I, Alisdair McNeill confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Date: 01/03/2013
Acknowledgements

Thank you to all the study participants who kindly agreed to participate in the clinical study and undergo skin biopsy. This work would not have been possible without their forebearance and generosity of spirit.

I gratefully acknowledge the help of the Gaucher disease clinical team (Dr Hughes, Prof Mehta, Prof Cox, Dr Deegan and all the specialist nurses) in recruiting study participants.

I would like to extend my thanks to Dr Matthew Gegg for providing me with outstanding teaching on how to perform the laboratory techniques utilised in this thesis.

I acknowledge the supervision of Professor A Schapira and Professor T Warner.

Last, but not least, thanks to my wife Ruth for supporting me.
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List of abbreviations used
ADP: adenosine diphosphate
ATP: adenosine triphosphate
ATF: activating transcription factor
ATP: adenosine triphosphate
CHOP: CCAAT-enhancer-binding protein homologous protein
CI: confidence interval
DAPI: 4',6-diamidino-2-phenylindole
DHE: dihydroethidium
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
dH2O: distilled water
Endo H: endoglycosidase H
ER: endoplasmic reticulum
ERAD: endoplasmic reticulum associated degradation
ERT: enzyme replacement therapy
FCCP: Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FCS: Fetal calf serum
GA: giberalling acid
GBA: glucocerebrosidase gene
GCC: ganglion cell complex thickness
Gcase: glucosylceramidase enzyme
GD: Gaucher disease
GM: monosialodihexosylganglioside
GRP78: Glucose regulatory protein 78 (also known as BiP)
HA: haemagluttinin
HDACi: histone deacetylase inhibitor
HSP: heat shock protein
8-OHG: 8-hydorxy guanyl
IIF: indirect immunofluorescence
IQR: interquartile range
IRE1: inositol requiring enzyme-1
LAMP1: Lysosomal associated membrane protein-1
LIMP2: Lysosomal integral membrane protein-2
4-HNE: 4-hydroxynonenal
NMC: non-manifesting carriers
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PD: Parkinson's disease
PERK: PKR-like endoplasmic reticulum kinase
PSI: proteasome inhibition
MCB: monochlorbamine
MCI: mild cognitive impairment
MMSE: minmental state examination
MOCA: Montreal cognitive assessment
MPS: mucopolysaccharidoses
LBD: Lewy body dementia
LRRK2: Leucin rich repeat kinase-2
LSD: lysosomal storage disorder
NCL: Neuronal ceroid-lipofuscinoses
NMSS: non-motor symptoms scale
OCR: oxygen consumption rate
Optical coherence tomography: OCT
OR: Odds ratio
PINK1: PTEN induced putative kinase-1
PD – GBA: Parkinson's disease with heterozygous GBA mutation
PD – S: Parkinson's disease without GBA or LRRK2 G2019S mutation
RBD: Rapid eye movement sleep behaviour disorder
RNA: ribonucleic acid
RNFL: retinal nerve fiber layer
ROS: reactive oxygen species
SNCA: alpha-synuclein
SRT: substrate reduction therapy
TFEB: transcription factor EB
MDS-UPDRS: Movement Disorders Society Unified Parkinson's Disease Rating Scale
TMT: timed motor tests
UMSARS: unified Multiple Systems Atrophy Rating scale
UPR: unfolded protein response
UPSIT: University of Pennsylvania Smell Identification Test
XBP1: x-box binding protein 1

Abstract

Objectives - Gaucher disease (GD) is caused by bi-allelic mutations in the glucocerebrosidase gene (GBA). GD and heterozygous carriage of GBA mutations significantly increase the risk of developing Parkinson's disease (PD). Here we studied GD patients and carriers to identify a cohort of individuals with clinical signs of prodromal PD and generated fibroblast lines from them to study why GBA mutations cause PD.

Methods - 83 patients with Type I GD and 41 of their heterozygous carrier relatives were recruited from lysosomal storage disorder clinics at the Royal Free Hospital and Addenbrooke's Hospital Cambridge, along with 30 mutation negative matched controls. They were clinically screened for hyposmia (University of Pennsylvania Smell Identification Test), cognitive impairment (Montreal Cognitive Assessment), autonomic dysfunction, REM sleep behaviour disorder and motor signs of PD. Two hundred and thirty cases of sporadic PD were screened for GBA gene mutations. Fibroblasts were generated from skin biopsies taken from a selection of patients. GBA metabolism (Western blotting for protein levels, enzyme activity, immunofluorescent localisation), mitochondrial metabolism, endoplasmic reticulum and oxidative stress markers were assayed in the cell lines.

Results – GD patients and heterozygous carriers had significantly lower olfactory and cognitive function scores than controls. Several GD patients and carriers had motor signs of PD (e.g. rest tremor) while controls did not. Thirteen PD patients with heterozygous GBA mutations were identified. Their clinical phenotype was similar to mutation negative PD cases. GD fibroblast lines (n=5), and lines from heterozygous GBA mutation carriers with (n=4) and without PD (n=2) had reduced GBA enzyme activity due to endoplasmic reticulum retention of GBA protein. This was associated with upregulation of endoplasmic reticulum stress markers and oxidative stress (increased rate of dihydroethidium oxidation).

Conclusions – a subset of GD patients and carriers express clinical markers of prodromal PD. Study of fibroblasts from these individuals indicates that endoplasmic reticulum and oxidative stress may contribute to increased PD risk in these individuals.
Chapter I.

GENERAL INTRODUCTION.
**General Introduction**

Parkinson’s disease (PD) is the second most common neurodegenerative disease in the United Kingdom (U.K), with an age dependent prevalence of 1/150-1/350 (Lees *et al*, 2009). PD is characterised by rest tremor, rigidity, bradykinesia and postural instability. The classical motor syndrome of PD becomes apparent once greater than 50% of dopaminergic neurons in the *substantia nigra pars compacta* have degenerated. Neuronal degeneration in PD is associated with the presence of eosinophilic inclusions in surviving neurons known as Lewy bodies. Lewy bodies are composed of alpha-synuclein (SNCA), a synaptic protein of uncertain function. Before the motor syndrome of PD occurs there is a long prodromal period during which non-motor signs and symptoms of PD predominate (Schapira & Tolosa, 2010). Olfactory dysfunction, autonomic dysfunction, depression and subtle motor slowing are well recognised as clinical markers of the prodromal phase. The aetiology of PD is complex; <5% of cases can be explained by monogenic inheritance (dominant e.g. *SNCA* mutations *LRRK2*, recessive e.g. *Parkin, PINK1, DJ-1* mutations) with 95% of PD thought to be a complex disorder caused by interactions between genetic and environmental factors (Lees *et al*, 2009).

Genetic causes of PD have been identified through studies of dominant and recessive PD families and Genome Wide Association Studies (GWAS) of many thousands of patients. However, the genes identified in these studies account for only a small fraction of PD risk. The recent observation that patients with Gaucher disease (GD), a recessive lysosomal storage disorder caused by mutations in glucocerebrosidase (*GBA*), and their obligate carrier relatives, are at increased risk of PD has provided important new insight into the causes of PD (Sidransky *et al*, 2009). GD patients have a 9-12% chance of developing PD before age 80 (Rosenbloom *et al*, 2011) and between 5-30% of sporadic PD cases have a heterozygous mutation in the *GBA* gene (Sidransky *et al*, 2009). Mutations in *GBA* are therefore the numerically most important risk factor for PD.

Individuals with GD or heterozygous *GBA* mutations represent a group at increased risk of developing PD. By studying them for clinical markers of prodromal PD (e.g. olfaction, cognition, subtle motor signs) it should be possible to identify a cohort of individuals in the earliest stages of PD. Biological samples from these individuals can then form the basis for biomarker studies to identify markers which may help aid early diagnosis of PD. Fibroblast cell lines generated from this cohort will also form the basis of a cell biology study to define the cellular processes causing increased PD risk.
Parkinson’s disease: clinical features

The core features of the movement disorder caused by idiopathic PD are bradykinesia, rigidity, rest tremor and postural instability (Lees et al, 2009). The onset of idiopathic PD is slow and insidious with non-specific symptoms, such as reduced manual dexterity, fatigue or stiffness, being common at onset (Lees et al, 2009). Subtle early motor features will often not be noted by the patient or their relatives, explaining why there can be a delay of several months or even years between onset of symptoms and correct diagnosis of PD. The diagnosis of PD is chiefly clinical, based upon the Queen Square Brain Bank criteria. Bradykinesia refers to slowness of movements with decremental speed and amplitude evident with repetitive movements, and PD can not be diagnosed without the presence of definite bradykinesia. Bradykinesia can also manifest as reduced frequency of blinking and “mask like” facies. Tremor is also a characteristic feature of PD (Lees et al, 2009). Classically the tremor of PD occurs at rest, with a “pill rolling” quality and is coarse (4-6 Hz). However, a faster postural and kinetic tremor, resembling essential tremor can also occur in PD (Lees et al, 2009). The resting tremor of PD can affect the arms, legs, head and jaw/tongue. Rigidity, as assessed by passive movements of the limbs, classically has a “cog wheel” nature due to superimposed rest tremor. PD has a characteristic posture with truncal flexion and festinant gait. Clinical features which help to support a diagnosis of PD include unilateral onset, persistent asymmetry, hyposmia and excellent response to L-DOPA. Clinical features that exclude a diagnosis of PD include a history of repeated head injury, cerebellar signs, early severe autonomic failure and early severe cognitive impairment. In the majority of cases (around 90%) a diagnosis of PD can be made after clinical evaluation.

There are several parkinsonian syndromes which are distinct from idiopathic PD, so called “parkinsons plus syndromes” (Lees et al, 2009). In progressive supranuclear palsy (PSP) parkinsonism is symmetrical, with a tendency to lean backwards accompanied by falls early in the course of the disease. PSP also features a supranuclear gaze palsy and the underlying pathology is a tauopathy (4 repeat tau) not synucleinopathy. Multiple systems atrophy (MSA) presents with atypical symmetrical parkinsonism with prominent cerebellar ataxia or autonomic dysfunction. The pathology of MSA is oligodendroglial alpha-synuclein accumulation and MSA risk is associated with SNCA polymorphisms (Scholz et al, 2009) but not GBA mutations (Segarane et al, 2009). Corticobasal syndrome is a rare syndrome. The classic description of corticobasal syndrome includes clumsiness/ loss of function of one hand due to combinations cortical sensorimotor dysfunction (Ling et al, 2010). On examination ideomotor and limbkinetic apraxia, cortical sensory loss leading to an alien limb, limb dystonia, focal action or stimulus-sensitive myoclonus may be found. Levo-dopa responsive par-
Kinisonism may also occur. A variety of pathologies can present with corticobasal syndrome, including cerebrovascular disease, Alzheimer’s disease and prion disease. Conversely corticobasal syndrome may be mistaken for progressive supranuclear palsy or frontotemporal dementia. Corticobasal syndrome is only diagnosed correctly in life in around 25% of cases, at post-mortem the predominant pathology is 4-repeat tau (Ling et al, 2010). This compares to a 90% diagnostic accuracy for PD when Queens Square brain bank criteria are met. Nonetheless it is usually possible to clinically differentiate between idiopathic PD and “Parkinsons plus syndromes”.
Parkinson’s disease: epidemiology and environmental risk factors

The overall prevalence of PD in industrialised countries is reported to be 0.3% (reviewed in de Lau & Breteler, 2006). Studies in multiple countries have confirmed that PD is a strongly age related condition, being very rare under the age of 50. Between age 60 and 70 prevalence ranges from 0.25-1%, while by age 80 prevalence is 1-4.5 % (reviewed in de Lau & Breteler, 2006). It is unclear whether ethnicity affects prevalence of PD with some studies suggesting that PD is less prevalent in black and Asian groups than whites (van den Eeden et al, 2003; Mayeux et al, 1995). Idiopathic PD is more common in men than women. Given the high prevalence of GBA mutations (1:10-1:18) and LRRK2 mutations in Ashkenazi Jews it might be expected that this population would have a significantly higher prevalence of PD than other populations. However, there are no studies comparing the prevalence of PD in Ashkenazi Jews with other ethnic groups, and further research might usefully address this issue.

Less than 5% of cases of PD are monogenic with the remainder caused by interactions between genetic and environmental risk factors (Lees et al, 2009). Environmental risk factors will be discussed here and genetic factors in a later section. A comprehensive systematic review and meta-analysis of 173 studies investigating environmental risk factors for PD has recently been published (Noyce et al, 2012). Table 1 summarises the environmental factors with a positive and negative association with PD risk. Several postulated environmental risk factors did not show any association with PD in Noyce’s analysis: oral contraceptives, statin use, diabetes, tea, paracetamol, aspirin (Noyce et al, 2012).

Table 1. Environmental risk factors for PD

<table>
<thead>
<tr>
<th>Positive association with PD</th>
<th>Odds Ratio (Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pesticide exposure</td>
<td>OR 1.78 (CI 1.5-2.10)</td>
</tr>
<tr>
<td>Head injury</td>
<td>OR 1.58 (CI 1.3-1.91)</td>
</tr>
<tr>
<td>Rural living</td>
<td>OR 1.43 (CI 1.13-1.81)</td>
</tr>
<tr>
<td>Beta-blockers</td>
<td>OR 1.28 (CI 1.19-1.39)</td>
</tr>
<tr>
<td>Farming</td>
<td>OR 1.26 (CI 1.1 – 1.44)</td>
</tr>
<tr>
<td>Well water</td>
<td>OR 1.21 (CI 1.04-1.40)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Negative association with PD</th>
<th>Odds Ratio (Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td>OR 0.44 (CI 0.39-0.50)</td>
</tr>
<tr>
<td>Coffee consumption</td>
<td>OR 0.67 (CI 0.58-0.76)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>OR 0.74 (CI 0.61-0.90)</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>OR 0.90 (CI 0.82-0.99)</td>
</tr>
<tr>
<td>Alcohol</td>
<td>OR 0.90 (CI 0.84-0.96)</td>
</tr>
</tbody>
</table>

The mechanisms of effect of the above environmental factors are unclear. The protective effect of coffee is stronger in men than women. Caffeine is thought to be the active ingredient, possibly by inhibiting the adenosine A2 receptor, which can protect against dopaminergic neurodegeneration.
Nicotine has been shown to protect against dopaminergic neurodegeneration in animal models by agonising nicotinic cholinergic receptors (Quik et al, 2010). Farm work, well water drinking and pesticide exposure are postulated to be linked to PD by ingestion of compounds which induce neurodegeneration. NSAID exposure may protect against dopaminergic neurodegeneration by its anti-inflammatory effects, since inflammation has been postulated as a pathogenic mechanism in PD. Hypertension, alcohol and NSAID use may protect against PD by their association with elevated urate levels. It should be noted that the Odds Ratios for all of these risk factors is low, and therefore these factors can only explain a small fraction of the aetiology of idiopathic PD.

Elevated serum urate has long been associated with a reduced risk of PD. Jain et al (2011) demonstrated a protective effect for raised serum urate on PD risk in men but not women. O’Reilly et al (2010) confirmed that serum urate does not seem to protect women against PD. Schwarzschild et al (2011) demonstrated that higher serum urate levels reduced the likelihood of a dopaminergic neuronal loss on dopamine transporter imaging in early PD. Higher levels of serum and cerebrospinal fluid urate have also been associated with slower progression of PD (Ascherio et al, 2009). Urate is an antioxidant and has been shown to protect cultured dopaminergic neurons from MPP+ induced cell death (Cipriani et al, 2009).
**Parkinson’s disease: neuropathology**

Neuropathological diagnosis of PD requires loss of dopaminergic neurons in the *substantia nigra pars compacta* and intraneuronal inclusions formed of alpha-synuclein (Lewy bodies) (Dickson *et al.*, 2009). Neurodegeneration is not limited to the *substantia nigra* and neuronal death and dysfunction in other brain areas contributes to the non-motor symptoms which occur in PD and the clinical heterogeneity of PD. Braak and co-workers propose that alpha-synuclein pathology begins in the dorsal motor nucleus of the vagus nerve and olfactory bulb (Braak *et al.*, 2003). According to Braak staging pathology then spreads through the brainstem in a caudal to rostral manner (Braak stage 1-3) before involving cerebral neocortex and limbic system (Braak stage 4-6). In several pathological studies of aged individuals who did not have known neurological disease it has been reported that Lewy body pathology is largely confined to the dorsal motor nucleus of the vagus and olfactory bulb (van Berg *et al.*, 2012). This is so called incidental Lewy body disease. This pathological diagnosis is proposed to be representative of the pre-motor phase of PD. There is limited correlation between Braak stage and clinical stage (van Berg *et al.*, 2003). Braak stage does not correlate with disease duration or Hoehn and Yahr but the extent of cortical Lewy bodies does correlate with cognitive impairment. The Braak stages of PD patients with non-tremor dominant disease were lower than those who had tremor predominant disease (van Berg *et al.*, 2012). This may be related to the higher prevalence of dementia in this subtype.

While Lewy body pathology is clearly of critical importance in PD there is recent evidence that Lewy body deposition may not be the initial step in PD pathogenesis. For example, Milber *et al.* (2012) demonstrate that loss of tyrosine hydroxylase staining and depletion of neurons occurs in the *substantia nigra* in the very early Braak stages (i.e. before Lewy body deposition in the nigrostriatal system). In addition, Lin *et al.* (2012) report an increase in mitochondrial DNA deletions in substantia nigra neurons in brain tissue from idiopathic Lewy body disease with minimal synuclein deposition in the nigra. These studies highlight the contribution of factors such as oxidative stress, which can cause mitochondrial DNA deletions, to early PD pathogenesis.

The pathological feature which best correlates with bradykinesia in PD patients is loss of dopaminergic neurons in the *substantia nigra pars compacta* (reviewed in Lees, 2009). There is also neuronal loss in the raphe nucleus, dorsal motor nucleus of the vagus, nucleus basalis of Meynert and locus coeruleus. Neuronal loss is accompanied by 3 intraneuronal inclusions: the Lewy body, pale body and Lewy neurite. Lewy body and Lewy neurites are composed of
abnormally folded alpha-synuclein. Lewy bodies are classified as cortical or brainstem based upon their morphology. At all clinical stages of PD between 3-4 % of nigral neurons contain Lewy bodies, suggesting that Lewy bodies are being constantly formed and degraded. A small number of cortical Lewy bodies are seen in almost all cases of PD at autopsy, but extensive cortical Lewy body deposition associated with Alzheimer disease type changes are usually only observed in demented PD patients (Dickson et al, 2009).

**Parkinson’s disease pathogenesis: mitochondrial dysfunction**

Several cellular processes have been identified as contributing to neuronal death in PD. The discovery that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a toxin which induces a parkinsonian syndrome (Langston et al, 1983), inhibits complex I of the respiratory chain first stimulated interest in the role of mitochondrial dysfunction in the neuropathogenesis of PD. Multiple studies have since confirmed the importance of mitochondrial dysfunction in PD. Loss of mitochondrial complex I subunits has been demonstrated by immunohistochemistry in substantia nigra from PD patients, and reduced complex I activity demonstrated in PD brain tissue (substantia nigra and frontal cortex) (Schapira et al, 1990), PD platelets (Kringe et al, 1992) and fibroblasts (Grunewald et al, 2010).

Moreover, primary mitochondrial disorders can present with parkinsonism, especially Polymerase-gamma (POLG) mutations (Davidzon et al, 2006). The cause of mitochondrial dysfunction in sporadic PD is unclear. However, studies of substantia nigra from aged brain and PD brain demonstrated clonally expanded mitochondrial DNA (mtDNA) deletions, with associated loss of respiratory chain subunits and mitochondrial dysfunction (Reeve et al, 2008). There is also evidence implicating a role for alpha-synuclein in mitochondrial dysfunction. Mice overexpressing wild type or mutant alpha-synuclein develop mitochondrial fragmentation (Martin et al, 2006). Devi et al (2008) report that alpha-synuclein can interact directly with mitochondria in neuroblastoma cells and impair complex I. There is also evidence that alpha-synuclein can impair mitochondrial fusion thus leading to fragmentation (Kamp et al, 2010). Thus, though the precise mechanism is unclear evidence suggests that alpha-synuclein accumulating in PD can interact with mitochondria and influence their function in a manner which may predispose to neurodegeneration.
Mitochondrial dysfunction has also been reported in several types of monogenetic PD. Fibroblasts from LRRK2 G2019S mutation associated PD demonstrate reduced ATP synthesis and increased mitochondrial proton leak due to altered expression of uncoupling proteins (Papkovskaia et al., 2012). Both fibroblasts carrying bi-allelic ATP13A2 mutations and neuronal cell lines with ATP13A2 knock down demonstrate altered mitochondrial physiology compatible with defective mitophagy, such as increased maximal oxygen consumption rate after treatment with mitochondrial depolariser and altered mitochondrial morphology (Grunewald et al., 2012). It is postulated that loss of ATP13A2 function causes lysosomal dysfunction and accumulation of dysfunctional mitochondria (Grunewald et al., 2012). Mitochondrial dysfunction has also been reported in models of Parkin and PINK1 associated PD. Grunewald et al. (2010) demonstrated reduced ATP synthesis and increased mitochondrial mass in fibroblasts from PD patients with bi-allelic Parkin mutations. Abramov et al. (2012) demonstrate a number of mitochondrial abnormalities in fibroblasts from PINK1 patients including reduced mitochondrial membrane potential with the mitochondria consuming ATP via the F1F0-ATPase in order to maintain mitochondrial membrane potential. Thus mitochondrial dysfunction plays a role in the pathogenesis of both sporadic and monogenetic PD.
Parkinson’s disease neuropathogenesis: oxidative stress

There is much evidence that oxidative stress plays an important role in PD neuropathogenesis (Gandhi and Abramov, 2012). The oxidative stress hypothesis postulates that with ageing there is cumulative exposure to oxidative stress, causing damage to mitochondrial DNA and mitochondrial membranes resulting in further oxidative stress and bioenergetic compromise which contributes to neurodegeneration (Gandhi and Abramov, 2012). Oxidative stress occurs when the ability of the cell’s anti-oxidant defences to buffer the damaging effects of reactive oxygen species (ROS) are overwhelmed. ROS include superoxide (O2-), hydrogen peroxide (H2O2), and the hydroxyl radical (·OH). ROS may damage several different components of the cell; causing protein, DNA or RNA oxidation or lipid peroxidation (Beal, 2002). The oxidation products of polyunsaturated fatty acids, especially arachidonic acid and docosahexanoic acid which are found at high levels in the brain, are malondialdehyde and 4-hydroxynonenal. ROS attacks protein, oxidising both the backbone and the side chain. This in turn reacts with amino acid side chains to form carbonyl groups, which can be detected with special Western blotting techniques (discussed in Chapter 4). ROS attack nucleic acids in a number of ways: causing DNA-protein crosslinks, breaks in the DNA strand, and modifying purine and pyridine bases resulting in DNA mutations. In general, ROS are produced from a number of sources in the cell: monoamine oxidase, mitochondrial complex I and III, NADPH oxidase and xanthine oxidase (Gandhi and Abramov, 2012). There are a number of cellular antioxidant defence systems. These are described in detail in the introduction to Chapter 4.

