote the chapter.

Caroline Rick: Project conception, study design

Irena Begaj: Study design, data extraction and analysis

Natalie Ives: Statistics advisor for the project, performed the capture recapture analysis

Felicity Evison: Data extraction

Rebecca L Woolley: performed the capture recapture analysis

Carl E Clarke: Project conception, study design

Chapter 5: Development of a Magnetic Resonance Imaging (MRI) sensitive probe for imaging in Parkinson's disease

Sharon Muzerengi: Sample preparation, performed all fluorescence microscopy experiments, data interpretation, and wrote the chapter

Manuel Tropiano and Stephen Faulkner: Synthesised the lanthanide compounds

Sarah Newton and Melanie Britton: Performed NMR spectroscopy

Chapter 6: Human dopaminergic cell line (LUHMES) in the developments of an MRI sensitive probe

Sharon Muzerengi

Chapter 7: Development of a ^{19}F Magnetic resonance imaging probe

Sharon Muzerengi: Sample preparation, data interpretation, wrote chapter

Sarah Newton and Melanie Britton: performed NMR spectroscopy

Chapter 8: Conclusion and future direction

Sharon Muzerengi

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