Several post-mortem studies of PD brain have demonstrated accumulation of products of oxidative damage to membranes (reduced polyunsaturated fatty acid levels, elevated 4-hydroxy nonenal) (Cheng et al, 2011), proteins (protein carbonyls) and DNA (8-hydroxy guanine)(Alzam et al, 1997) in the neurons of the substantia nigra along with loss of the antioxidant reduced glutathione. A high prevalence of mitochondrial DNA deletions in substantia nigra neurons has also been described – which is thought to be due to oxidative damage (Bender et al, 2006). Elevated levels of oxidative stress markers have also been found in blood (8-OHG) and cerebrospinal fluid (4-HNE, 8-OHG) samples from PD patients (Abe et al, 2003). There are several possible sources for ROS production in PD. Complex I inhibition with MPTP or rotenone is associated with increased superoxide radical formation (Smith and Bennett, 1997). Mutations in a number of PD genes cause mitochondrial dysfunction associated with oxidative stress. The cellular pathophysiology of these genes is discussed below in the section “Parkinson’s disease: neurogenetics”. Alpha-synuclein can also interact with mitochondria and induce oxidative stress in some models. Increased ROS
production by NADPH oxidase occurs in microglia in rotenone and MPTP treatment models (Gao et al, 2003), while PINK1 deficiency results in NADPH activation and ROS production (Gandhi et al, 2009). Dopaminergic neurons are also exposed to additional oxidative stress produced by the metabolism of dopamine by monoamine oxidase (which generates hydrogen peroxide) and the auto-oxidation of dopamine (which generates superoxide). This may partly explain the selective vulnerability of dopaminergic substantia nigra neurons. In turn Oxidation of alpha-synuclein contributes to its aggregation and Lewy body formation. Thus there is much evidence that oxidative stress is a key mediator of neuronal injury and death in PD.

Glutathione is a thiol which plays a vital role in antioxidant defence, especially in the brain (Johnson et al, 2012). Glutathione is synthesised intracellularly with a typical intracellular concentration of 0.2-10 mM. Glutathione synthesis begins with gamma-glutamylcysteine synthetase forming an amide bond between glutamic acid and cysteine, before glutathione synthetase adds glycine to complete synthesis of glutathione. Astrocytes have higher levels of glutathione than neurons, and can secrete glutathione to serve as a source of glutathione precursors for neuronal uptake (Gegg et al, 2005). Inside cells reduced glutathione serves as an antioxidant; acting as a substrate for antioxidant enzymes, inactivating electrophilic species, and restoring reduced cysteine-thiol moieties on proteins, with the concomitant production of oxidized GSSG. Oxidised glutathione is recycled to reduced glutathione by glutathione reductase with NADPH as a co-factor (Johnson et al, 2012).

Multiple studies have demonstrated depletion of reduced glutathione in the substantia nigra of PD patients (Perry et al, 1982; Sian et al, 1994). Moreover, reduced glutathione levels are also depleted in the substantia nigra of patients with incidental Lewy body disease; suggesting that this might be an early event in PD pathogenesis (Dexter et al, 1994). There was no significant alteration of mitochondrial respiratory chain activity in these samples (Dexter et al, 1994). This suggests that oxidative stress causes glutathione depletion in PD brain, preceeding any mitochondrial dysfunction in sporadic PD (Dexter et al, 1994). Recent evidence suggests that glutathione depletion alone does not cause mitochondrial dysfunction (Heales et al, 2011), but it is probable that loss of antioxidant capacity will render mitochondria vulnerable to ROS mediated damage (Heales and Bolanos, 2002).
**Parkinson’s disease neuropathogenesis: dysfunctional autophagy and ubiquitin-proteasome system**

Impaired turnover of alpha-synuclein is of key importance in PD pathogenesis (Schapira and Tolosa, 2012). Intra-cellular proteins are disposed of by 2 cellular pathways: the ubiquitin-proteosome system (UPS) and autophagy-lysosome pathway (ALP)(Benbrook et al, 2012). There is evidence that alpha-synuclein can be degraded by both of these with a complex interplay of physiological and pathological factors determining which is involved.

The proteasome is a cytoplasmic organelle which degrades misfolded or unwanted proteins into short peptides of around 8 amino acids which can be recycled for protein synthesis (Benbrook et al, 2012). Proteins are tagged for degradation by the proteasome by attachment of ubiquitin molecules by the ubiquitin ligase system. In addition to disposing of misfolded proteins the proteasome regulates the cell cycle and gene expression by degrading proteins such as transcription factors. The proteasome is a barrel shaped structure composed of 20S subunits and 19S regulatory subunits. The 19S regulatory subunit functions by restricting access of proteins to the enzymatically active site in the 20S subunit (Benbrook et al, 2012). Under conditions of cellular stress the expression of certain Heat Shock Proteins is upregulated and these target misfolded proteins to the proteasome for degradation.

Autophagy consists of 3 distinct pathways: macroautophagy, microautophagy and chaperone mediated autophagy (Hetz, 2012; Benbrook et al, 2012). Macroautophagy involves formation of double-membraned autophagosomes, which fuse with lysosomes to deliver cytoplasmic contents (e.g. misfolded proteins). Microautophagy involves lysosomal pinocytosis of cytoplasmic contents and is involved in the turnover of long half-life cytosolic proteins. Chaperone-mediated autophagy is dependent on the heat shock cognate 70 (hsc70) protein and its binding to lysosomal-associated membrane protein 2A (LAMP2A), a lysosomal surface receptor (Alvarex et al, 2010). A highly specific subset of proteins containing the KFREQ motif are recognized by the hsc70 chaperone and internalized for degradation by LAMP2A lysosomal membrane receptors. A further form of autophagy is mitophagy, which involves delivery of damage mitochondria to the lysosome for degradation.
Rott et al (2011) describe how alpha-synuclein can be targeted to either the UPS or lysosome (autophagy) for degradation. Alpha-synuclein is monoubiquitinated by the enzyme SIAH. This targets alpha-synuclein to the UPS for degradation. The enzyme USP9X removes ubiquitin from alpha-synuclein and therefore directs it to the lysosome/macroautophagy for catabolism. Knock down of USP9X leads to accumulation of monoubiquitinated alpha-synuclein. In brain tissue from sporadic PD Rott et al (2011) demonstrated reduction in USP9X levels and activity and accumulation of USP9X in Lewy bodies (where it likely to have reduced activity due to the nature of the aggregated alpha-synuclein). Thus USP9X inhibition, possibly related to age related changes, may predispose to reduced ubiquitination and degradation of alpha-synuclein and Lewy body accumulation.

Ebrahimi-Fakhari et al (2011) used an in vivo mouse model (which involved applying UPS and autophagy inhibitors to mouse cortex through a cranial window) to demonstrate that the UPS degrades alpha-synuclein under conditions of normal, physiological turnover but that the autophagy-lysosome pathway is the chief route for degradation in pathological conditions. They suggest that age related accumulation of alpha-synuclein in alpha-synuclein transgenic mice treated with proteasome inhibitor demonstrates that age related loss of UPS activity will predispose to alpha-synuclein accumulation. Moreover, aged alpha-synuclein transgenic mice have significantly reduced UPS activity suggesting that alpha-synuclein accumulation can inhibit the UPS. They report that the autophagy-lysosome system is recruited to degrade alpha-synuclein only under pathological conditions of pre-existing elevated alpha-synuclein levels. For example, autophagy inhibitors lead to elevated alpha-synuclein levels only in mice transgenic for alpha-synuclein (which have high basal levels of alpha-synuclein) and not in wild type controls. They also demonstrated that in alpha-synuclein transgenic mice inhibition of the UPS lead to upregulation of autophagy-lysosome activity and vice versa.

In addition to defects of macroautophagy, there is evidence that chaperone mediated autophagy is defective in sporadic PD. Levels of HSC70 and LAMP2 are reduced in the substantia nigra of PD brain; suggesting a mechanism which could lead to impaired chaperone mediated autophagy (Alvarez et al, 2010). Knock down of LAMP2a resulted in increased half life of alpha-synuclein and reduced chaperone mediated autophagy activity (Alvarez et al, 2010).

Mutations in several genes associated with monogenetic PD result in disruption of the lysosome-autophagy pathway. Bi-allelic mutations in ATP13A2 cause Kufor-Rakeb syndrome (discussed
below). Dehay et al (2012) show that in fibroblasts from Kufer-Rakeb syndrome patients there is impairment of lysosomal acidification, decreased proteolytic processing of lysosomal enzymes and reduced degradation of lysosomal substrates. They also demonstrated reductions in ATP13A2 protein levels in substantia nigra from sporadic PD brain; suggesting this protein may have a wider role in PD pathogenesis than simply as part of Kufer-Rakeb syndrome. Usenovic et al (2012) demonstrated accumulation of alpha-synuclein in cortical neurons from ATP13A2 null mice and showed that silencing of alpha-synuclein in these cells ameliorated the toxicity associated with ATP13A2 loss of function. Grunewald et al (2012) demonstrated deficient mitophagy in fibroblasts from Kufer Rakeb syndrome patients providing another mechanism by which lysosomal dysfunction can lead to dopaminergic cell loss. Several studies have implicated dysfunctional autophagy as a mechanism by which LRRK2 mutations cause PD. Increased basal autophagy levels have been demonstrated in G2019S LRRK2 mutation carrying fibroblasts, possibly relating to phosphorylation of MAPK/ERK kinases by LRRK2 (Bravo-San Pedro et al, 2012). Gomez-Suaga et al (2011) showed that overexpression of LRRK2 in HEK293T cells causes increased autophagy induction through calcium dependent activation of a CaMKK/adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway. Pink1 and Parkin have been shown to play specific role in mitophagy, as discussed further in other parts of this chapter. Mutations in GBA clearly will have an impact on lysosomal function, though the relevance to PD is still being elucidated, and the biochemical effects of GBA mutation are discussed in more detail below.
Parkinson’s disease: neurogenetics

The genetic basis of PD has been elucidated by positional cloning studies of kindreds with dominant and recessive PD alongside Genome Wide Association Studies (GWAS) of thousands of patients with sporadic PD. The first PD associated gene identified was alpha-synuclein (SNCA), cloned in a large dominant PD kindred from Southern Italy (Polymeropoulos et al, 1997). The mutation in the initial report (A53T) was found on a common haplotype in families from Italy, Greece and North America who all had Mediterranean ancestry. Two other SNCA point mutations have been identified: A30P in a German family (Kruger R et al, 1998) and E46K in Spain (Zaranz et al, 2004). The importance of SNCA in PD pathogenesis has been confirmed by the discovery that duplications and triplications of SNCA cause dominant PD (Ibanez et al, 2004). PD caused by SNCA point mutations and triplications has a very similar phenotype to sporadic late onset L-DOPA responsive PD, but with earlier onset (mean 12 years earlier) and possibly more severe cognitive impairment. Pathologically SNCA mutation/duplication associated PD is similar to sporadic PD with loss of nigral neurons and LB accumulation, but with more frequent tau pathology than typical sporadic PD (Ikeuchi et al, 2008). SNCA is the major component of the Lewy body and it is postulated that SNCA point mutations/copy number variations facilitate aggregation into Lewy bodies.

The second dominant PD gene was identified at the PARK8 locus – LRRK2 (Gilks WP et al, 2005; Di Fonzo A et al, 2005). LRRK2 is a large 51 exon protein with over 20 sequence changes identified in PD families, only six of which (R1441C, R1441G, R1441H, Y1699C, G2019S, I2020T) are proven to be pathogenic (Zimprich A et al, 2004). The G2019S mutation has been identified in up to 7% of dominant PD families and 1-2% of sporadic PD in Caucasian populations. The prevalence of G2019S associated familial and sporadic PD can be 30-40% in some ethnic groups such as Ashkenazi jews and North African Berber Arabs. Europe wide 1.5% of sporadic cases and 4% of familial cases have the LRRK2 G2019S mutation. A large study of over 300 LRRK2 mutation positive PD cases demonstrated that the phenotype was very similar to mutation negative PD, but with a wide range of age of onset from mid-20s through to 90s (Healy DG et al, 2008). The majority of pathological studies of LRRK2 associated PD have demonstrated typical Lewy body pathology, but also rare cases with pure nigral degeneration or progressive supranuclear palsy like tau pathology (Zimprich A et al, 2004).
Vacuolar sorting protein 35 (VPS35) has recently been identified as another cause of dominant PD. Vilarino-Guell et al (2011) identified the p.Asp620Asn VPS35 mutation in a Swiss family with dominant PD. Zimprich et al (2011) identified the same mutation in an Austrian dominant PD family and a second mutation p.Arg524Trp in a sporadic PD case. These mutations were associated with typical late onset, dopa responsive PD. Sharma et al (2012) performed a large multi-centre study of VPS35 in PD. Fifteen thousand PD patients from around the world were genotyped for VPS35 mutations; p.Asp620Asn was detected in 5 familial and 2 sporadic PD cases and not in healthy controls, p.Leu774Met in 6 cases and 1 control and p.Gly51Ser in 3 cases and 2 controls. The p.Leu774Met and p.Gly51Ser mutations did not occur more frequently in PD than controls. This study confirms VPS35 mutations as a cause of familial and sporadic PD, albeit a rare one. The clinical characteristics of the cases were reported as being characteristic of idiopathic PD. The role of VPS35 in PD is unclear, but it is proposed to play a role in recycling membrane proteins from the endosome to the golgi.

In 2012 Chartier-Harlin et al identified mutations in eukaryotic translation initiation factor 4-gamma (EIF4G1) as a cause of dominant PD in a large French family. They also identified 9 PD patients from amongst 4 000 sporadic PD cases screened. Clinical features were very similar to sporadic PD (late onset, dopa-responsive, asymmetric onset, typical motor features of PD) and 2 cases that came to autopsy had Lewy body pathology. Mutant EIF4G1 protein acts in a dominant negative manner, binding to wild type protein and disrupting the transcription initiation complex (Chartier-Harlin et al, 2012) being associated with increased cellular vulnerability to oxidative stress. It is clear that EIF4G1 mutations are a rare cause of sporadic PD. In a series of Asian PD patients the frequency of EIF4G1 sequence variants was not different to that of controls (Zhao et al, 2012). Lesage et al (2012) screened 251 dominant PD cases from France; they could only confirm segregation of EIF4G1 mutations with PD in 1 family and concluded that this gene was not a common cause of dominant PD in France. Tucci et al (2012) did not identify any EIF4G1 pathogenic mutations in dominant PD and point out the large number of benign polymorphisms in this gene suggesting that one of the originally reported mutations (p.A502V) may be a polymorphism as it was only found in a control. EIF4G1 mutations have been reported in pathologically confirmed Lewy body dementia, but it is not clear if this report represents coincidence and larger studies are required to examine whether or not mutations in this gene predispose to Lewy body dementia (Fujioka et al, 2012). At present it is not clear if EIF4G1
sequence variants are benign polymorphisms co-incidentally associated with PD and Lewy body dementia or if they are disease causing mutations which are very rare.

Three main genes have been identified as causing autosomal recessive Early Onset Parkinson’s Disease (EOPD); Parkin, PINK1 and DJ-1. Several much rarer causes of EOPD syndromes are also known such as PLA2G6, FBX07, ATP13A2, SLC30A10 and spatacsin. Unlike the dominant causes of familial PD recessive EOPD presents with atypical parkinsonism. The frequency of Parkin, PINK1 and DJ-1 mutations in EOPD varies widely depending on the study. For example Lucking et al report Parkin mutations cause up to 50% of EOPD cases and 5-10 % of sporadic PD cases with age of onset before 50 years while other studies reported frequencies as low as 1.6% (Brooks et al, 2009). A recent study comprehensively assessed mutation frequency in a UK EOPD population and systematically reviewed reported mutation frequencies (Kilarski et al, 2012). In 136 UK EOPD cases 3.7% had compound heterozygous Parkin mutations, there was 1 PINK1 patient with bi-allelic mutations (Filipino) and no DJ-1 mutations. They identified 1 EOPD case with the LRRK2 G2019S mutation. In the systematic review encompassing 3952 unique published cases 8.6% of EOPD cases had Parkin mutations (44% homozygous, 56% compound heterozygous). Mutation of PINK1 was identified in 3.7% of 2324 reported EOPD cases, 80% of PINK1 mutations were homozygous and PINK1 mutations were much more common in Asian patients. Just 0.4% of 1351 EOPD cases reported had bi-allelic DJ-1 mutations.

The first locus for recessive EOPD was identified on chromosome 6 in 13 Japanese families, with several homozygous deletions in the Parkin gene then found (Hattori et al, 1998). Subsequently a spectrum of mutations (including nonsense, missense and rearrangements) was identified, confirming that Parkin is the most common cause of EOPD (Abbas et al, 1999). EOPD due to Parkin mutations has a mean age of onset of 32 years, is L-DOPA responsive but with severe early fluctuations in response and slow progression. Symmetry at onset and early lower limb dystonia distinguish this form of PD from sporadic PD. Moreover, non-motor symptoms in Parkin associated PD are much less severe than sporadic PD and olfaction is normal (Kagi et al, 2010). The neuropathology of Parkin associated PD differs from sporadic PD in that there are often no LB, and a pure nigral degeneration (Sasaki et al, 2004). Parkin is thought to function as a ubiquitin ligase, and loss of its function may result in the accumulation of aberrantly folded proteins and damaged mitochondria (Shimura et al, 2000).
A second, less common gene for EOPD is *Pink1* (Valente *et al*, 2004). Samaranch *et al* (2010) provide a detailed clinic-pathological description of EOPD in a Spanish family with compound heterozygous *PINK1* mutations. They report 6 affected individuals, most of whom had an asymmetric onset of a parkinsonian syndrome dominated by bradykinesia-rigidity with only 2 having rest tremor. Atypical features included anxiety, psychosis and hyporeflexia. Lower limb incoordination and falls were prominent early features. Other studies also report a high frequency of neuropsychiatric disorder in *PINK1* EOPD (Kasten *et al*, 2010). Histopathology demonstrated loss of neurons in the *substantia nigra*, and Lewy bodies in the substantia nigra and nucleus basalis of Meynert. The lack of pathology in the locus coereleus is atypical however. The authors suggest that sparing of this structure could account for the slow progression of *PINK1* EOPD (Samaranch *et al*, 2010).

In 2003, Bonifati and colleagues carried out homozygosity mapping and positional cloning to identify a homozygous deletion in PARK7 causing early onset PD in a Dutch family and a missense mutation in PARK7 causing disease in an Italian EOPD family (Bonifati *et al*, 2003). Patients have an age of onset in their 20s or 30s with Levodopa-responsive parkinsonism. Atypical features of dystonia and neuropsychiatric manifestations have been reported. Functional imaging of the brain, showed significant evidence for a presynaptic dopamine deficit. Neuropathological descriptions of EOPD patients with bi-allelic *DJ-1* mutations have not been reported.

Bi-allelic mutations in *PLA2G6*, *ATP13A2*, *FBX07* and *spatacsin* cause complex dystonia-parkinsonism syndromes which do not resemble sporadic PD (Paisan-Ruiz *et al*, 2010). *PLA2G6* bi-allelic mutations cause L-dopa responsive dystonia-parkinsonism with an onset age ranging from 10 to 26 (Yoshino *et al*, 2010; Lu *et al*, 2012). The main clinical features are akinesia and rigidity, generalized dystonia and cognitive impairment. These patients generally have no evidence of brain iron accumulation despite having identical mutations to that which cause infantile neuronal axonal dystrophy (INAD). Bi-allelic *ATP13A2* mutations cause a pyramidal-parkinsonian syndrome with facio-facial-finger minmyoclonus and cognitive impairment with some cases having increased brain iron on MRI (Kufor-Rakeb syndrome)(Ramirez *et al*, 2006). *FBX07* bi-allelic mutations also cause a pyramidal-parkinsonian syndrome (Di Fonzo *et al*, 2009). *SPG11* (spatacsin) causes a spastic paraparesis with thinning of the corpus callosum and a small minority of such patients also develop parkinsonism (Paisan-Ruiz *et al*, 2010). A further recently identified cause of dystonia-parkinsonism is *SLC30A10* mutations, which cause dystonia-parkinsonism, manganese accumulation
in the basal ganglia and liver disease (Quadrie et al, 2012). This condition is potentially treatable with chelation using disodium calcium edetate.

These rare recessive genes seem to play little role or no in sporadic PD/EOPD. For example in a Taiwanese cohort of EOPD no homozygous \( ATP13A2 \) variants were found (Chen et al, 2011). Other groups have also failed to find an association between EOPD and \( ATP13A2 \) variants (Dos Santos et al, 2010; Fei et al, 2010). Di Fonzo et al (2007) found a single EOPD case with bi-allelic \( ATP13A2 \) mutations and a relatively mild phenotype, ie not classical Kufor Rakeb syndrome. Data on an association between \( PLA2G6 \) polymorphisms and PD is inconsistent. Lv et al (2012) could demonstrate no association between 4 \( PLA2G6 \) SNPS and PD. Lu et al (2012) found that bi-allelic \( PLA2G6 \) mutations were the second most common cause of EOPD in Taiwan after Parkin mutations, but did not find any association of \( PLA2G6 \) polymorphisms with sporadic PD. However these EOPD cases were highly selected and had atypical PD phenotypes. Again in a highly selected series of Japanese EOPD cases Yoshino et al (2010) confirm that bi-allelic \( PLA2G6 \) mutations can cause a complicated EOPD with dopa responsive dystonia-parkinsonism, dementia and frontal lobe atrophy with or without evidence of brain iron accumulation on MRI. These genes clearly are rare causes of EOPD and likely make little, if any, contribution to classic late onset sporadic PD. The reported phenotypes for these genes are clearly distinct from sporadic PD and so it would not be expected that a significant proportion of sporadic PD cases would have bi-allelic mutations.
Lysosomes and lysosomal storage disorders: general introduction

Lysosomes are small membrane bound organelles found in the cytoplasm with an acidic internal pH (Cox and Cachón-González, 2012). Lysosomes contain 50-60 soluble enzymes and at least 7 integral membrane proteins. Lysosomal storage disorders (LSD) are caused by recessive mutations in lysosomal genes (apart from Fabry disease, Hunter disease and Danon disease which are X-linked), leading to storage of undegraded substrate. LSD are classified based upon the type of substrate accumulating. LSD are also classified as infantile, juvenile and adult onset.

Lysosomes have a primary role in degradation of substrate and damaged organelles and in the autophagy pathway (Hetz, 2012). The lysosomal enzymes act in concert to degrade a range of macromolecules which may be lipid, polysaccharide, protein or nucleic acid in nature. These enzymes act in sequence, so if one is deficient then there is an interruption to the catabolic process and substrate accumulation occurs (Cox and Cachón-González, 2012). As such lysosomes also play a key role in autophagy since they digest the contents of autophagosomes delivered to lysosomal lumens by autophagosomes (Hetz, 2012). Lysosomes also play a role in apoptosis, with breach of the lysosomal membrane resulting in release of cathepsin proteases which trigger apoptotic pathways (Hetz, 2012).

Though LSD are individually rare, between 1:5 000 and 1:7 500 live births are affected by an LSD (Cox and Cachón-González, 2012). The most common LSD is GD and the epidemiology of GD is discussed later in this introduction. A summary of the main LSD is presented in the table below. LSD can be grouped according to the material which accumulates. The mucopolysaccharidoses (MPS) include Hurler-Scheie syndrome (Type I) and Hunter syndrome (Type II), the main sphingolipidoses are Tay-Sachs, GD and Fabry disease and the glycoproteinoses include alpha-mannosidosis. Other LSD feature accumulation of diverse materials, for example some of the NCL accumulate subunit C of the mitochondrial ATP synthetase while cathepsins accumulate in others.
Table 2. Summary of human lysosomal storage disorders.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Enzyme</th>
<th>Storage material</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD</td>
<td>Glucosylceramidase</td>
<td>glucosylceramide</td>
</tr>
<tr>
<td>GM1-gangliosidosis</td>
<td>Beta-galactosidase</td>
<td>GM1</td>
</tr>
<tr>
<td>GM2-gangliosidosis</td>
<td>Beta-hexosaminidase</td>
<td>GM2</td>
</tr>
<tr>
<td></td>
<td>A (Tay-Sachs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B (Sandhoff disease)</td>
<td></td>
</tr>
<tr>
<td>MPS II (Hunter)</td>
<td>Iduronate-2-sulfatase</td>
<td>Dermatan/heparan sulphate</td>
</tr>
<tr>
<td>MPS IIIA</td>
<td>Sulfaminidase</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>MPS IIIIB</td>
<td>N-acetyl-alpha-glucosaminidase</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>Morquio A</td>
<td>N-acetylgalactosamine-6-sulphate-sulphatase</td>
<td>Keratan sulphate, chondroitin-6-sulphate</td>
</tr>
<tr>
<td>Morquio B</td>
<td>Beta-galactosidase</td>
<td>Keratan sulphate</td>
</tr>
<tr>
<td>Niemann-Pick type C</td>
<td>NPC 1 and 2</td>
<td>Cholesterol and sphingolipids</td>
</tr>
<tr>
<td>NCL1</td>
<td>Protein palmitoyl thioesterase1</td>
<td>Lipidated thioesters</td>
</tr>
<tr>
<td>NCL2</td>
<td>Tripeptidyl amino peptidase1</td>
<td>Subunit C of mitochondrial ATP synthetase</td>
</tr>
<tr>
<td>NCL3</td>
<td>CLN3 protein</td>
<td>Subunit C of mitochondrial ATP synthetase</td>
</tr>
<tr>
<td>NCL4B</td>
<td>Not known</td>
<td>Subunit C of mitochondrial ATP synthetase</td>
</tr>
<tr>
<td>NCL10</td>
<td>Cathepsin D</td>
<td>Saposin A and D</td>
</tr>
<tr>
<td>Metachromatic leukodystrophy</td>
<td>Arysulfatase A</td>
<td>Sulphated glycolipids</td>
</tr>
<tr>
<td>Beta-galactosialidosis</td>
<td>Cathepsin A</td>
<td>Sialyl-oligosaccharides</td>
</tr>
<tr>
<td>Multiple sulfatase deficiency</td>
<td>Formylglycine-generating enzyme</td>
<td>Sulfatides</td>
</tr>
<tr>
<td>Niemann-Pick type B</td>
<td>Sphingomyelinase</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>Fabry disease</td>
<td>Alpha-galactosidase A</td>
<td>Gb3</td>
</tr>
</tbody>
</table>
Parkinsonism in lysosomal storage disorders other than GD

The strongest association between a LSD and PD is that of Gaucher disease (GD), and heterozygous GBA mutations, and PD. This is discussed later on in this general introduction. The association between other LSD and PD is weaker. This may reflect a truly weak association or may be an artefact due to the rarity of these conditions and difficulty of systematic study of sufficient numbers of cases to define an association. Shachar et al (2011) review reports of PD in LSD. Of 48 cases of adult onset GM1-gangliosidosis reported almost half had signs of akinetic-rigid parkinsonism (Roze et al, 2005; Muthane et al, 2004; Yoshida et al, 1992). In 1 case this was reported as being levo-dopa responsive (Shachar et al, 2011). A few case reports of parkinsonism in GM2-gangliosidosis exist (Tay-Sachs and Sandhoff diseases)(Inzelberg and Korczyn, 1994) and a post-mortem on a 67 year old woman with suspected adult onset GM2-gangliosidosis demonstrated Lewy bodies in the substantia nigra (Suzuki et al, 2007). Murine models of GM2-gangliosidosis demonstrate neuronal Lewy body accumulation (Suzuki et al, 2003).

Niemann-Pick C disease (NPC) has been associate with Lewy body deposition in the substantia nigra in human brain (Shachar et al, 2011). Interestingly a single case of atypical PD has been reported with a heterozygous NPC mutation (Josephs et al, 2004). Parkinsonian manifestations are reported in patients with a variety of types of Neuronal Ceroid-lipofuscinoses (NCL)(Aberg et al, 2001). In both juvenile and adult onset NCLs levo-DOPA responsiveness has been reported and loss of uptake on dopamine transporter uptake described (Aberg et al, 2000). Significant loss of neurons in the substantia nigra was reported in an adult case of NCL4 (Shachar et al, 2011).

There is no clear link between metachromatic leukodystrophy but arylsulfatase-A (the causative enzyme for this disease) has been reported to have reduced activity in blood in a series of atypical PD cases (Martinelli et al, 1994). Mucopolysaccharidoses (MPS) are a group of LSD associated with glycosaminoglycan accumulation. There are no clinical reports of convincing parkinsonism in MPS but brain tissue from MPSII had loss of nigral neurons and increased phosphorylation of SNCA (Hamano et al, 2008). Two cases of Fabry disease with parkinsonism have been reported, but these were associated with extensive subcortical infarctions and may represent vascular PD rather than a lysosomal aetiology for PD in this LSD (Shachar et al, 2011). The mechanisms linking these diverse LSD and increased PD risk remain to be completely elucidated, but may relate to increased propensity to accumulate alpha-synuclein in neurons.
**Gaucher disease: epidemiology**

Gaucher disease (GD) is an autosomal recessive lysosomal storage disorder caused by homozygous or compound heterozygous mutations in the glucocerebrosidase gene (GBA) (reviewed in Hughes and Pastores, 2010). GD is most common amongst Ashkenazi jews, with a carrier frequency of 1/10-1/14 and a disease prevalence of up to 1:855. Prevalence is much lower in other ethnic groups, for example in the Netherlands prevalence is around 1:100,000. Over 300 GBA mutations associated with GD have been reported, these are discussed in more detail in the introduction to Chapter 3 (Hruska et al., 2008). However, 4 mutations (N370S, L444P, 84GG and IVS2+1) account for 90% of GBA mutations in Ashkenzi Jews and 60% of mutations in non-Jewish GD patients. The most common genotype causing GD is N370S either in homozygous or compound heterozygous form (Duran et al., 2012). Given the large number of rare mutations it is common that the N370S mutation is identified with the second mutation not being found. There is a founder effect for mutation N370S in Ashkenzi Jews, D409H in Eastern Europeans and L444P in Swedish nationals. Presentations of GD form a spectrum from a perinatal lethal variant through to asymptomatic disease, and the link between genotype and phenotype will be discussed in the introduction to Chapter 3. Within this spectrum 3 major subtypes are recognised Type I, classically without primary CNS disease, and Types II and III, with primary CNS disease. An additional rare form is cardiovascular GD which presents with oculomotor apraxia and calcification of mitral and aortic valves in the presence of homozygous D409H mutations (George et al., 2001).
Gaucher disease: phenotype of Type I Gaucher disease (non-neuronopathic)

Type I GD is characterised by bone disease, with between 70-100% of patients affected by clinical or radiological bone disease (Hughes and Pastores, 2010). Bone disease ranges from asymptomatic osteopenia, to osteoporosis with pathological fractures and “bone crises” were there is deep bone pain with fever and leukocytosis (Khan et al, 2012). Bone marrow infiltration with Gaucher cells can be extensive, leading to marrow failure. Hepatosplenomegaly occurs due to Gaucher cell infiltration, splenomegaly can cause pancytopenia but liver involvement does not cause cirrhosis (Hughes and Pastores, 2013). Pulmonary hypertension or interstitial lung disease are rare complications of Type I GD. There is an increased incidence of peripheral neuropathy in Type I GD (Biegstraaten et al, 2010) and, until the association of GD with PD was discovered, Type I GD was not thought to involve the CNS.

The association between Type I GD and cancer remains to be completely elucidated. Taddei et al (2009) studied 403 Type I GD patients retrospectively and report a relative risk of 25 (95% CI 9.17-54.40) for multiple myeloma, a relative risk of 3.45 (95% CI 1.49-6.79) for other haematological malignancy and increased overall cancer risk (relative risk 1.80, 95% CI 1.32-2.40). Landgren (2007) describe an increased risk of non-Hodgkin lymphoma (RR, 2.54; 95% CI, 1.32-4.88), malignant melanoma (RR, 3.07; 95% CI, 1.28-7.38), and pancreatic cancer (RR, 2.37; 95% CI, 1.13-4.98) in 1500 Type I GD patients but no significant association with other solid tumours. De Fost et al (2006) describe increased risk of haematological malignancy in Western European GD patients (relative risk 12.7 [95% CI 2.6-37.0]), they also found an increased risk of hepatocellular carcinoma in the absence of cirrhosis (relative risk 141.3 [95% CI 17.1-510.5]). The mechanism for increased haematological malignancy risk in GD is unclear; it is postulated to be related to substrate accumulation and chronic inflammation and activation of immune cells. Lo et al (2012) describe 2 siblings with Type I GD and acute T-cell lymphoblastic lymphoma. These patients were shown by next generation sequencing to have bi-allelic MSH6 mutations, which causes DNA mismatch repair deficiency and increased cancer risk. It may be that genetic variation in cancer genes in GD patients contributes to increased cancer risk but it seems unlikely that co-inheritance of mutations in cancer genes will explain the increased risk of cancer for GD in general. The association between Type I GD and PD is discussed later in this introductory chapter.
Gaucher disease: phenotypes of Type II and Type III Gaucher disease (neuronopathic)

Type II GD (acute neuronopathic) is characterised by the onset of a neurological syndrome before the age of 2 years of age. There is limited psychomotor development and death typically occurs by age 4. Severe forms of Type II GD are associated with ichthyotic lamellar desquamation of the newborn (“collodion baby”), typically with bi-allelic null mutations in GBA (Lu et al, 1988). Examination of skin biopsies has confirmed that only Type II GD with null mutations is associated with the collodion baby phenotype and skin pathology (Sidransky et al, 1996). Though the neurodevelopmental phenotype dominates the clinical picture patients with Type II GD also have visceromegaly and haematological abnormalities similar to Type I.

Individuals with Type III disease (subacute neuronopathic) have onset in later childhood or teenage years with a slowly progressive course with lifespan into the 20s and 30s in some cases (Benko et al, 2011). Brain stem signs are common; including oculomotor apraxia, failure to initiate saccades, stridor and squint. Pyramidal signs such as spasticity and opisthotonus are frequent. Seizures, dementia and ataxia may occur in neuronopathic GD. In Type III GD eye movement abnormalities may be the only neurological manifestation in patients with otherwise severe systemic involvement. Benko et al (2011) prospectively followed the neurological phenotype of 15 Type 3 GD patients over 4 years. All patients studied had impaired saccadic eye movements, with horizontal movement more severely affected than vertical. Only 4 of the patients had normal brainstem auditory evoked responses, there was a wide range of cognitive function from severe learning disability through to above average intellect and all had slow performance on the Purdue pegboard. Two had evidence of progressive myoclonic encephalopathy of Gaucher disease. Kraoua et al (2011) describe a French series of 10 Type 3 GD patients. The most common initial neurological abnormalities were oculomotor with 1 patient presenting with epilepsy, though a further 3 eventually developed epilepsy. Cerebellar, pyramidal and dystonic features were also common. Six of the patients had neurological signs rated as stable or improved with treatment with enzyme replacement therapy or substrate reduction. Tajima et al (2009) report a series of Japanese Type 3 GD patients. Again the major neurological findings at diagnosis were oculomotor apraxia or seizures. This group described a relatively good response to enzyme replacement therapy in GD Type 3 patients who presented initially with non-neurological signs and who subsequently were reclassified after developing them. In contrast those patients with neurological signs at the outset had a poor response to treatments. It seems likely that enzyme replacement therapy is of limited efficacy for neurological manifestations of Type 3 GD, given that it cannot cross the blood brain barrier.
**Gaucher disease: treatment options**

Treatment with recombinant glucocerebrosidase enzyme (ERT) has revolutionised the management of Type I GD (Desnick and Schuchman, 2012). Regular intravenous infusions result in reversal of hepatosplenomegaly and protect against bone disease but is not associated with improved quality of life scores (Wyatt et al, 2012). ERT does not appear to be affective for neurological manifestations of GD; Type I patients have developed PD whilst on ERT and brain stem auditory evoked responses have been reported to deteriorate in Type III patients on ERT (Tajima et al, 2009). An alternative to ERT is substrate reduction therapy (SRT), which reduces production of glucosylsphinigosine, permitting clearance by mutant glucocerebrosidase enzymes with reduced activity. The most commonly used SRT is Miglustat (Venier and Igdoura, 2012), which is effective at reducing hepatosplenomegaly and bone disease. Miglustat does not prevent/treat neurological manifestations of GD, indeed postural tremor and memory impairment have been reported as possible side effects.
**Gaucher disease: biochemistry & pathogenesis**

The central process in GD is failure of glucosylceramidase to hydrolyse glucosylceramide to glucose and ceramide. This results in the accumulation of toxic substrates in lysosomes within macrophages, which are histologically identifiable as Gaucher cells. Physical accumulation of Gaucher cells results in hepatomegaly and splenomegaly, characteristic features of GD. Figure 1 illustrates the glycosphingolipid metabolic pathway affected in GD (after Xu et al, 2010). Enzymes which catalyse each reaction are in red text.

**Figure 1. Simplified outline of glycosphingolipid metabolism and disorders.**

- **Sphingosine**
  - Sphingomyelinase: Niemann-Pick
  - Beta-galactosidase: Krabbe disease

- **Ceramide**
  - Arylsulfatase: Metachromatic leukodystrophy

- **Glucosylceramide**
  - Glucosylceramidase: Gaucher disease

- **Ganglioside**
  - Beta-hexosaminidase: Tay-sachs

- **Sulfatide**
  - Alpha-galactosidase: Fabry disease

Sphinolipid metabolites re in black and the enzymes which metabolise them, and disorders associated with their deficiency are in red text.
Figure 2. Sequential degradation of glycosaminoglycans which accumulate in mucopolysaccharidoses (from Kowalewski et al, 2012).

The diagram to the right of the figure shows the chemical structure of glycosaminoglycans being sequentially degraded. On the left of the diagram the enzyme responsible for each step of catabolism is given with the disorder caused by its deficiency in brackets underneath.

The development of murine GD models has proved problematic. Germline deletion of both GBA genes results in perinatal lethality (Mistry et al, 2010). Mistry and colleagues (2010) conditionally knocked out GBA in haematopoietic and mesenchymal lineages using the Mx1 promoter. This mouse recapitulates human visceral pathology (e.g. splenomegaly), haematological dysfunction and osteopaenia. In this model osteopaenia was shown to be secondary to inhibition of Protein Kinase C in osteoblasts. Murine models have provided important insight into the neuropathogenesis of GD. Homozygous V394L, saposin C null mice develop neurological signs (waddling gait) and die from progressive neurodegeneration around 1-2 months of age. Brain tissue from this model has elevated glucosylceramide and glucosylsphingosine, with neuronal loss and evidence of inflammation (CD68 positive microglia, astrogliosis)(Sun Y et al, 2010). Other groups have confirmed a spatial and temporal relationship between substrate accumulation, neuroinflammation and neuronal loss. In the L444P/L444P mouse there is marked multi-system inflammation with minimal substrate accumulation, suggesting that inflammation in GD may not solely be a response to substrate accumulation (Mizukami et al, 2002). Taken together these studies suggest that substrate accumulation, cellular responses to it and neuroinflammation play roles in neuronal death in neuronopathic GD.

Gaucher disease: influence of GBA mutations on alpha-synuclein metabolism

Neuropathological examination (immunohistochemistry) of mice homozygous for D409H or V394L GBA mutations with hypomorphic prosaposin alleles demonstrated intra-neuronal alpha-synuclein and ubiquitin accumulation in hippocampus, cortex and cerebellum coincident with neurological deterioration and substrate accumulation (Xu YH et al, 2011). Cullen et al (2011) utilised enzyme linked immunosorbent assay (ELISA) to demonstrate increased membrane bound alpha-synuclein levels in forebrain, hippocampi and brainstem from 6 and 12 month old D409V/D409V knock in mice without neurological deficits. Immunohistochemical analysis of the same mice did not demonstrate elevated neuronal alpha-synuclein. Recently, Mazzulli and colleagues (2011) demonstrated that alpha-synuclein accumulates in human neuronal cell lines with GBA knocked down and in induced Pluripotent Stem Cells (iPS) differentiated into dopaminergic neurons. They also demonstrated that glucosylceramide accumulating in these cells facilitates formation of toxic
alpha-synuclein aggregates. These studies provide evidence that dementia and PD occurring in GD may be related to aberrant processing of alpha-synuclein, but it must be noted that the murine models utilised GBA mutations typically associated with neuronopathic GD rather than mutations frequently found in association with Type I GD or PD such as N370S. The clinical presentations of neuronopathic GD and of PD associated with GD differ significantly and it is unclear to what extent the underlying pathological mechanisms overlap.

The mechanisms by which heterozygous GBA mutations lead to synucleinopathy are unclear. Aged heterozygous D409V mice do not have neurological deficits or elevated alpha-synuclein levels as assessed by ELISA (Cullen et al, 2011). However, transfection of MES23.5 cells (a rodent midbrain cell line) and PC12 cells with mutant GBA alleles results in significant elevation of alpha-synuclein levels as detected by ELISA. This elevation of alpha-synuclein correlated with levels of mutant GBA protein expressed but not levels of GBA enzyme activity (Cullen et al, 2011). Treatment of these cell lines with Rapamycin significantly reduced alpha-synuclein levels in the transfected cells. This suggests that dysfunctional autophagy may be associated with GBA mutations and lead to alpha-synuclein accumulation, and that loss of GBA enzyme activity is less relevant to neurological disease in heterozygous carriers. However, Mazzulli and colleagues (2011) have demonstrated that alpha-synuclein aggregates can inhibit lysosomal GBA activity in neuronal cultures and in post-mortem sporadic PD brain higher levels of alpha-synuclein are associated with lower GCase activity. Thus loss of lysosomal GCase activity may occur secondary to alpha-synuclein accumulation in PD and produce a positive feedback effect by further inhibiting alpha-synuclein degradation. Heterozygous GBA mutation carriers would theoretically be much more susceptible to such an effect.
Gaucher disease: effects of glucosylceramidase inhibition on cellular metabolism

In preliminary work from our lab, Cleeter *et al* (2012) treated neuroblastoma cell lines with conduritol-b-epoxide, a specific inhibitor of GCase, and demonstrated inhibition of mitochondrial function. Complex I-linked ADP phosphorylation with glutamate/malate as substrate was significantly decreased by 47% at 20 days (*p* < 0.01) and by 33% at 30 days (*p* < 0.05). Complex II/III-linked ADP phosphorylation using succinate as substrate was significantly reduced by 30% at 20 days (*p* < 0.05) and 26% at 30 days (*p* < 0.05). There was no significant change in complex IV-linked ADP phosphorylation. This was associated with loss of mitochondrial membrane potential as assessed by TMRM fluorescence. There was no reduction in individual respiratory chain enzyme activities, but mitochondrial fragmentation was observed after 30 days of treatment. There was a significant increase in rate of dihydroethidium oxidation by 52% at 20 days (*p* < 0.01) and 71% at 30 days (*p* < 0.01). Treatment with conduritol-b-epoxide also caused a significant increase in alpha-synuclein accumulation, which was not related to lysosomal dysfunction or impaired autophagy. Clearly mitochondrial dysfunction, oxidative stress and alpha-synuclein accumulation are key processes in PD neuropathogenesis and this work illustrates that loss of GCase activity can trigger metabolic changes that might be associated with, and predispose to, neurodegeneration.

The Parkinson’s disease prodrome

The classical motor syndrome of PD occurs when greater than 50% of the dopaminergic neurons in the *substantia nigra pars compacta* have degenerated (Lees *et al*, 2009). However, the process of neuronal dysfunction and degeneration leading up to this is believed to last up to 20 years (Schapira & Tolosa, 2010). During this period it is the non-motor symptoms and signs of PD which predominate (Schapira & Tolosa, 2010). As discussed above, one model of PD neuropathogenesis postulates that alpha-synuclein accumulation and neuronal dysfunction begins in brainstem areas outside the substantia nigra, which can explain why some of the prodromal features of PD occur prior to the onset of the motor syndrome of PD.

Symptoms of autonomic dysfunction are common in PD. Constipation (defined as <1 bowel motion per day) is the best characterised of these and has been shown to precede the development of motor symptoms of PD (Savica *et al*, 2009). In the Honolulu ageing study the mean interval from administration of the bowel motion questionnaire to diagnosis of PD was 12 years for men, while in the Olmsted County case-control study constipation preceded onset of PD by 20 years or more in women (Savica *et al*, 2009). In the Honolulu aging study men 25 % of men with constipation had incidental lewy body disease (compared with 6.5% of controls) (Abbott *et al*, 2007) and constipated men who died without PD had a significantly lower density of substantia nigra neurons than non-constipated men (Petrovich *et al*, 2009).
REM sleep behaviour disorder (RBD), is characterised by loss of the normal muscular atonia seen during REM sleep, with the result that the individual acts out their dreams and complains of non-refreshing sleep. RBD has been associated with lewy body pathology in the brain at autopsy. In a prospective study RBD was found to precede development of PD by 12 years (Postuma et al, 2009).

Olfactory dysfunction occurs in up to 90% of cases of PD and is one of the best characterised clinical markers of prodromal PD (Schapira & Tolosa, 2010). In the Honolulu ageing study olfactory dysfunction was associated with an odds ratio of developing PD of 5.2 for the lowest quartile of olfactory function scores compared with the highest quartile of scores (Ross GW et al, 2008). This increased risk only applied for the first 4 years after testing however. In the Honolulu cohort olfactory dysfunction predicted the presence of lewy body pathology in those who died free from PD (Ross GW et al, 2006). The role of olfactory dysfunction in predicting PD risk was reinforced by a study demonstrating that 5 years after testing 12.5% of hyposmic individuals with a first degree relative with PD develop the disorder (Berendse & Ponsen, 2009). Hyposmia is associated with loss of dopaminergic neurons as assessed by DAT-scan in first degree relatives of PD patients who subsequently develop PD; suggesting that this clinical sign is a sensitive marker of the early stages of dopaminergic neuronal loss in PD before the motor syndrome becomes clinically apparent (Berendse & Ponsen, 2009).

Subtle bradykinesia may also occur in the years before the motor syndrome of PD becomes fully manifest (Schapira & Tolosa, 2010). This can be assessed by simple clinical evaluations known as “timed motor tests” (Haaxma et al, 2008). For the upper limbs one of the best characterised tests is the Purdue pegboard. This requires the participant to place as many pegs as they can into a row of holes in 30 seconds. Using their dominant then their non-dominant hand and then both hands simultaneously. For the lower limbs a timed 3-metre walk is commonly used. In this the participant sits in a chair, stands, walks 3 metres then turns and returns to sit. The number of pegs placed in the pegboard and the time taken to walk 3m have been shown to discriminate patients with PD from age matched controls (Haaxma et al, 2010). Moreover, when hemi-parkinsonian patients were assessed it has been shown that the number of pegs placed is lower for the unaffected limb than for normal controls, suggesting that a reduced number of pegs placed may detect PD before the full motor syndrome develops (Haaxma et al, 2010). To further support the contention that the peg board can detect bradykinesia in pre-motor PD there is evidence that a reduced number of pegs placed correlates with loss of dopaminergic neurons on DAT-scan (Bohnen et al, 2007) and
at post-mortem (Alder et al, 2002). In established PD the peg board score and 3m walk speed correlates with the UPDRS score, at baseline and disease progression, suggesting that these markers are sensitive to clinical progression in PD (Haaxma et al, 2008).

In our clinical study we will use validated clinical rating scales to assess for the presence of these prodromal markers of PD in a cohort of GD patients and their obligate carrier relatives. For example, we will use the commercially available British version of the University of Pennsylvania Smell Identification Test to screen for hyposmia.

**The role of glucocerebrosidase mutations in Parkinson’s disease**

The association between PD and glucocerebrosidase mutations was first noted by clinicians who recognised an increased frequency of PD amongst GD patients, and subsequently their obligate carrier relatives (Goker-Alpan et al, 2004). Two large cohort studies have estimated the risk of PD in patients with Type I GD. Bultron and colleagues (2010) identified 11 cases of PD amongst 444 Type I GD patients seen at a New York clinic and calculated a lifetime risk for PD in Type I GD of 21-24 times that of the general population. The study of Rosenbloom et al (2011) calculated a 4% risk of developing PD at age 70 and a risk of 8-12% by age 80 for Type I GD patients. The association of heterozygous GBA mutations with increased PD risk has been confirmed in multiple studies. The meta-analysis of Sidransky et al (2009), which included almost 6 000 PD patients, found an Odds Ratio of 5 for the presence of a heterozygous GBA mutation in a case of sporadic PD compared with a control. Increased frequencies of GBA mutations have been found in clinical cohorts of sporadic PD from several regions, including North America (Eblan, Walker & Sidransky, 2005), Japan (Sidransky et al, 2009), Greece (Kalinderi et al, 2009) and North Africa (Lessage et al, 2011). As expected, the highest frequency of GBA mutations is seen in Ashkenazi Jewish populations, with some studies from Israel detecting heterozygous GBA mutations in almost 1/3 cases of sporadic PD (Aharon-Peretz et al, 2005). In the Sidransky meta-analysis (2009) the most common mutations in Ashkenazi PD patients were N370S (72%), followed by 84GG (11%) and R496H (6%). In non-Ashkenazi patients L444P (35%) and N370S (18%) were the most common alleles. Importantly, G2019S LRRK2 mutations are not found in association with GBA mutations in cases of PD with heterozygous GBA mutations (Eblan et al, 2006). The lifetime risk of developing PD for a heterozygous GBA mutation carrier is not known.

PD associated with heterozygous GBA mutations (PD-GBA) has a younger age of onset than sporadic PD (mean age of onset 5 years younger) (Sidransky et al, 2009), with cases of PD with an
age of onset under 50 years old significantly more likely to have a *GBA* mutation and some studies suggesting that severe GBA mutations (e.g. L444P) have a younger age of onset than mild mutations (e.g. N370S) (Gan-Or et al, 2008). The phenotype of PD associated with GBA mutations is very similar to that of sporadic PD. In the study of Saunders-Pullman *et al* (2010) a series of 250 Ashkenazi patients with typical PD were screened for GBA mutations. Four individuals with bi-allelic *GBA* mutations were identified who had a phenotype of typical PD. This vividly illustrates that the movement disorder associated with GD can be indistinguishable from sporadic PD. Several case series of PD patients with heterozygous GBA mutations describe patients with dopamine responsive parkinsonism, more often akinetic rigid than tremulous, which is associated with the development of cognitive impairment later in disease. The Sidransky meta-analysis suggested that compared to sporadic PD, PD-GBA may be symmetrical at onset and more frequently associated with cognitive impairment. Crucially, pathological studies of brain tissue from sporadic PD patients with heterozygous GBA mutations display Lewy bodies and other neuropathological features characteristic of sporadic PD (Neumann *et al*, 2009). A case report has described loss of dopaminergic neurons on DAT scan in a GD patient with PD and his heterozygous parent with PD (Kono *et al*, 2007). Thus there is strong evidence that GBA mutations predispose to a parkinsonian disorder indistinguishable from sporadic PD which implies that studying PD-GBA will provide insights into the pathogenesis of sporadic PD.
The role of fibroblasts in studying human neurological disease

Despite fibroblasts being non-neuronal cells they are widely used as patient derived cell lines in the study of neurological disorders and have provided many useful insights into the pathogenesis of various diseases. Fibroblasts lack many of the specialised features of neurons (e.g. neurotransmitters) but contain the cellular machinery relevant to PD and GD pathogenesis such as the glucocerebrosidase enzyme, lysosomes, endoplasmic reticulum, mitochondria et al. In addition patient derived fibroblasts carry mutations associated with the genetic disease being studied. This permits more authentic modelling of recessive disorders than the alternative model of knocking down mRNA and protein expression, since many recessive mutations do not completely inhibit the gene and its protein’s function. Moreover it is possible to model heterozygous mutations in this way. It is very difficult to do this in other models, for example transfecting in a plasmid containing a mutant allele will result in a tri-allelic cell with 2 wild type alleles and one mutant allele which does not recapitulate the human disease. Using fibroblasts also avoids confounding by factors such as genes being disrupted during “Knock out” and “Knock in” procedures in mice.

Abnormalities occurring in these organelles due to mutations in \( GBA \) are likely to be present in all the cells of the body of a GD patient or carrier, even if some cell types, such as bone marrow stem cells, are more prone to manifest pathology than others, such as neurons. In this project fibroblasts from GD patients, heterozygous carriers of \( GBA \) mutations without PD and PD patients with heterozygous \( GBA \) mutations will be studied to identify cellular changes which may explain why \( GBA \) mutations increase risk of developing PD. Moreover, fibroblasts do have some relevance to GD as the skin is damaged in severe early onset Type II GD associated with null mutations in \( GBA \).

Published studies demonstrating oxidative stress in fibroblasts from Type II and Type III GD patients provides evidence to support the use of fibroblasts in studying the basis of neurological abnormalities in Type I GD (De Gunato, 2007). Fibroblasts have also been used to study several genetic types of PD. Fibroblasts from patients with the \( LRRK2 \) \( G2019S \) mutation have been reported to show defects in autophagy (Bravo-San Pedro et al, 2012) and increased mitochondrial uncoupling (Papovskia et al, 2012). Fibroblasts with bi-allelic \( Parkin \) mutations have features compatible with impaired mitophagy – loss of mitochondrial branching, elevated mitochondrial mass, impaired mitochondrial energy generation and elevated mitochondrial mass (Grunewald et al, 2009). Thus it is feasible that studying patient derived fibroblasts will provide useful insight into both the pathogenesis of GD and PD associated with \( GBA \) mutations.
Hypotheses to be tested and initial research plan

**Hypothesis 1:** A subset of GD patients and GBA mutation carriers will express clinical markers of prodromal PD.

GD patients and heterozygous GBA mutation carriers will be recruited from the Royal Free Hospital Lysosomal Storage Disorders clinic. Research participants will be screened for prodromal markers of PD using well validated tools such as the University of Pennsylvania Smell Identification Test (British version). The individuals recruited to the study will be invited to undergo skin biopsy to generate fibroblasts for use in addressing hypothesis 4 (see below).

**Hypothesis 2:** Age related risk of PD will be elevated in heterozygous GBA mutation carriers.

The age related risk of PD in heterozygous GBA mutation carriers (i.e. parents of GD patients) will be determined from family history studies of GD patients.

**Hypothesis 3:** the clinical phenotype of PD associated with GBA mutations will be very similar to that of sporadic PD.

PD patients with heterozygous GBA mutations will be phenotyped using standard rating scales of motor and non-motor symptoms.

**Hypothesis 4:** fibroblasts from GD patients and heterozygous GBA mutation carriers will manifest biochemical defects known to be associated with PD.

Fibroblasts will be generated from partial thickness punch biopsies of skin. These will be assayed for defects of lysosomal metabolism and mitochondrial function. Evidence of oxidative stress will be sought. A variety of methodologies including Western blotting, polymerase chain reaction based techniques, measurement of enzymatic activity and single cell imaging will be used as appropriate.
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Chapter II.

A CLINICAL STUDY OF PRODROMAL MARKERS OF PARKINSON’S DISEASE IN GAUCHER DISEASE PATIENTS AND HETEROZYGOUS CARRIERS OF GLUCOCEREBROSIDASE MUTATIONS
Ia. Introduction

Clinical markers of prodromal Parkinson’s disease

The classical motor syndrome of PD occurs when greater than 50% of the dopaminergic neurons in the substantia nigra pars compacta have degenerated (Lees et al, 2009). However, the process of neuronal dysfunction and degeneration leading up to this is believed to last up to 20 years (Schapira & Tolosa, 2010), a phase referred to as the Parkinson’s disease prodrome. During this period it is the non-motor symptoms and signs of PD which predominate (Schapira & Tolosa, 2010), these are termed prodromal markers of PD. As discussed in the general introduction, one model of PD neuropathogenesis postulates that SNCA accumulation and neuronal dysfunction begins in brainstem areas outside the substantia nigra, which can explain why some of the prodromal features of PD occur prior to the onset of the motor syndrome of PD.

Symptoms of autonomic dysfunction are common in both the prodromal and clinical phase of PD. Constipation (defined as <1 bowel motion per day) is the best characterised of these and has been shown to precede the development of motor symptoms of PD (Savica et al, 2010). In the Honolulu ageing study the mean interval from administration of the bowel motion questionnaire to diagnosis of PD was 12 years for men, while in the Olmsted County case-control study constipation preceded onset of PD by 20 years or more in women (Savica et al, 2009). In the Honolulu aging study 25 % of men with constipation had incidental lewy body disease (compared with 6.5% of controls) (Abbott et al, 2007) and constipated men who died without PD had a significantly lower density of substantia nigra neurons than non-constipated men (Petrovich et al, 2009). The mechanism of constipation in prodromal PD is unclear. However, recent studies have demonstrated alpha-synuclein accumulation in the enteric nervous system in colonic biopsies taken from PD patients 2-5 years before onset of the motor syndrome (i.e. in the prodromal phase). SNCA accumulation was mainly localised to substance-P positive neurons (Shannon et al, 2012). This suggests that Lewy body formation/SNCA accumulation in the enteric nervous system in the prodromal phase of PD may be causally related to the occurrence of constipation. In addition to constipation other autonomic symptoms are associated with PD.
However, in the majority of cases urinary and erectile dysfunction develop after the onset of motor Parkinsonism (reviewed in Sakakibara et al, 2011). In 1 study only 60% of PD patients had orthostatic hypotension as an early feature (reviewed in Goldstein et al, 2011). All of these autonomic symptoms have low sensitivity and specificity for prodromal PD and are more commonly associated with motor PD. There are several screening tools to detect the presence and severity of autonomic symptoms in PD. However, most of these are very detailed and time consuming to administer. In the context of this study of a range of prodromal markers of PD the autonomic component questionnaire of the Unified Multiple Systems Atrophy Rating Scale (UMSARS) was selected as a screening tool as it is easy to administer and validated for the detection of the 4 main autonomic symptoms (constipation, urinary dysfunction, orthostatic hypotension, erectile dysfunction)(Wenning et al, 2004).

Rapid eye movement (REM) sleep behaviour disorder (RBD) is a parasomnia and movement disorder described by Schenck in 1986 (reviewed in Arnulf, 2012). RBD is associated with loss of normal muscle atonia during REM sleep, leading to dreams being acted out. It is thought to be related to lesions of the REM sleep atonia centre in the pontomedullary brain stem. Patients with RBD classically experience violent/distressing dreams which are combined with purposeful limb movements and vocalisations during sleep (reviewed in Arnulf, 2012). Probable RBD is diagnosed based upon clinical screening, which can be done using validated questionnaires, with confirmation by formal sleep studies (video-polysomnography during sleep). RBD is present in up to 60% of PD patients and 80-100% of patients with Lewy body dementia. Interestingly 50-80 % of patients with RBD develop PD, LBD or MSA within 5-15 years (Schenck et al, 1996; Postuma et al, 2009). RBD is the most sensitive and specific marker of prodromal PD and RBD patients represent an important at risk cohort for studying the earliest phases of PD (see below). Several questionnaires have been developed to screen for RBD. The screening questionnaire designed by Stiasny-Kolster et al (2012) was chosen to screen for RBD as it was developed in a European population and validated in a large cohort of RBD patients with diagnosis confirmed
by sleep studies. Other RBD questionnaires available were not utilized as they had been validated in non-European populations or were general screening tools for sleep disorders in PD.

Olfactory dysfunction (hyposmia) occurs in up to 90% of cases of PD and is one of the best characterised clinical markers of prodromal PD (Schapira & Tolosa, 2010). It should be noted that hyposmia is not unique to PD and can be observed in other neurodegenerative conditions such as Alzheimer’s disease. Hyposmia occurs in Progressive supranuclear palsy, MSA, CBD and essential tremor but to a lesser degree than in PD (Doty RL, 2012). The pathological substrate of hyposmia in PD is Lewy body deposition in the olfactory bulb, anterior olfactory nucleus and olfactory cortices (Ubeda-Banon et al, 2012). In the Honolulu ageing study olfactory dysfunction was associated with an odds ratio of developing PD of 5.2 for the lowest quartile of olfactory function scores compared with the highest quartile of scores (Ross GW et al, 2008). This increased risk only applied for the first 4 years after testing however. In the Honolulu cohort olfactory dysfunction predicted the presence of central nervous system Lewy body pathology in those who died free from PD (Ross GW et al, 2006). The role of olfactory dysfunction in predicting PD risk was reinforced by a study demonstrating that 5 years after testing 12.5% of hyposmic individuals with a first degree relative with PD develop the disorder (Ponsen et al, 2009). Hyposmia is associated with loss of dopaminergic neurons as assessed by dopamine transporter imaging in first degree relatives of PD patients who subsequently develop PD themselves; suggesting that this clinical sign is a sensitive marker of the early stages of dopaminergic neuronal loss in PD before the motor syndrome becomes clinically apparent (Berendse & Ponsen, 2009). Several aspects of olfaction are abnormal in PD; including odour identification, discrimination and olfactory threshold (reviewed in Doty RL, 2012). In clinical practice the most commonly used tests of olfactory function are based upon odour identification, as these have been shown to be the most reliable and reproducible (Doty RL, 2012). One of the most extensively studied tests of olfactory function is the University of Pennsylvania Smell Identification Test (UPSIT). This features 40 odours contained in a microencapsulated form on a card. The card is scratched to release the odour and the study participant selects from 4 choices
to identify the odour. There is extensive age and sex adjusted normative data for the UPSIT to enable identification of hyposmic individuals. The UPSIT was selected as a screening tool for hyposmia as it has been used extensively in publications, is easy to administer, is portable and is available in a British format.

Subtle bradykinesia may also occur in the years before the motor syndrome of PD becomes fully manifest (Schapira & Tolosa, 2010). This can be assessed by simple clinical evaluations known as “timed motor tests” (Haaxma et al, 2008). For the upper limbs one of the best characterised tests is the Purdue pegboard, which is commercially available. This requires the participant to place as many pegs as they can into a row of holes in 30 seconds. Using their dominant hand, then their non-dominant hand and then both hands simultaneously. For the lower limbs a timed 3-metre walk is commonly used. In this the participant sits in a chair, stands, walks 3 metres then turns and returns to sit. The number of pegs placed in the pegboard and the time taken to walk 3m have been shown to discriminate patients with PD from age matched controls (Haaxma et al, 2010). Moreover, when hemi-parkinsonian patients were assessed it has been shown that the number of pegs placed is lower for the unaffected limb than for normal controls, suggesting that a reduced number of pegs placed may detect PD before the full motor syndrome develops (Haaxma et al, 2010). To further support the contention that the peg board can detect bradykinesia in pre-motor PD there is evidence that a reduced number of pegs placed correlates with loss of dopaminergic neurons on dopamine transporter imaging (Bohnen et al, 2007) and at post-mortem (Adler et al, 2002). In established PD the peg board score and 3m walk speed correlates with the UPDRS score, at baseline and disease progression, suggesting that these markers are sensitive to clinical progression in PD (Haaxma et al, 2008). The Purdue pegboard and 3m timed walk are well validated in the literature and easy to administer, therefore they were selected as screening tools to identify individuals with subtle bradykinesia.

Cognitive dysfunction (both Mild Cognitive Impairment, MCI, and dementia) has recently been recognised as being highly prevalent in incident, untreated PD. Reported rates of cognitive dys-
function in newly diagnosed PD range from 20-35% (Foltynie et al, 2004). Most commonly cognitive deficits are either frontostriatal or temporal lobe in nature (Foltynie et al, 2004). Functional imaging with 18F-fluorodeoxyglucose (FDG) and PET (FDG-PET) demonstrated posterior or cortical hypometabolism in patients with early PD and MCI. There are no studies demonstrating that MCI is a prodromal marker of PD, but given the evidence from incident PD cohorts it seems probable that MCI occurs in prodromal PD. The Montreal Cognitive Assessment (MoCA) is a well validated bedside screening tool for detection of cognitive impairment. The MoCA has been shown to be significantly more sensitive in detection of cognitive impairment in PD than the more commonly used Mini Mental State Examination (MMSE)(Dalrymple-Alford et al, 2010; Hoops et al, 2009). The MoCA is simple to use and quick and does not require specialized equipment making it suitable for clinical screening studies of prodromal PD.

**Physiology of the human olfactory system and anatomical basis of hyposmia**

The major anatomical components of the human olfactory system are described here to provide a context for the discussion of causes of hyposmia later in this chapter (Gottfried and Zald, 2005). Odor-evoked responses are conducted from the first order neurons in the olfactory mucosa to synapse in the second order neurons (mitral and tufted cells) in the olfactory bulb. The axons of the mitral and tufted cells then form the olfactory tracts ipsilateral to the olfactory bulb. The olfactory tract lies in the olfactory sulcus of the basal forebrain and conveys olfactory stimuli to a variety of brain structures (Gottfried and Zald, 2005). These projection sites are collectively called the “primary olfactory cortex” and include the anterior olfactory nucleus, anterior and posterior piriform cortex, amygdala and rostral entorhinal cortex. The piriform cortex is the largest area receiving olfactory projections and is often used synonymously with primary olfactory cortex. Higher order projections from these primary olfactory areas converge on secondary olfactory cortical regions in the orbitofrontal cortex, agranular insula, hypothalamus, thalamus and hippocampus (Gottfried and Zald, 2005). This complex network of cortical regions forms the basis for odour guided regulation of behaviour, autonomic function, feeding and memory.
Functional neuroimaging studies demonstrate that distinct cortical networks underly different aspects of odour perception (Reviewed in Savic, 2002). In the majority of studies, the piriform cortex, amygdala, thalamus, right orbitofrontal cortex, insula and cingulum are activated in all types of olfactory task. However, when comparing the intensity of different odours the left insular cortex and right cerebellum are activated in addition. In discriminating the quality of different odours the right prefrontal cortex, bilateral frontal operculum and brainstem areas are activated. While odour recognition/memory tasks involve the right temporal neocortex.

Clearly the human olfactory system is vulnerable to damage at a number of anatomical locations. Damage to the olfactory bulb is associated with hyposmia after head injury while sinonasal disease (e.g. polyps) can damage the sensory nerves in the nasal mucosa. Several different central nervous system diseases are associated with hyposmia as discussed elsewhere in this chapter (Doty, 2012). Thus when hyposmia is identified in a particular neurological disease further characterisation, e.g. with functional neuroimaging is required to identify the cortical structures involved in the pathogenesis of hyposmia.

**Retinal thinning as an ocular manifestation of early Parkinson’s disease**

A variety of ocular features have been reported in Parkinson’s disease. Visual symptoms range from dry eyes, through to perceptual abnormalities and complex visual hallucinations (reviewed in Archibald, 2011). The retina contains dopaminergic cells in the form of amacrine cells which are found in the inner nuclear layer and to a lesser extent the retinal ganglion cell layer (reviewed in Archibald, 2011). The main physiological role of dopamine in the retina is to facilitate light adaptation. Loss of dopaminergic cells in the retina of PD patients has been demonstrated at post mortem and this is proposed to explain the loss of contrast and colour sensitivity displayed by PD patients.

Optical coherence tomography (OCT) is a non-invasive technique which utilises reflected light to measure retinal nerve layer thickness with a resolution of 3-5 micrometres. Several OCT
studies have demonstrated retinal nerve fiber loss in PD patients. Hajee et al (2009) reported reduced retinal nerve fiber layer thickness in early PD. La Morgia et al (2012) demonstrated loss of temporal retinal nerve fiber layer thickness in PD patients compared to controls; they point out that a similar pattern is observed in mitochondrial disease. Aaker et al (2010) describe loss of macular thickness in PD but normal retinal nerve fiber layer thickness. However, Archibald et al (2011) could not demonstrate retinal pathology using OCT in sporadic PD. Some of the variability of studies of OCT in PD reflects the different OCT techniques used and the heterogeneity of PD patients. Nonetheless, it seems that OCT can demonstrate retinal nerve fiber loss as a biomarker of both early and established PD.

There are no studies of OCT in GD. However, the retina is involved in GD with reports of pre-retinal white spots (probable retinal Gaucher cells) described in GD patients before the advent of ERT (Wollstein et al, 1999; Cogan et al 1980). Seidova et al (2009) describe reduced electroretinogram responses in a GD patient. The retina is involved in other LSDs, for example, the cherry red spot in Tay-Sach’s disease. Given that OCT can detect subtle retinal degeneration in both early and advanced PD and that the retina is likely to be involved in GD it was decided to investigate the ability of OCT to detect retinal degeneration in GD patients.

The role of dopamine transporter imaging in diagnosis of Parkinson’s disease
Parkinson’s disease is clinically diagnosed based upon the Queen Square Brain Bank criteria (Hughes et al, 1992). In the majority of cases diagnosis is straightforward with no need for ancillary tests. However, in certain circumstances imaging of the dopaminergic nigrostriatal system is required to aid diagnosis. For example, in cases where there is monosymptomatic tremor to help differentiate essential or dystonic tremor from PD (Kagi et al, 2010a). In clinical practice dopaminergic nigrostriatal imaging is performed using radioligands which bind the dopamine transporter [DAT, 123I-FP-CIT, 123I-beta-CIT or 99m-TRODAT-1], or less commonly 18-F-dopa [an analogue of L-DOPA which is taken into dopaminergic neurons], and visualising uptake with positron emission tomography (PET) or single photon emission computed
tomography (SPECT)(Kagi et al, 2010a). At the Institute of Neurology 123I-FP-CIT SPECT [DaTSCAN] is utilised to image the nigrostriatal dopaminergic system; compared to other radioligands it is taken up rapidly permitting imaging 3-6 hours after injection and binds exclusively to DAT on dopamine synthesising neurons. Factors such as age, gender and smoking which can theoretically influence DAT density are of limited impact on DaTSCAN use in clinical practice (Kagi et al, 2010a). It should be noted that several drugs can influence DAT availability and should be stopped at an appropriate time before DaTSCAN. For example, cocaine, selective serotonin uptake inhibitors and modafinil (Kagi et al, 2010a).

The primary use of DaTSCAN in clinical practice is to assess patients with early or clinically uncertain PD. In a small minority of cases with clinically probable PD (6.7-14.7%) DaTSCAN is normal. However, detailed clinical analysis of patients with so called “SWEDDs” (scans without evidence of dopaminergic dysfunction in patients with signs of PD) demonstrated that the majority have subtle clinical signs which point to a diagnosis other than PD (e.g. evidence of dystonic tremor). Most authors conclude that DaTSCAN is unlikely to be normal in bona fide cases of early PD, and that a normal DaTSCAN should prompt consideration of another diagnosis in patients with clinically uncertain PD. In this regard it is of note that DAT ligand binding intensity correlates with bradykinesia and not tremor in PD (kagi et al, 2010a).

DaTSCAN is of limited value in distinguishing PD from Parkinson’s plus syndromes such as Multiple Systems Atrophy and Progressive Supranuclear Palsy (Kagi et al, 2010a). However, DaTSCAN is normal in essential or dystonic tremor, vascular Parkinsonism and psychogenic PD. In PD DAT ligand binding is characteristically reduced in an asymmetric pattern, with greatest reduction contralateral to the affected side, and with relative sparing of the caudate early in disease (Kagi et al, 2010).

DaTSCAN has also been used to demonstrate dopaminergic nigrostriatal dysfunction in conditions which are associated with a high risk of developing PD. For example, DaTSCAN studies of REM sleep behaviour disorder have revealed loss of dopaminergic nigrostriatal
neurons (Iranzo et al, 2011), which could be taken as evidence of pre-motor substantia nigra dysfunction. In support of this, DAT ligand binding decreases over time in RBD patients and in one study baseline reduction in DaTSCAN ligand binding predicted development of clinical PD over a 2.5 year period (Iranzo et al, 2011). DaTSCAN ligand binding is also reported as being reduced in patients with hyposmia and in the contralateral basal ganglia in hemi-parkinsonian patients. This suggests that DaTSCAN imaging may be of use in tracking progression from pre-motor to motor PD in individuals who are in populations at increased risk of developing PD (e.g. GBA mutation carriers).

There are relatively few studies of DaTSCAN or 18-F-DOPA –PET in monogenetic PD (Guo et al, 2011). In PD with bi-allelic Parkin mutations there is, compared to sporadic PD, a more symmetrical and generalised loss of DAT ligand binding, with a slower rate of loss of DAT ligand binding over time than in sporadic PD (Ribeiro et al, 2009). Likewise, in PD patients with bi-allelic PINK1 mutations reduction in DAT-ligand binding is more symmetrical than in sporadic PD, though some patients have an asymmetrical pattern similar to sporadic PD (Samaranch et al, 2010). DAT-SPECT in SNCA mutation associated PD is similar to sporadic PD but reported as being more symmetrical (Bostantjopoulou et al, 2008; Sammi et al, 1999; Ahn et al, 2008; Nishioka et al, 2006) while in LRRK2 G2019S mutation associated PD the pattern is asymmetrical and identical to sporadic PD (Isaias et al 2006). DaTSCAN imaging has been reported in 2 GD patients with PD and 1 heterozygous GBA mutation carrier with PD, documenting DAT-ligand binding loss similar to sporadic PD but not reporting region of interest analysis of DAT-ligand binding intensity with comparison to sporadic PD (Kono et al, 2007; Sunwoo et al, 2011). Thus, to provide a description of DaTSCAN imaging in PD-GBA we performed a retrospective analysis of DaTSCANs from cases of PD-GBA and other monogenetic PD cases.
**Studies of prodromal markers of PD in “at risk” cohorts**

There are a number of clinical cohorts who can be considered to be at increased risk of developing PD. The most obvious of these are individuals who carry pathogenic mutations in PD associated genes (e.g. *LRRK2*) but who have not developed PD. As discussed above individuals with RBD are also at significantly increased risk of developing PD. Several studies have investigated clinical markers of prodromal PD in these “at risk” cohorts.

Asymptomatic carriers of the *LRRK2 G2019S* mutation have been extensively studied. Saunders-Pullman *et al* (2011) demonstrated hyposmia in a subset of non-manifesting carriers of the *LRRK2 G2019S* mutation suggesting it to be a prodromal marker of PD in these subjects. Marras *et al* (2011) identified a higher prevalence of constipation, postural tremor and reduced colour discrimination in asymptomatic *LRRK2 G2019S* carriers.

Several clinical markers of prodromal PD have been identified in patients with RBD (Postuma *et al*, 2009). Including hyposmia, orthostatic hypotension and mild cognitive impairment. A subset of individuals with RBD have depletion of dopaminergic neurons on dopamine transporter imaging. Longitudinal studies of RBD have demonstrated that this loss of dopaminergic neurons is progressive and that individuals with prodromal markers can convert to overt motor PD (Iranzo *et al*, 2011).

These studies clearly demonstrate that clinical markers of the PD prodrome can be identified in “at risk” cohorts and that these markers can identify individuals who subsequently go on to develop PD. It is more efficient to identify at risk individuals carrying PD associated mutations rather than those who have RBD, given that accurate diagnosis of RBD requires cumbersome sleep studies. These points indicate that our study of clinical markers of prodromal PD in Gaucher disease patients and heterozygous *GBA* mutation carriers is feasible, with the potential to efficiently identify a cohort of individuals in the prodromal phase of PD.
Phenotypic variability of sporadic and genetic PD

Both sporadic and genetic PD show considerable variability in the motor and non-motor phenotype (reviewed in Kasten et al, 2010; Chaudhuri et al, 2011). The major motor phenotypic groups in sporadic PD are tremor predominant and postural instability-gait disorder. In addition rare genetic forms of PD have unusual motor features. For example, in Kufor-Rakeb syndrome facio-facual-finger minimyoclonus is a characteristic feature (Santoro et al, 2011) whilst lower limb dystonia is common in Parkin mutation associated PD (Kagi et al, 2010b). In the rare pallido-pyramidal syndromes (e.g. FBX07 mutations) parkinsonism is accompanied by upper motor neuron signs.

The heterogeneity is even more apparent in the non-motor phenotype of PD (reviewed in Kasten et al, 2010; Chaudhuri et al, 2011). The proportion of PD patients who develop PD-dementia varies, but longitudinal studies indicate that up to 85% of PD patients will eventually develop dementia (Foltynie et al, 2004). Neuropsychiatric features such as anxiety and depression are also common in sporadic PD, but by no means uniform. Symptoms of autonomic dysfunction are prevalent in sporadic PD affecting a variable proportion of patients (Sakakibara et al, 2011). Constipation affects the majority of PD patients (circa 60%), and many develop urinary and erectile dysfunction. Orthostatic hypotension is a feature of sporadic PD, often occurring late in the disease course.

The explanation for the phenotypic heterogeneity in PD is unclear. The study of monogenetic PD has shown that genotype plays a clear role (reviewed in Kasten et al, 2010). For example, cognitive dysfunction has been shown to be more frequent and more severe in PD associated with GBA or SNCA mutations (Kasten et al, 2010). Neuropsychiatric problems may occur more frequently in PINK1 associated PD. Interestingly, PD with recessive Parkin mutations has a very mild non-motor symptom profile (Kagi et al, 2010b). Given the evidence that mutations in PD associated genes can influence the phenotype of PD it was decided to undertake a detailed
phenotypic study of PD patients with *GBA* mutations in order to examine the hypothesis that
*GBA* mutations can influence the phenotype of PD.

**Ib. Methods**

**Patient Cohort**

Consecutive patients with GD, without a previous diagnosis of PD or dementia, from the Royal Free Hospital Lysosomal Storage Disorders unit (LSDU) and Lysosomal Disorders clinic at Addenbrookes hospital, Cambridge, were invited to participate in the clinical study. The GD patients had been previously diagnosed with Type I disease by the Consultant Haematologists of the LSDU (Prof A Mehta & Dr D Hughes [Royal Free], Dr P Deegan & Prof TM Cox [Addenbrookes Hospital]) on the basis of their clinical features, molecular genetics and blood biochemistry. A detailed family history was taken from each proband to identify relatives who were either obligate carriers of heterozygous *GBA* mutations (parents of probands) or at risk of carriage (uncles, aunts, siblings). Each proband was also asked about a family history of dementia or movement disorders. Relatives were then contacted by letter and invited to participate in the study with the proband’s permission. *GBA* mutation status was confirmed in probands and carriers by direct sequencing of the *GBA* gene (see below).

To identify a cohort of PD patients with *GBA* mutations 220 patients with typical PD were selected from the Royal Free Hospital Movement Disorders database, which is a prospective collection of DNA and clinical data from movement disorders patients seen at the Royal Free Hospital. The DNA from these individuals was sequenced to detect mutations in exons 1-11 of the *GBA* gene (sequenced by Rob Baker and Dr Raquel Duran-Ogalla). Controls who were age, sex and ethnicity matched to the Gaucher disease patient and carrier groups were recruited from relatives of the GD patients and PD patients. Absence of a pathogenic *GBA* mutation was confirmed by gene sequencing. A cohort of PD patients who did not carry a *GBA* mutation nor the *G2019S LRRK2* mutation (n=20) was recruited from the Royal Free Hospital movement disorders database to act as a mutation negative PD control group. All PD patients in the
Movement Disorders DNA database had previously had the \textit{LRRK2 G2019S} mutation sequenced by a previous research fellow.

The project has been granted ethical approval by the North West London Research Ethics Committee (REC number 10/H0720/21) and hospital Research and Development approval granted by the Royal Free Hospital (project ID 7995, CSP 42224) and Cambridge University Hospitals NHS Trusts.

\textbf{Glucocerebrosidase gene sequencing}

Sequencing of exons 1 – 11 of the \textit{GBA} gene was performed by Rob Baker in the Molecular Haematology laboratory of the Royal Free Hospital, where diagnostic sequencing of the \textit{GBA} gene is performed as an NHS service, and Dr Raquel Duran PhD of the Molecular Neuroscience group, UCL Institute of Neurology. GBA sequencing was performed with a previously reported protocol (Neumann \textit{et al}, 2009) using polymerase chain reaction (PCR) primers designed exclusively for regions of the GBA gene not found in the pseudogene. After amplification by PCR the product was run on a 1\% agarose gel with ethidium bromide and size checked to ensure it was not the pseudogene. Sanger sequencing was performed for each exon and flanking intronic sequences using the Dye Terminator Sequencing Kit (Applied Biosystems) and run on an ABI 3700xl genetic analyzer. All identified mutations were confirmed by a second reaction and by checking the forward and reverse sequences.

\textbf{Assessment of Clinical Markers Of Prodromal Parkinson’s Disease}

All testing procedures were performed identically in GD patients, carriers, PD patients and controls. It was not possible to blind as to the disease or mutation status of the participants.

Olfactory function was assessed using the British version of the University of Pennsylvania Smell Identification Test (SIT), which has been used to assess olfaction in several published U.K cohorts. The test was administered using the manufacturer’s protocol, both nostrils were used
for olfactory function testing. Individuals with an upper respiratory tract infection (URTI) or who had an anatomical lesion of their upper airways known to cause hyposmia were excluded as were current smokers. The SIT has 40 items, for each item the participant is asked to scratch a pad, which releases an odour and select from a choice of 4 what the odour is. If the individual cannot recognise the smell then they are instructed to make a best guess, as the SIT is a “forced choice” test. Hyposmia was defined as a score lower than the 15th centile for age and sex. It should be noted that none of the odours tested activate the trigeminal nerve, i.e. this is truly a test of olfactory nerve and olfactory system function. Training in administration of the UPSIT was provided by the outpatient department of the Royal National Ear, Nose and Throat hospital (UCL).

Cognitive function was assessed with 2 well recognised questionnaires, the Mini Mental State Examination (MMSE) and the Montreal Cognitive Assessment (MoCA). Each questionnaire was administered according to the author’s protocols. Mild cognitive impairment on the MoCA was a score less than 26 while dementia was taken as a score less than 24, as recommended by the authors of the test. Dementia on the MMSE was taken as a score less than 24, as recommended by the original authors of the test.

Each participant was assessed for features of parkinsonism by a structured physical examination using the Movement Disorders Society-Unified Parkinson’s Disease Rating Scale (MDS-UPDRS). The participants’ facial expression, speech and general movements are assessed for parkinsonian features such as bradykinesia. The participant is asked to make a series of repetitive movements of their arms and legs to assess for bradykinesia (slowed movements, interrupted movements, decremental amplitude). The limbs are assessed for rest, postural and kinetic tremor. Posture, gait and balance are rated. Each item is scored from 0 (normal) – 4 (most severely affected). Individuals found to have signs of PD then had a complete neurological examination (including reflexes, cerebellar signs, cranial nerves et al). In addition the presence of isolated motor signs which did not meet the full diagnostic criteria for
Parkinson’s disease were noted: for example isolated rest tremor, isolated rigidity or clear gait/postural disturbances. Training on administration of the UPDRS was obtained by viewing the training videos on the Movement Disorders Society website (http://www.movementdisorders.org/).

Subtle motor dysfunction was assessed using 2 timed motor tests. For the upper limbs the Purdue pegboard was used. This requires the participant to place as many pegs as they can into a row of holes in 30 seconds. Using their dominant hand then their non-dominant hand and then both hands simultaneously. This was performed with the participants seated comfortably in front of a desk. For the lower limbs a timed 3-metre walk was used. In this the participant sits in a chair, stands, walks 3 metres then turns and returns to sit. The participant is instructed to walk at a normal brisk pace for them. Patients with painful bone or joint disease inhibiting movement were excluded from the timed motor tests.

Symptoms of autonomic dysfunction were screened for using the autonomic subscale of the Unified Multiple Systems Atrophy Rating Scale (UMSARS). This asks about the presence of constipation, urinary dysfunction, erectile dysfunction and orthostatic symptoms. The REM Sleep Behaviour (RBD) questionnaire was administered to detect symptoms of RBD in the study cohort.

**Determination of age related risk of Parkinson’s disease in heterozygous GBA mutation carriers**

Family history data on the health status and current age or age and cause of death was obtained for parents of patients with Type I GD using standard clinical family history technique. A Kaplan-Meier analysis was performed using PASW (version 20.1, IBM) to define the age related risk of PD in these obligate carriers.

**Clinical phenotyping of PD cases with GBA mutations**
Study participants with PD who did or did not carry a heterozygous $GBA$ mutation were assessed identically. All had a full neurological history and neurological examination to assess age of onset and clinical features of the Parkinsonian syndrome affecting them. The full UPDRS, UPSIT, MoCA, RBD questionnaire and the non-motor symptoms scale were applied to assess the motor and non-motor phenotype of these individuals in detail.

**Optical Coherence Tomography**

OCT was performed by Dr Gloria Roberti and Dr Gerassimos Lascaratos (under Prof Garraway-Heath) at Moorfields Eye Hospital. OCT was performed using Fourier domain OCT (RTVue-100, Optovue Inc, Fremont, CA). OCT was performed with dilated pupils in both eyes, after ensuring that intra-ocular pressure was normal and visual fields were full. The ganglion cell complex thickness (GCC), which extends from the internal limiting membrane to the inner nuclear layer, and retinal nerve fiber layer (RNFL) thickness were measured as markers of retinal degeneration. Exclusion criteria were intraocular pressure $\geq 22$ mmHg in either eye; evidence of a reproducible visual field defect in either eye; a myopic refractive error exceeding -8.00 D; intraocular surgery in the study eye; history of ocular trauma in the study eye. Mean GCC and RNFL thickness were compared between groups using a student's t-test (IBM, PASW version 20.0). Both eyes from each individual were included in the analysis.

**DaTSCAN imaging in monogenetic PD**

We formed a consortium of Movement Disorders and Neurogenetics centres from Western Europe, South America and Asia to identify a large series of monogenetic PD cases. Mutations in $GBA$, $SNCA$, $LRRK2$, Parkin, or $PINK1$ were identified by molecular genetic sequencing according to standard clinical protocols. From amongst such patients individuals who had been imaged with DaTSCAN as part of their initial clinical evaluation were identified by retrospective chart review.

A region of interest (ROI) analysis was performed using Image J (NIH, Bethesda, MD). Four ROIs were anatomically defined: right and left caudate, right and left putamen. To permit calculation of background signal a region of occipital cortex was designated as a 5th ROI. The
outcome measure was the specific-to-nondisplaceable binding ratio V3” (ROIstriatum –
ROIoccipital/ROIoccipital). For each ROI, 4 transaxial slices were analysed. Four slices
showing the most intense radiotracer uptake were selected for analysis from each case. For each
case the right:left asymmetry index (ASI, most severely affected ROI V3”/least severely affected
ROI V3”) was calculated. The whole striatal asymmetry index was also calculated (SASI:
(ipsilateral-contralateral striatum V3”)/((ipsilateral+contralateral striatumV3”)/2)*100%)
Images were analysed blind to the genetic diagnosis by a single investigator (AM), apart from
the SNCA scans which were reported by SB. Ethical approval was granted by the relevant bodies
from each participating centre. Written informed consent was taken from each participant.

Statistical analysis was performed with PASW 20.1 (version 20, IBM). Differences in ASI
between genotypes were sought using the Mann-Whitney U-test. Significance was taken at the
5% level (p=0.05), with bonferroni correction (i.e. p=0.05/n where n=number of comparisons).
The SASI was compared between genotypes with a linear regression analysis. Correlations
between disease duration and with SASI were sought using Spearman's correlation.

**General statistical analysis of prodromal markers**

All analysis were performed using PASW (SPSS version 20.1, IBM). Statistical advice was
provided by Dr Fox, Education Unit UCL Institute of Neurology.

UPSIT, MoCA, MMSE, UPDRS, RBD and UMSARS scores are not normally distributed.
Differences between groups in the assessed marker were tested for with a Kruskall-Wallis test
(the non-parametric equivalent of ANOVA). Post-hoc testing with a Mann-Whitney U-test was
then performed. Since multiple comparisons were made Bonferroni correction was applied,
being 0.05/n (where n is the number of comparisons made). Two comparisons were made for
each null hypothesis tested (controls versus GD and controls versus carriers) so significance at
the 5% level was taken as a p value of 0.025.
Categorical variables were compared using the chi-squared test. Bivariate correlations between prodromal markers were investigated using Spearman's test. Bonferroni correction was not applied to chi-squared or correlation statistics. All results are quoted as mean +/- 1 SD or median and interquartile range.

Ic. Results

Demographic, Clinical & Molecular Genetic Characteristics Of Patient Cohort

A total of 83 previously diagnosed GD Type I patients (female 37: male 46, mean age 49.1 +/- 15 years, range 18-89 years) were initially recruited. Of these the following 6 individuals were excluded from the study of prodromal markers: GD7 and GD9 were both recent immigrants from Eastern Europe and so not familiar with the U.K SIT and there was a prohibitive language barrier, GD15 and GD43 were brothers and excluded as they had the R262G/R262G genotype not typical of Type I GD, GD43 as he had L444P/L444P genotype typical of Type II GD and GD26 as he was under the age of 18 years. Additionally 2 GD patients gave family history data and blood but refused the phenotyping study. The GD patients with atypical genotypes were enrolled in the family history study as they could provide family history data and had parents who were willing to participate as obligate carriers.

Thus for the study of prodromal markers 75 patients with Type I GD were enrolled (female 40: male 35, mean age 50.9 +/- 14 years, range 22-89 years) and 41 carriers (female 24: male 17, mean age 61 +/- SD 11.9 years, range 40-81 years). The mean age of the carriers was significantly older than that of the GD patients (students t-test, P<0.0001), as expected given that the carriers were the parents of the GD patients. Thirty four age/sex matched controls (male 16 : female 18, mean age 56.6 +/- 14 years, range 23 – 88 years) were recruited. The controls were age matched to the GD cases (students t-test, p=0.062) and carriers (students t-test, p=0.1). The controls were sex matched to both the GD cases and controls. None of the study cohort or controls had been previously diagnosed with PD or dementia. The controls were free of any neurological or systemic disease which might impair cognitive or motor function. Controls were
recruited from the following sources: non-carrier relatives of GD patients (7/34, 20%), relatives of PD patients (10/34, 29%), non-carrier members of the U.K. Gaucher Disease association (2/34, 5.8%) and volunteers from laboratory and office staff at the Department of Clinical Neurosciences, UCL (15/34, 44%). Of the GD patients 17/83 (20%) were of Ashkenazi Jewish descent, 5/83 (6.25%) were Eastern European, 2/83 (2.5%) South African, 1/83 (1.25%) Afro-Carribean, 1/83 (1.25%) Sri Lankan and the remainder white UK born who had no recorded Ashkenazi heritage (55/83, 68.75%). Among carriers 6/41 (17%) were Ashkenzi Jewish, 1 Eastern European and 1 Afro-Carribean with the remainder (33/41, 75%) white UK born individuals with no recorded Ashkenazi heritage. Among controls 7/34 (20%) were Ashkenazi Jewish while the remainder where White, UK citizens with no reported Jewish ancestry. The controls were matched by ethnicity to both the GD cases and carriers.

For GD patients the majority (65/83, 78%) had at least one N370S allele, with the most common genotype being N370S/L444P (17/83, 20.5%) followed by N370S/N370S (14/83, 16.8%). The remainder were compound heterozygous for N370S and a rare point mutation on the second allele. In addition 5 patients carried novel GBA mutations with the following genotypes: N370S/IVS9+1, R262G/L444P, N370S/A341V, N370S/V447E, N370S/G250V. None of the GD patients had clinical features of Type III disease such as generalised seizures or progressive myoclonic epilepsy, but formal oculomotor testing was not available. Table 2 summarises the clinical and molecular genetic features of the GD patients. For carriers the most common genotype was N370S (15/41, 36%). Clinical and molecular genetic data for the carriers are summarised in table 3. The presence of a GBA mutation in carriers and absence of a GBA mutation in control subjects was confirmed by sequencing of the GBA gene.
Table 3. Clinical, demographic and genetic characteristics of GD patient cohort.

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<th>Bone disease</th>
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Key: PD = Parkinson’s disease, D=dementia, (f) = father, (m)=mother, (gf)=grandfather, (gm)=grandmother, (s)=sibling. *=excluded as genotype not typical for Type I GD, ^=excluded as language barrier prohibited assessment, #=excluded as aged under 18 years. Fhx = family history only and no phenotyping performed.
Table 4. Clinical, demographic and genetic characteristics of carriers

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<td>C20</td>
<td>72, F</td>
<td>White</td>
<td>N370S</td>
</tr>
<tr>
<td>C21</td>
<td>65, F</td>
<td>White</td>
<td>N370S</td>
</tr>
<tr>
<td>C22</td>
<td>65, M</td>
<td>White</td>
<td>L444P</td>
</tr>
<tr>
<td>C23</td>
<td>62, M</td>
<td>White</td>
<td>N370S</td>
</tr>
<tr>
<td>C24</td>
<td>53, F</td>
<td>White</td>
<td>RecNcil</td>
</tr>
<tr>
<td>C25</td>
<td>66, M</td>
<td>White</td>
<td>RecNcil</td>
</tr>
<tr>
<td>C26</td>
<td>64, F</td>
<td>White</td>
<td>N370S</td>
</tr>
<tr>
<td>C27</td>
<td>74, F</td>
<td>White</td>
<td>G250V</td>
</tr>
<tr>
<td>C28</td>
<td>74, M</td>
<td>White</td>
<td>N370S</td>
</tr>
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<td>C29</td>
<td>74, F</td>
<td>Ashkenazi</td>
<td>N370S</td>
</tr>
<tr>
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<td>62, M</td>
<td>White</td>
<td>L444P</td>
</tr>
<tr>
<td>C31</td>
<td>41, F</td>
<td>Ashkenazi</td>
<td>N370S</td>
</tr>
<tr>
<td>C32</td>
<td>42, F</td>
<td>White</td>
<td>L444P</td>
</tr>
<tr>
<td>C33</td>
<td>61, M</td>
<td>White</td>
<td>N370S</td>
</tr>
<tr>
<td>C34</td>
<td>52, F</td>
<td>White</td>
<td>RecNcil</td>
</tr>
<tr>
<td>C35</td>
<td>65, M</td>
<td>N370S</td>
<td>RecNcil</td>
</tr>
<tr>
<td>C36</td>
<td>64, F</td>
<td>White</td>
<td>N370S</td>
</tr>
<tr>
<td>C37</td>
<td>73, F</td>
<td>White</td>
<td>G250V</td>
</tr>
<tr>
<td>C38</td>
<td>73, M</td>
<td>White</td>
<td>N370S</td>
</tr>
</tbody>
</table>
Prodromal markers of Parkinson's disease in GD patients compared to controls

Results are summarised in figures 2-4. Compared with controls GD patients demonstrated an impairment of olfaction (control median 35.1 [interquartile range 34-37] vs GD median 31.5 [interquartile range 30 – 36] Mann-Whitney U-test, P=0.004). Three GD patients were excluded from the smell identification test as they were current smokers. The median MoCA score was lower in GD patients versus controls (GD median 27 [IQR 24.5-28] vs control median 28 IQR [26.75-28.7], p=0.022). There was no difference in MMSE scores (GD median 30 [IQR 29-30] vs control median 29 [IQR 29 – 30], P=0.251).

Four GD patients had signs compatible with early parkinsonism upon clinical examination. GD18 had bilateral rigidity with activation manoeuvre, postural tremor and gait impairment. GD35 had an asymptomatic left upper limb rest tremor and bilateral upper limb rigidity with activation manoeuvre. GD52 had a flexed posture, bilateral rigidity, and a postural and kinetic tremor of the upper limbs. GD76 had asymmetrical rigidity of the upper limbs with activation manoeuvre and dragged the left leg when walking. In addition the UPDRS part III (P=0.006) scores were significantly higher in GD patients (median 0 [IQR 0 – 2.75]) than age matched controls (median 0 [IQR 0-0]). There was no difference in the UPDRS Part II scores (GD median 0 [IQR 0-0] vs control median 0 [IQR 0-0], P=0.035).

There was no difference on the score from the RBD questionnaire (GD median 0 [IQR 0 – 0] vs control median 0 [IQR 0 – 0], P=0.45) nor the UMSARS score between GD cases and controls (GD median 0 [IQR 0 – 0.5] vs control median 0 [IQR 0 - 0], P=.059). Five GD patients gave a
positive response to the RBD questionnaire using a cut off score of 5, which is suggestive of possible RBD, compared with none of the control group. This was not significant on the chi-squared test.

**Prodromal markers of Parkinson's disease in heterozygous carriers compared to controls**

Results are summarised in figures 2-4. Compared with controls heterozygous *GBA* mutation carriers demonstrated an impairment of olfaction (carrier median 32 [IQR 27 - 35] vs control median 35.1 [IQR 34 - 37], P<0.001). One Eastern European carrier was excluded from the smell identification test as he was not sufficiently familiar with the odours used for UK populations, 2 were excluded due to coryzal illness, 1 excluded as they were blind and unable to read the test card. The MoCA demonstrated impaired cognition in carriers versus controls (carrier median 26 [IQR 24 - 27] vs control median 28 IQR [26 - 28.7], P=0.004), but there was no overall difference in MMSE scores (carrier median 29.5 [IQR 28.75 – 30] vs control median 29 [IQR 29 – 30], P=0.60). One carrier was excluded from cognitive function testing due to blindness.

Two carriers had physical signs compatible with early parkinsonism. C23 had bilateral rigidity, mask like facies and slow movements without decremental amplitude while C34 had minimal masked facies, bilateral rigidity with activation manoeuvre, left arm kinetic tremor and flexed posture. The UPDRS part III score was significantly higher in carriers than controls (carrier median 0 [IQR 0 – 2] vs control median 0 [IQR 0-0], P=0.004). There were no significant differences in the UPDRS part II (carrier median 0 [IQR 0 – 0] versus control median 0 [0-0], P=0.1), the UMSARS (carrier median 0 [IQR 0 – 0] vs control median 0 [IQR 0 – 0], P=0.1) or the RBD questionnaire scores (carrier median 0 [IQR 0 – 0] vs control median 0 [IQR 0 – 0],
P=0.57) between carriers and controls. No carrier gave a positive response to the RBD questionnaire using 5 as the cut off score.

**Timed motor tests in GD patients and carriers**

The average number of pegs placed in the Purdue pegboard in 30 seconds (number of pegs placed with right hand plus number placed with left hand divided by 2) differed significantly between controls, carriers and GD patients (Kruskal-Wallis, p=0.01). *Post-hoc* testing demonstrated a trend for GD patients to place fewer pegs than controls (GD patients median 13.74 [IQR 13 – 15] vs control median 14.5 [IQR 13.4 – 15.5], Mann-Whitney, p=0.047) while carriers placed significantly fewer pegs than controls (carrier median 13 [IQR 11.5 – 14.5] vs control median 14.5 [IQR 13.4 – 15.5] Mann-Whitney, p=0.001).

The time taken to complete the 3-metre walk test differed significantly between the control, carrier and GD groups (Kruskal-Wallis, p=0.001). *Post-hoc* testing revealed that it took significantly longer for carriers (median 8 seconds [IQR 7 – 8 seconds, Mann-Whitney, p<0.001) and GD patients (median 8 seconds [IQR 7-8], Mann-Whitney, p<0.0001) to complete the 3-metre walk than controls (median 6 seconds [IQR 6 - 7]).
Figure 3. Box plot of UPSIT scores in Gaucher disease patients and carriers versus controls.

GD patients (median 31.5, IQR 30-36) and heterozygous mutation carriers (median 32, IQR 27-35) demonstrated a significant impairment of olfaction compared to controls (median 35.1, IQR 34-37).
Figure 4. Box plot of UPDRS Part III score in Gaucher disease patients and carriers versus controls.

GD patients (median 0, IQR 0-2.75) and heterozygous carriers (median 0, IQR 0-2) had significantly higher UPDRS part III scores than controls (median 0, IQR 0-0)
Figure 5. Box plot of MoCA scores in Gaucher disease patients and carriers versus control. GD patients (median 27, IQR 24.5-28) and heterozygous carriers (median 26, IQR 24-27) scored significantly less on the MoCA than controls (median 28, IQR 26.75-28.8).
Comparison of Montreal cognitive assessment domains between GD, carriers and controls
The MoCA subscale scores (given as median and IQR) for GD patients and carriers compared to control are given in Table 4. Differences in subscale scores were examined using the Mann-Whitney U-test. Significant differences are highlighted in bold.

**Table 5. Montreal cognitive assessment subscale scores.**

<table>
<thead>
<tr>
<th>Subscale</th>
<th>Control</th>
<th>GD</th>
<th>Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Executive</td>
<td>5, 4-5</td>
<td>5, 4-5, P=0.159</td>
<td>5, 3-5, P=0.129</td>
</tr>
<tr>
<td>Memory</td>
<td>3, 3-3</td>
<td>3, 3-3, P=0.558</td>
<td>3, 3-3, P=0.236</td>
</tr>
<tr>
<td>Naming</td>
<td>3, 2-4</td>
<td>3, 1-4, P=0.304</td>
<td>3, 1-4, P=0.409</td>
</tr>
<tr>
<td>Attention</td>
<td>6, 6-6</td>
<td>6, 5-6, P=0.004</td>
<td>6, 5-6, P=0.007</td>
</tr>
<tr>
<td>Language</td>
<td>3, 3-3</td>
<td>3, 2-3, P=0.029</td>
<td>3, 2-3, P=0.042</td>
</tr>
<tr>
<td>Abstraction</td>
<td>2, 2-2</td>
<td>2, 2-2, P=0.879</td>
<td>2, 1-2, P=0.016</td>
</tr>
<tr>
<td>Orientation</td>
<td>6, 6-6</td>
<td>6, 6-6, p=0.32</td>
<td>6, 6-6, P=0.236</td>
</tr>
</tbody>
</table>

Correlations between olfactory, cognitive and motor function in GD patients and carriers
Several GD patients and carriers had more than one clinical marker of neurodegeneration, statistical associations were therefore sought between them. Statistically the UPSIT score was correlated negatively with UPDRS part III in GD patients (Spearman's rho -0.46, p<0.001) and carriers (Spearman's rho -0.41, p=0.011), ie lower olfactory function scores were associated with higher UPDRS part III scores. The UPSIT and MoCA were positively correlated in GD patients (rho=0.257, p=0.034) and the correlation approached significance in carriers (rho=0.326, p=0.052).

In GD the number of pegs placed in the purdue pegboard was negatively correlated with the UPDRS III score (Spearman's rho -0.44, P<0.001) and approached a significant negative correlation in carriers (Spearman's rho = -0.339, P=0.058). The number of pegs placed correlated positively with UPSIT scores in GD (rho = 0.288, P=0.03) and carriers (rho = 0.549, P=0.002).
The time taken for the 3 minute walk correlated positively with UPDRS III scores in GD (rho = 0.434, P=0.004) and carriers (rho = 0.381, P=0.024). There was no correlation of the 3 minute walk time with UPSIT scores in GD (rho = -0.37, p=0.8) or carriers (rho =-0.179, p=0.3). The 3-minute walk was correlated negatively with the pegboard score in GD (rho =-0.478, p=0.002) but there was no correlation in carriers (rho = -0.21, p=0.234).

Table 5 describes the numbers of individuals who expressed one prodromal marker in isolation or who manifested combinations of markers. Hyposmia was defined as an UPSIT score less than the 15th centile expected for age and sex and dementia as a score <24/30 on the MoCA. No controls scored in the hyposmia or dementia range.

**Table 6. Summary of prodromal markers of Parkinson’s disease occurring in isolation and combination.**

<table>
<thead>
<tr>
<th></th>
<th>GD</th>
<th>Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated hyposmia</td>
<td>5/75 (6.6%)</td>
<td>5/41 (12%)</td>
</tr>
<tr>
<td>(score &lt;15th centile for age/sex)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated dementia</td>
<td>4/75 (5.3%)</td>
<td>2/41 (4.8%)</td>
</tr>
<tr>
<td>(score &lt;24/40 on MoCA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated clear motor sign</td>
<td>2/75 (2.6%)</td>
<td>1/41 (2.4%)</td>
</tr>
<tr>
<td>(e.g. rest tremor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyposmia &amp; cognitive impairment</td>
<td>1/75 (1.3%)</td>
<td>2/41 (4.8%)</td>
</tr>
<tr>
<td>Hyposmia &amp; clear motor sign</td>
<td>1/75 (1.3%)</td>
<td>0/41</td>
</tr>
<tr>
<td>Hyposmia, cognitive impairment and clear motor sign</td>
<td>1/75 (1.3%)</td>
<td>0/41</td>
</tr>
<tr>
<td>Cognitive impairment &amp; clear motor sign</td>
<td>1/75 (1.3%)</td>
<td>1/41 (2.4%)</td>
</tr>
</tbody>
</table>
Correlations between age and neurodegenerative markers in Gaucher disease

Since Parkinson's disease is strongly age related, correlations between age of GD patients and abnormalities in neurodegenerative markers were sought. UPSIT scores demonstrated a significant negative correlation with age in GD patients (rho = -0.269, P=0.030). Higher UPDRS part III scores were strongly correlated with increasing age in GD patients (rho = 0.50, P=<0.001) while MoCA scores had a significant negative correlation with age (rho = -0.44, P <0.001). The UPSIT scores in carriers had a significant negative correlation with age (rho = -0.43, p=0.009). Neither the UPDRS part III score (rho = 0.251, p=0.124) nor the MoCA score correlated with age (rho = -0.25, p=0.124) in carriers. The UPSIT score did not correlate with age in controls (rho = 0.15, p=0.4), neither did UPDRS III (rho =0.07, p=0.7) score or the MoCA score (rho =0.04, p=0.8).
Optical coherence tomography in Gaucher disease patients

Eleven GD (5 female, mean age 63.7 years, range 45-89 years, 6/11 N370S/N370S genotype and remainder N370S/L444P) and 7 healthy controls with no history of neurological disease matched for age (mean age 67, range 51-85, p=0.9 vs GD) and sex (4 female) were studied. None had ocular pathology. Four of the GD patients had clinical markers of prodromal PD; 1 with hyposmia, 1 with cognitive impairment, 1 with hyposmia and cognitive impairment, 1 with rigidity, flexed posture and postural tremor. None of the participants met diagnostic criteria for PD.

The mean GCC thickness for all GD patients (mean 92 +/- 7 uM) did not differ from controls (mean 93.4 +/- 8 uM, p=0.6). Mean GCC thickness for GD patients with neurological signs (86+/−7 uM) was significantly less than that of controls (p=0.044) or GD patients without neurological signs (93.6+/−5 uM, p=0.024) (figure 5). Mean RNFL thickness was significantly less in GD patients with a prodromal sign (91.28 +/-6.5uM, p=0.03) than controls (mean 105+/−16 uM) but not GD patients without a sign (100+/−8 uM, p=0.3). The difference in the mean GCC and RNFL thickness remained significant after correcting for the participants’ refractive error.
Figure 6. Mean Ganglion Complex Thickness in GD compared to control.
Bars represent mean and 95% confidence intervals. Note decreased mean ganglion complex thickness in GD patients with a prodromal sign of PD compared to both controls and GD patients without a sign.
Demographic, Clinical & Molecular Genetic Features of Parkinson's disease patients with GBA mutations

Sequencing of the GBA gene in 220 cases of typical PD from the Royal Free Hospital Movement Disorders database identified 12 PD patients with a heterozygous GBA mutation (designated PD-GBA) of whom 9 were available for enrolment into this study. Three parents of GD patients were identified who had PD. Giving a cohort of 12 PD-GBA for phenotyping (5/12 female, mean age 65 (range 49-81 years), 1/12 (8%) Ashkenazi Jewish, mean disease duration 8 years (range 2-15). Twenty of the 208 GBA mutation negative PD cases were selected as controls (7/20 female, mean age 65 (range 50-83), 2/12 (10%) Ashkenazi Jewish, mean disease duration 9 years (range 2-20years), designated as sporadic PD (PD-S). All PD cases in the Royal Free Hospital Movement Disorders database had previously had the G2019S LRRK2 mutation excluded by direct sequencing. Table 6 summarises the characteristics of the PD-GBA cases. Mutations in the PD-GBA group who underwent clinical phenotyping were: N370S (5/12, 42%), L444P (2/12, 16%), recombinant alleles (2/12, 16%), R496H (1/12, 8.3%), V460L (1/12, 8.3%) and IVS2+1 (1/12, 8.3%).
Table 7. Clinical and genetic characteristics of PD-GBA and PD-S patients

<table>
<thead>
<tr>
<th></th>
<th>PD-GBA (n=12)</th>
<th>PD-S (n=20)</th>
<th>Significance</th>
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<tbody>
<tr>
<td>Sex</td>
<td>5F:7M</td>
<td>7F:13M</td>
<td>P=0.067</td>
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<tr>
<td>GBA Mutation (number)</td>
<td>N370S (5)</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L444P (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recombinant (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IVS2+1 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>V460L (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R496H (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mean, range)</td>
<td>65, 49-81</td>
<td>65, 50-83</td>
<td>P=0.90</td>
</tr>
<tr>
<td>Family history (%)</td>
<td>2/12, 16%</td>
<td>2/20, 10%</td>
<td>P=0.06</td>
</tr>
<tr>
<td>Disease duration</td>
<td>8, 2-15</td>
<td>9, 2-20</td>
<td>P=1.0</td>
</tr>
<tr>
<td>UPDRS Part I score (median, interquartile range)</td>
<td>8, 5-14</td>
<td>9, 5-14</td>
<td>P=0.95</td>
</tr>
<tr>
<td>UPDRS Part II score (median, interquartile range)</td>
<td>17, 9-24</td>
<td>18, 9-20</td>
<td>P=0.67</td>
</tr>
<tr>
<td>UPDRS part III score (median, interquartile range)</td>
<td>33, 26-35</td>
<td>34, 30-46</td>
<td>P=0.13</td>
</tr>
<tr>
<td>UPDRS Part IV score (median, interquartile range)</td>
<td>1, 0-5</td>
<td>0, 0-1</td>
<td>P=0.135</td>
</tr>
<tr>
<td>On L-dopa treatment</td>
<td>8/12, 75%</td>
<td>12/20, 60%</td>
<td>P=0.067</td>
</tr>
<tr>
<td>On dopamine agonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIT score (median, interquartile range)</td>
<td>16</td>
<td>15</td>
<td>P=0.50</td>
</tr>
<tr>
<td>MoCA score (proportion &lt;26/30)</td>
<td>7/12, 58%</td>
<td>5/20, 25%</td>
<td>P=0.0014*</td>
</tr>
<tr>
<td>NMSS score (median, interquartile range)</td>
<td>104, 69-134</td>
<td>38.5, 16.25-60</td>
<td>P=0.002*</td>
</tr>
<tr>
<td>RBDQ score (proportion score &gt;6/10)</td>
<td>7/12, 58%</td>
<td>1/20, 5%</td>
<td>P=0.0016*</td>
</tr>
</tbody>
</table>
All PD patients enrolled had a classical phenotype of idiopathic PD which met Queen Square Brain Bank criteria (Hughes et al, 1992). Two of the PD-GBA patients had an autosomal dominant family history of PD. All PD patients were assessed in the “on” state. The motor phenotype of these individuals was very similar to sporadic PD, and none had features of a “Parkinson’s plus” syndrome. None had cranial nerve palsies, pyramidal signs or clinical evidence of peripheral neuropathy. Seven of the 10 PD-GBA patients had a tremor predominant phenotype while 3/10 had a predominant bradykinetic-rigid phenotype. Of the 7 tremulous patients 4 had typical rest tremor of the upper limbs while 3 had action tremor of the upper limbs. The PD-GBA and PD-S cases were matched for use of levo-dopa (75% vs 60%, p=0.067) and UPDRS parts I (PD-GBA median 8 vs PD-S 9, p=0.95), II (17 vs 18, p=0.067), III (33 vs 34, p=0.13) and IV scores (1 vs 0, p=0.13). No patient had deep brain stimulation but two PD-GBA patients were on selective serotonin reuptake inhibitors for depression. The degree of hyposmia in the PD-GBA and PD-S cases was identical (UPSIT score median 16/40 vs 15/40, Mann-Whitney U-test p=0.503). Using a cut off score of 26/30 on the MoCA, significantly more PD-GBA had cognitive impairment than PD-S (7/12 [58%] vs 5/20 [25%], chi-squared test p = 0.014). PD-GBA scores for abstraction (p=0.034) and orientation (p=0.022) were significantly lower. The overall NMSS score was higher for PD-GBA than PD-S (median 104 [interquartile range 69-134] vs 38 [16-60], p = 0.002). PD-GBA reported more symptoms per participant than PD-S (median 13 [range 1-19] versus median 7 [range1-14], p=0.0012). The following were more common in PD-GBA: falls with loss of consciousness (25% vs 0%, p=0.04), fatigue (92% vs 30%, p=0.001), unexplained pain (58% vs 10%, p=0.005), loss of interest in life (66% vs 5%, p=0.0004), anxiety (66% vs 20%, p=0.02), apathy (50% vs 10%, p=0.03). Using the recommended cut off score of 5/10, more PD-GBA participants screened positive for REM sleep disorder than PD-S (7/12 [58%] vs 1/20 [5%], p= 0.0016).

The MoCA subscale scores were compared between PD-GBA and PD-S in table 7. Results are given as median with IQR. Differences in MoCA subscale scores were examined with the Mann-Whitney U-test.
Table 8. Montreal cognitive assessment subscale scores in PD-GBA compared to PD-S

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PD-S Controls</th>
<th>PD-GBA</th>
<th>Significance GBA vs sporadic PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Executive</td>
<td>5, 4-5</td>
<td>4, 3.75 – 4.25</td>
<td>3.5, 2-5</td>
<td>P=0.59</td>
</tr>
<tr>
<td>Memory</td>
<td>3, 3-3</td>
<td>2.5, 2-4</td>
<td>3, 2-3</td>
<td>P=0.89</td>
</tr>
<tr>
<td>Naming</td>
<td>3, 2-4</td>
<td>3, 3-3</td>
<td>3, 3-3</td>
<td>P=1.0</td>
</tr>
<tr>
<td>Attention</td>
<td>6, 6-6</td>
<td>6, 5-6</td>
<td>5, 4-6</td>
<td>P=0.2</td>
</tr>
<tr>
<td>Language</td>
<td>3, 3-3</td>
<td>3, 3-3</td>
<td>3, 3-3</td>
<td>P=0.58</td>
</tr>
<tr>
<td>Abstraction</td>
<td>2, 2-2</td>
<td>2, 2-2</td>
<td>3, 2-3</td>
<td>P=0.034</td>
</tr>
<tr>
<td>Orientation</td>
<td>6, 6-6</td>
<td>6, 6-6</td>
<td>5, 4-6</td>
<td>P=0.022</td>
</tr>
</tbody>
</table>

**Determination of age related risk of Parkinson’s disease in carriers of heterozygous GBA mutations**

Data on 166 parents of Type I GD patients was available (17/83 [20%] families Ashkenazi Jewish, 5/83 [6%] Eastern European and the remainder White UK citizens, no consanguineous families). Eighty-three families were enrolled in the family history study as 2 GD probands consented to give family history but not to other parts of the study. A PD case was defined by the proband reporting a definite medical diagnosis in their parents. Ten cases of PD were identified in obligate carriers, 3 were examined in person to confirm diagnosis (these are included in the cohort of 12 PD-GBA described above). Of the 10 cases of PD in relatives 5 were male (50%), and mean age of onset was 67 (standard deviation 8.6 years). Figure 6 shows a Kaplan-Meier analysis for developing PD for men and women combined. Age related risk of PD peaked at age 80 (15%). Based on the London population (5) 0.33 cases of PD would be expected in our cohort of 166 (derived from lifetime prevalence of 2/1 000 for men and women). This gave a life time relative risk of 30 (95 % CI 7-122).
Figure 7. Kaplan-Meier analysis of age related risk of developing Parkinson’s disease. Risk is derived from family history data on 166 obligate carriers of glucocerebrosidase mutations (parents of Type I Gaucher disease patients), combined for men and women. Overall lifetime RR of Parkinson’s disease was 30 (95% CI 7 to 122) in carriers compared to the London population.
DaTSCAN imaging in monogenetic PD

Clinical and genetic characteristics of the 36 monogenetic PD patients enrolled are given in Table 8. Control DaTSCAN images were available from 12 individuals without neurological disease from the European Nuclear Medicine Consortium (provided by Dr John Dickson, Nuclear Medicine Department University College London Hospital). All scans were performed according to standard clinical protocols. All of the DaTSCANS utilised in the current study had previously been reported as showing reduction of radioligand binding compatible with PD.

Ethical approvals were obtained at each centre and written informed consent taken as required. The PINK1 (Samaranch et al, 2010), heterozygous GBA mutation associated PD (McNeill et al, 2012b), Brazilian Parkin and LRRK2 patients (Barsottini et al, 2009), SNCA cases (Bostantjopoulou et al, 2008) and Italian Parkin cases (Varrone et al, 2004) have been reported previously.

The age at assessment of the GBA group (50+/-13 years) was not significantly older than the Parkin group (44+/-14, p=0.14), PINK1 (42+/-17, p=0.07), LRRK2 (40+/-7, p=0.40) or SNCA (47 +/-7, p=0.58) groups. The proportion of men in the GBA mutation group (7/7, 100%) did not differ from that of the Parkin (9/12, 75%), PINK1 (5/7, 71%, p>0.05), LRRK2 (2/2, 100%, p>0.5) or SNCA (5/8, 62.5%) groups. The disease duration did not differ between groups (one way ANOVA, F=2.3, p=0.076). The UPDRS part III scores for the PINK1 group were significantly lower than the other genetic subgroups (p<0.05 for all comparisons with Bonferroni correction). There was no difference between the proportion of patients in each genetic subgroup taking levo-dopa. The control group were all of Western European ancestry but were matched to the PD group for age (52+/-12 years, p>0.05) and sex (7/12 male, chi-squared test p>0.05).
A comparison of right:left asymmetry index (ASI) and whole striatal asymmetry index (SASI) between genotypes was performed. Table 9, figure 7 and figure 8 describe the ASI for putamen and caudate and SASI for each group. Figure 9 shows representative DaTSCANS from monogenetic PD cases.

The caudate ASI for the PD cases with GBA mutations (Mann-Whitney U-test, median ASI 0.42 [0.33-0.56], p<0.001) or LRRK2 G2019S mutations (0.75 [0.59-0.86], p=0.005) was significantly different to the control value of 0.92 (interquartile range 0.89-0.92). The caudate asymmetry indices for PD with bi-allelic Parkin (median 0.88 [0.72-0.93], p=0.098), PINK-1 (median 1.0 [0.9-1.13], p=0.065) or SNCA (median 1.02 [0.9-1.13], p=0.91) did not differ from the control value. This indicates an asymmetric reduction of radioligand uptake in the caudate of PD associated with GBA and LRRK2 mutations with a relatively symmetrical reduction in ligand uptake in the caudate of PINK1, Parkin and SNCA mutation associated PD.

The putamen ASI for PD cases with GBA mutations (median 0.18 [0.08-0.33], p<0.001) and LRRK2 (median 0.49 [0.13-0.5], p=0.01) differed significantly from the control value (median 0.89 [0.82-0.96]). The putamen ASI for bi-allelic Parkin (median 0.6 [0.1-1.0], p=0.68), PINK1 (median 1.05 [0.9-1.4], p=0.14) and SNCA (median 1.05 [0.92-1.22], p=0.30) mutation associated PD did not differ from control values (median 0.89 [0.82-0.86]). This indicates an asymmetric reduction of radioligand uptake in the putamen of PD associated with GBA and LRRK2 mutations with a relatively symmetrical reduction in ligand uptake in the putamen of PINK1, Parkin and SNCA mutation associated PD.
To further explore these findings, the whole striatum asymmetry index (SASI) was calculated. A linear regression analysis demonstrated a significant difference in the SASI between genotypes (r-squared = 0.233, p<0.001). The highest SASI value was for PD-GBA (mean 80.5+/−5.1), followed by LRRK2 G2019S (44.6+/−5.3), Parkin (32.7+/−5.6), PINK1 (13.16+/−4.4), SNCA (13.4+/−3.9) and controls (8.3+/−2.7). This supports the results from the caudate and putamen ASI which demonstrate that loss of radioligand uptake is relatively asymmetrical in PD associated with GBA and LRRK2 G2019S mutations and relatively symmetrical in Parkin, PINK1 and SNCA.

To investigate clinical variables which may confound the relationship between genotype and dopaminergic neuronal imaging symmetry, correlations between SASI and patient characteristics were sought. There was no correlation between disease duration (Spearmans rho 0.09, p=0.60) or patient age at evaluation (Spearmans rho 0.15, p=0.2) and SASI.
Table 9. Clinical, demographic & genetic characteristics of Parkinson’s disease patients who underwent DaTSCAN.

<table>
<thead>
<tr>
<th></th>
<th>GBA</th>
<th>PARKIN</th>
<th>PINK1</th>
<th>LRRK2</th>
<th>SNCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>7/7(100%)</td>
<td>9/12 (75%)</td>
<td>5/7(55%)</td>
<td>½ (50%)</td>
<td>5/8 (62.5 %)</td>
</tr>
<tr>
<td>Age at scan</td>
<td>50+/-13 years</td>
<td>44+/-14 years</td>
<td>42+/- 17 years</td>
<td>32 and 53</td>
<td>47.1+/-7.5</td>
</tr>
<tr>
<td>Disease duration</td>
<td>7+/- 4 years</td>
<td>14.5+/-10</td>
<td>12.3+/- 11 years</td>
<td>5 and 11</td>
<td>7.4+/-2 years</td>
</tr>
<tr>
<td>Mutations</td>
<td>N370S/L444P (1/7), N370S (1/7), L444P (1/7), IVS2+1G&gt;A(1/7), E326K(1/7), T369M (1/7), R496H (1/7), Del exon 3/del exon 3 (1/12), Del exon 3-4/del exon 3-4 (1/12), Del exon 3/del exon 2-3 (1/12), Del exon 2/del exon 2-4 (1/12), C820T/del exon 2 (1/12), G96C/C1305T (1/12), R42P/R42P (3/12), G429EfsX5/G429EfsX5 (3/12), 1573insTTAG/1573insTTAG (1/7), 1488 + 1G&gt;A/1488 + 1G&gt;A (1/7), 1488+1G&gt;A/1252_1488del (5/7), G2019S (2/2), G209A (8/8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-DOPA use</td>
<td>4/7 (57%)</td>
<td>8/12 (66%)</td>
<td>5/9 (55%)</td>
<td>2/2 (100%)</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>UPDRS III</td>
<td>13.7 +/-2</td>
<td>28.2 +/-12.7</td>
<td>12.8 +/- 6</td>
<td>41 and 43</td>
<td>36.2 +/-14</td>
</tr>
</tbody>
</table>
Table 10. Summary of asymmetry index results for monogenetic Parkinson’s disease cases.

<table>
<thead>
<tr>
<th></th>
<th>GBA</th>
<th>Parkin</th>
<th>PINK1</th>
<th>SNCA</th>
<th>LRRK2</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASI</td>
<td>0.42*</td>
<td>0.88 [0.72-0.93]</td>
<td>1.0 [0.9-1.13]</td>
<td>1.02 [0.9-1.13]</td>
<td>0.75* [0.59-0.86]</td>
<td>0.92 [0.89-0.92]</td>
</tr>
<tr>
<td>PASI</td>
<td>0.18*</td>
<td>0.6 [0.1-1.0]</td>
<td>1.05 [0.9-1.4]</td>
<td>1.05 [0.92-1.2]</td>
<td>0.49* [0.13-0.5]</td>
<td>0.89 [0.82-0.96]</td>
</tr>
<tr>
<td>SASI</td>
<td>80+/−5.1</td>
<td>32.7+/−5.6</td>
<td>13.1+/−4.4</td>
<td>13.4+/−3.9</td>
<td>44.6+/−5.3</td>
<td>8.3+/−2.7</td>
</tr>
</tbody>
</table>

Key: CASI, PASI = right/left asymmetry index quoted as median and interquartile range for caudate and putamen, SASI = striatal asymmetry index quoted as mean and standard error. *= significantly different than ASI from control population (Mann-Whitney u-test, p<0.001).
Figure 8. Box plots of right:left asymmetry indices by genotype.

Top panel. Median right:left asymmetry indices for caudate nucleus for each genotype.
Lower panel. Median right:left asymmetry indices for the putamen for each genotype. The caudate and putamen values for *GBA* and *LRRK2* were significantly different to the control value (p<0.001).
Figure 9. Striatal Asymmetry index by genotype.

Striatal asymmetry index reported as mean +/- standard error. The higher the index the greater the degree of asymmetry between right and left striatum. A linear regression analysis demonstrated a significant effect of genotype on SASI (p<0.001). As can be seen from the graph GBA and LRRK2 genotypes have the highest SASI and so the greatest asymmetry of radioligand uptake.
Figure 10. Representative DaTSCANS from monogenetic PD cases.

10a. DaTSCAN from 50 year old man with PD-GBA, note marked loss of tracer uptake in right striatum.

10b. DaTSCAN from 67 year old woman with PINK1 mutation associated PD, note bilateral absence of tracer uptake in the putamina and symmetric reduction in the caudate heads.
10c. DaTSCAN from a 45 year old man with Parkin mutation associated PD, not symmetrical reduction of tracer uptake in caudate heads.

10d. DaTSCAN from a 32 year old man with LRRK2 G2019S mutation associated PD, note loss of tracer uptake in left caudate compared to right.

Id. Discussion

Discussion of significance of clinical markers of prodromal PD detected in GD patients and heterozygous GBA mutation carriers

Here neurological features relevant to increased risk of developing a neurodegenerative disorder such as PD were studied in the largest cohort of GD patients and heterozygous GBA mutation carriers yet reported (McNeill et al, 2012a). We demonstrate that hyposmia, cognitive dysfunction and parkinsonian motor signs are more frequent amongst GD patients and heterozygous GBA mutation carriers than age matched controls. Both GD patients and carriers also performed significantly more slowly on the timed motor tests than control individuals. These clinical markers of prodromal PD were heterogeneously distributed, with a subset expressing more than one and the majority of individuals having no abnormalities on clinical testing. This may reflect a mixed population, with some individuals being in the early stages of a neurodegenerative disorder and others not.

The potential markers of neurodegeneration studied have diverse aetiologies and a poor ability to predict development of PD. Therefore, only a minority of study participants with abnormal markers will be in the prodrome of a neurodegenerative disorder. Hyposmia has been described in GD patients with PD (Saunders-Pullman et al, 2010), but there are no reports of hyposmia in non-parkinsonian GD patients or as a side effect of GD treatment. Only a minority of individuals with idiopathic hyposmia go on to develop a neurodegenerative condition. In the Honolulu ageing study 10/549 hyposmic individuals developed PD (Ross et al, 2008) while Ponsen et al (2004) found 10% of hyposmic first degree relatives of PD patients developed PD themselves.
Thus only a fraction of hyposmic GD patients and heterozygous carriers will have hyposmia due to a neurodegenerative prodrome.

The cause, or causes, of hyposmia in the GD patients and heterozygous GBA mutation carriers could not be elucidated in the current study. However, current smokers and those with symptomatic disease of their upper respiratory tracts were excluded, thus minimising these potential confounding variables and there is no reported association between GD and upper respiratory tract pathology. Obviously, there is an association between impaired cognition and impaired odour indentification. While there was a correlation between UPSIT and MoCA score in our cohort, the fact that several GD patients and heterozygous GBA mutation carriers had isolated hyposmia demonstrates that hyposmia in our cohort was not solely due to cognitive impairment. In theory pathology involving any part of the olfactory tract from olfactory bulb to olfactory cortical areas could contribute to hyposmia. Alpha-synuclein pathology in the olfactory bulb has been suggested as a cause of hyposmia in PD and it is possible that this process also occurs in GD and heterozygous GBA mutation carriers, since increased neuronal alpha-synuclein accumulation has been described in association with GBA mutations (Gegg et al, 2012). It is also interesting that in 2 GD mouse models infiltration of the olfactory bulb with activated microglia/macrophages is clearly described (Cabrera-Salazar et al, 2012; Farfel-Becker et al, 2011). Suggesting that the primary pathological process of GD might contribute to hyposmia in GD in addition to/instead of alpha-synuclein deposition. However, this would not explain hyposmia in the heterozygous GBA mutation carriers. Pathology involving olfactory cortical areas might also occur in GD and heterozygous GBA mutation carriers. Clearly in the context of the current study, the most attractive hypothesis is that olfactory cortical areas become affected by alpha-synuclein pathology in GD patients and heterozygous mutation carriers. In support of this, patients with Lewy-body dementia have been shown to develop hyposmia which is more marked than that observed in Alzheimer's disease, independent from naming defects associated with cognitive impairment (Williams et al, 2009). Moreover, alpha-synuclein/Lewy body accumulation and neuronal loss has been described in the hippocampus and temporal lobe
of GD patients (Wong et al, 2004). The pathological basis of hyposmia in GD and heterozygous GBA mutation carriers remains to be defined. This issue could be resolved by histopathological examination of olfactory bulb, olfactory tract and olfactory cortex tissue from GD patients and heterozygous GBA mutation carriers. However a lack of appropriate post-mortem tissue is likely to be a barrier to this. Functional neuroimaging studies of cortical responses to olfactory stimuli could help detect olfactory cortex pathology/dysfunction contributing to hyposmia, but such studies are complex to perform and interpret. In any event, the pathology causing hyposmia in GD and heterozygous GBA mutation carriers is perhaps of less interest than the issue of whether hyposmia identifies individuals at increased risk of developing PD in the future.

Cognitive dysfunction had not been reported in Type I GD at the time of this study but substrate reduction therapy (SRT) has been reported to induce memory problems (Hollak et al, 2009). However, only 2 GD patients were receiving SRT when assessed. Neither of whom had cognitive impairment. Multiple studies have demonstrated that PD patients with heterozygous GBA mutations have a greater prevalence and severity of cognitive dysfunction than PD patients without mutations. For example Brockmann et al (2012) conducted a study of 20 PD patients carrying the N370S or L444P mutation found cognitive impairment by MoCA and other neuropsychiatric features to be more common than in PD patients without GBA mutations, but they did not provide details of performance on the individual domains of the MoCA. Alcalay et al (2012) detail the cognitive profile of PD-GBA compared to PD-S. They describe defects of non-verbal memory and visuo-spatial function using the Wechsler memory scale-revised and Benton Visual Retention Test in PD-GBA compared to PD-S. Our findings on the non-motor manifestations of PD-GBA are broadly in agreement in that we identified more frequent cognitive impairment in PD-GBA using the MoCA (McNeill et al, 2012b). However, we did not identify specific defects in non-verbal memory and visuo-spatial function; this probably reflects both the small sample size of our study and the non-sensitivity of the MoCA for certain types of cognitive dysfunction. There are no reports of cognitive dysfunction in non-manifesting carriers of heterozygous GBA mutations. However, given the association of PD-GBA with marked
cognitive dysfunction it seems reasonable to suggest that cognitive dysfunction may occur in the pre-motor stages of PD associated with GBA mutation carriage. In support of this it is interesting to note that in our cohort both non-manifesting GBA carriers and PD-GBA participants had lower scores on the abstraction domains of the MoCA compared to controls. This broad similarity in cognitive deficits suggests that the cognitive impairment present in non-manifesting carriers may be a non-motor feature of individuals who are in the prodromal phase of PD-GBA. The cognitive deficits identified in the GD patients and non-manifesting GBA carriers may thus represent a feature of neurological disease associated with GBA mutations rather than solely being part of the neurodegenerative process of Parkinson’s disease.

It is well recognised that the UPDRS part III differentiates poorly between subtle parkinsonian signs and non-specific motor features occurring as part of normal ageing. Amongst study participants who scored 0-10 on the UPDRS part III there was a mixture of individuals with signs which were highly likely to reflect early motor features of PD (e.g. rigidity with activation manoeuvre) and those whose motor features overlapped with normal physiology (e.g. bilateral postural tremor) or might reflect bone disease in GD (e.g. postural abnormalities). Despite this, there were 4 GD patients and 2 carriers with UPDRS part III scores >10 with motor phenotypes falling short of diagnostic criteria for PD (e.g. rest tremor with asymmetric rigidity but no bradykinesia) but clearly distinct from normal. We do not propose that all subjects with elevated UPDRS part III scores will develop PD but hypothesise that those with clear motor abnormalities (e.g. rest tremor) are more likely to be in the prodromal phase of PD. In addition, the correlation between the timed motor test results and the UPDRS part III scores supports the contention that GD patients and heterozygous GBA mutation carriers with elevated UPDRS part III scores may be in the early phases of PD (Haaxma et al, 2008). In both GD patients and carriers higher UPDRS Part III scores were associated with slower performance on the timed motor tests (Purdue pegboard and 3m walk). Moreover, 3 of the 4 PD patients with an isolated motor sign of PD had the lowest 3 pegboard scores of all GD patients, whilst the 4th had a score in the bottom quartile of GD patients. The score on the Purdue pegboard has been shown to correlate with
density of ligand binding on dopamine transporter imaging (Bohnen et al, 2007; Troiano et al, 2010). This relationship between decline in ligand binding on dopamine transporter imaging and pegboard performance occurs in both PD and normal ageing, but with greater severity in PD. Moreover, slower performance on the Purdue pegboard in normal elderly individuals has been associated with development of clinically probable PD over a 4 year period, though the sensitivity and specificity were poor (Adler CH, 2002). This correlation between poor performance on the timed motor tests (which is associated with dopaminergic neuronal loss and increased risk of developing PD) and higher UPDRS part III scores thus implies that the isolated motor signs identified in the GD patients and non-manifesting carriers may reflect prodromal PD in this cohort.

The motor syndrome of PD is associated with cognitive dysfunction and hyposmia (Schapira & Tolosa, 2010). Thus it is expected that those individuals with combinations of prodromal markers will be most likely to be in the prodromal phase of PD. The fact that higher UPDRS part III scores are statistically associated with lower MoCA and UPSIT scores demonstrates that there is a subset of GD patients and carriers manifesting multiple prodromal markers who are most likely to be in the prodromal phase of PD (see table 5).

We did not identify an increased frequency of symptoms of autonomic dysfunction in the GD patients and carriers, and whilst more GD patients gave a positive response to the REM sleep behaviour disorder questionnaire than controls this did not reach significance. This does not undermine our contention that we identified early markers of neurodegeneration in this cohort. In the majority of cases urinary and erectile dysfunction develop after onset of motor PD (Goldstien et al, 2011). In one study of PD patients with autonomic dysfunction, orthostatic hypotension was only present in 60% as an early feature (Goldstien et al, 2011). Constipation is well recognised as a marker of increased PD risk, but it has low specificity (Petrovich et al, 2009). Thus, autonomic symptoms are often more associated with clinical PD than the prodromal phase and their absence does not preclude increased risk of neurodegeneration. REM behaviour
disorder correlates with risk of developing neurodegeneration (Postuma et al., 2009). There is evidence REM sleep behaviour disorder may define an endophenotype of PD, with 25 – 50% of PD patients having clinical features of the disorder (Arnulf, 2012). Thus, since not all PD patients have this disorder, it is logical that not all individuals in the prodrome of neurodegeneration will have REM sleep behaviour disorder and so its absence does not indicate a low risk of neurodegeneration. Moreover, the screening tools used to identify autonomic dysfunction and RBD are not “gold standard” tests and have suboptimal sensitivity and specificity. Thus some individuals with these prodromal markers will not have been identified by the screening tools. This is especially true of REM sleep behaviour disorder in which the research participant’s bed partner was usually not present to help answer the questionnaire.

Both bi-allelic and heterozygous GBA mutations predispose to PD and Lewy body dementia (LBD) (Sidransky et al., 2009). The olfactory deficits, cognitive dysfunction and parkinsonian signs in our cohort may thus represent the prodrome of one of these disorders. Olfactory dysfunction is well recognised in established and prodromal PD. In the Honolulu Ageing study, hyposmia (lowest quartile of odour identification scores) gave an OR of 5.2 for the development of PD over a 4 year period and correlated with idiopathic Lewy body pathology at autopsy (Ross et al., 2008; Ross et al., 2006). Hyposmia also occurs in LBD, distinct from odour identification/naming difficulties associated with dementia (Williams et al., 2009). Olfactory dysfunction in PD and LBD is associated with Lewy body deposition in the olfactory bulb, nucleus and cortex but not the olfactory mucosa. Several GD patients and carriers had isolated cognitive impairment (dementia), defined by the MoCA (score of <24/30). Mild cognitive impairment is common in newly diagnosed PD (18 – 30%). A distinct MCI phenotype is proposed as a prodrome to LBD. Cortical Lewy body pathology is associated with cognitive impairment in PD and LBD. Poor performance on the Purdue pegboard has been correlated with
loss of dopaminergic neurons at autopsy and dopamine transporter imaging. Thus a subset of the GD patients and carriers have clinical signs associated with Lewy body deposition or neuronal loss which have been linked to future development of PD or LBD. However, it needs to be recognised that there are multiple neurological causes for the clinical signs we have identified, including Alzheimer’s disease and cerebrovascular disease. We did not perform diagnostic tests such as brain MRI to rule these pathologies out, but as there is no known association between GBA mutations and these pathologies it seems less likely that they are responsible for our findings.

Discussion of optical coherence tomography findings in GD patients at risk of PD

Given the lack of sensitivity and specificity of the clinical markers of prodromal PD it was decided to examine GD patients with OCT. Retinal nerve fibre loss as demonstrated by OCT has been shown in early and advanced PD (Hajee et al., 2009; Garcia-Martin et al., 2012). We utilised OCT to measure GCC and retinal nerve fibre layer thickness in GD patients in order to examine the hypothesis that clinical markers of prodromal PD in this cohort would be associated with loss of retinal thickness. This would support the contention that the clinical markers of prodromal PD were associated with underlying neurodegeneration. There was no significant difference in the mean GCC or RNFL thickness of GD patients compared to controls. However, the mean GCC and RNFL thickness of GD patients who expressed at least one prodromal marker of PD was significantly lower than that of both the control group and the GD patients without prodromal markers. The OCT data from GD patients suggests that the clinical markers of prodromal PD reflect underlying neurodegeneration and may help to stratify Type I GD patients at high and low risk of developing PD or another neurodegenerative condition. The study group of GD patients utilised in this part of the project was by necessity small given the time consuming nature of OCT. Our results suggest that the clinical markers of prodromal PD detected in our cohort are associated with the presence of underlying neurodegeneration. This preliminary study indicates that OCT may be clinically useful in stratifying GD patients into
those at high and low risk of neurodegeneration. This requires confirmation in larger study cohorts with longitudinal follow up.

The main cells found in the GCC are ganglion cells and dopaminergic amacrine cells. The cells of the GCC have been shown, in cell culture models, to be sensitive to mitochondrial toxins and oxidative stress (Almasieh et al, 2012). Thus it might be that the loss of GCC thickness in our cohort reflects metabolic derangements in the central nervous system associated with the presence of GBA mutations. In chapters 3 and 4 the cell biology of GD and the heterozygous carrier state is described in detail using fibroblasts as a model. Fibroblasts derived from GD patients and heterozygous GBA mutation carriers both with and without PD have evidence of oxidative stress (McNeill et al, in preparation). Thus chronic oxidative stress in these individuals may contribute to degeneration of cells in the GCC. Moreover, in a mouse model of the lysosomal storage disorder Niemann-Pick Type C there is retinal ganglion cell death associated with markers of dysfunctional autophagy (Claudepierre et al, 2010). Thus, the retina is clearly vulnerable to the pathological process associated with bi-allelic carriage of GBA mutations. Future studies might profitably focus on examining the cell biology of retinal ganglion cells derived from murine models of GD held at University College London or studying the effect of GCase inhibition or knockdown in cultured retinal ganglion cells in order to understand the mechanisms underlying retinal degeneration in GD and carriers.

**Discussion of GBA mutation spectrum detected in the Royal Free-Addenbrookes GD Cohort**

All of the GD patients included in the phenotyping study had a typical Type I GD phenotype and genotypes. Two Type I GD patients with the R262G/RecNeil genotype were excluded from phenotyping as their genotype was more commonly associated with early onset neuronopathic GD. Our Type I GD cohort was multi-ethnic. Most of the families were of white UK extraction with only 20% having Ashkenazi Jewish heritage. The literature suggests that greater than 75% of GBA mutations in Type I GD should be N370S (Duran-Ogalla et al, 2012). The fact that 78%
of alleles in our GD patients were N370S is thus entirely congruent with this. The presence of clinical markers of prodromal PD was not restricted to GD patients of Ashkenazi origin and our data indicates that these clinical features are not due to genetic background restricted to any one particular ethnic group. The function consequences of the novel mutations were studied using \textit{in silico} prediction tools (Duran-Ogalla et al, 2012). The A341V mutation was predicted to destabilize the GBA-substrate binding site and so disrupt GBA catalytic activity. The V447E mutation was predicted to disrupt the binding site for the GBA activator protein saposin C. The R262G change results in loss of a positively charged residue with potential destabilisation of GBA structure, an R262H mutation which affects the same site has been linked with PD. G250V was not predicted to affect GBA function, however the heterozygous carrier it was found in had prodromal markers of PD suggesting it may be pathogenic and it was present with N370S in a GD patient. The mutation IVS9+1G>A was strongly predicted by HSF finder to destroy the canonical donor splice site at +1 position of intron 9. This was likely to affect splicing and produce aberrant transcripts and a non functional GBA protein.

\textbf{Discussion of the non-motor phenotype of PD in heterozygous \textit{GBA} mutation carriers}

The motor phenotype of PD-GBA is very similar to that of PD-S, but the non-motor phenotype is less well studied (Sidransky \textit{et al}, 2009). We identified that the degree of hyposmia in PD-GBA and PD-S is not significantly different. This suggests that the neuropathology in PD-GBA involves olfactory pathways to a similar degree to that seen in PD-S. This is interesting as olfactory dysfunction in \textit{LRRK2} and \textit{Parkin} associated PD is reported to be less severe than PD-S, suggesting that the neuropathological process in these genotypes may involve olfactory pathways to a lesser degree than in PD-S. Using the non-motor symptoms scale we also identified a greater number and severity of non-motor symptoms in PD-GBA than PD-S. This is in keeping with other studies which utilised different clinical screening tools to study the phenotype of PD-GBA. Brockmann \textit{et al} (2011) used the Neuropsychiatric inventory to show that PD-GBA patients have a greater prevalence and severity of depression and anxiety, which agrees with our findings from the non-motor symptoms scale. As discussed above several
groups have found more severe cognitive impairment in PD-GBA, as did we with the MoCA. However, we did not identify more severe autonomic symptoms in our PD-GBA cohort; but this may reflect the relatively small size of our study. The more severe non-motor symptom profile of PD-GBA has been suggested to be due to more widespread Lewy body pathology in PD-GBA, this has been confirmed by some neuropathological studies but not by all (Parkkinen et al, 2011). Interestingly we found that more PD-GBA than PD-S patients had possible REM sleep behaviour disorder as classified by the REM sleep behaviour disorder questionnaire. This has not been previously reported in PD-GBA and needs confirmation with formal sleep studies, but correlates with the observation that reduced echogenicity of the brainstem raphe on transcranial sonography occurs more frequently in PD-GBA than PD-S (Brockmann et al, 2011). This area is potentially involved in sleep regulation and so this observation could be of mechanistic relevance to our findings. Our study of PD-GBA further supports the notion that genotype influences the non-motor phenotype of PD and that genetics may explain part of the phenotypic heterogeneity in PD.

Despite these differences in non-motor phenotype between PD-GBA and PD-S the motor phenotype appears identical on clinical examination. PD-GBA patients manifest the same spectrum of both motor and non-motor features reported in PD-S. But in general PD-GBA patients report a greater number and severity of non-motor symptoms. This is in contrast to other genetic forms of PD which less closely resemble PD-S. For example, Parkin mutation associated PD presents with very mild non-motor manifestations and normal olfaction (Kagi et al, 2010b). While the motor phenotype of ATP13A2 and FBX07 mutations is very different to that of PD-S. Based on our detailed clinical phenotyping, and the reports of typical Lewy body pathology in PD-GBA, PD-GBA sufficiently resembles PD-S to represent a good genetic model to study the aetiology of PD.
Discussion of age related risk of PD in heterozygous GBA mutation carriers

Data on the age related risk of PD in GD and heterozygous GBA mutation carriers is clearly of interest to both clinicians and patients. Bultron et al (2010) demonstrated that Type I GD patients have a lifetime relative risk of PD of around 20 fold greater than control populations. Rosenbloom et al (2010) studied the registry of the International Gaucher Disease Collaboration to define age related incidence of PD in GD. For men at age 80 the incidence of PD was 12% while for women it was 9%. This risk is very similar to the age related risk of PD we defined in the heterozygous GBA mutation carriers in our study.

In heterozygous mutation carriers from families with autosomal dominant PD associated with GBA mutations Anheim et al (2012) described an age related risk of PD of 7.6% at 50 years and 29.7% at 80 years. This is significantly greater than the age related risk we identified amongst parents of Type I GD patients (the parents are obligate heterozygotes), in whom there is a risk of PD of 5% at age 60 and 15% at age 80 years. It is also greater than the risk of PD calculated by Rana et al (2012), who found risk of PD in obligate GBA mutation carriers to be 2.2% at age 65 years and 10.9% at 85 years. A family history of PD is associated with an elevated risk of the disorder in first degree relatives, thus other genetic and environmental factors present in PD families may account for the increased risk of PD in heterozygous carriers reported by Anheim et al (2012). There may also be ascertainment bias when studying age related PD risk in families in which the index case has PD. Thus it is likely that our estimate of age related PD risk in the obligate carriers from Type I GD families is more accurate given the absence of confounding factors relating to an autosomal dominant family history of PD.

Discussion of DaTSCAN features of monogenetic PD

Here we report the DaTSCAN features of a large set of cases of monogenetic PD. Our key finding is an asymmetric pattern of loss of striatal dopaminergic neurons in PD with GBA or LRRK2 mutations as assessed by both the ASI and SASI. However, by comparison, the pattern of dopaminergic neuronal loss in PD with SNCA mutations or bi-allelic Parkin or PINK1
mutations was relatively symmetrical. There was no correlation between measures of asymmetry and disease duration or patient age at examination. Indicating that the differences in symmetry of dopaminergic neuronal loss are related to genotype and not differences in disease stage between groups.

The results of our comparison of dopaminergic neuronal imaging features are in keeping with previous reports of imaging of single PD genotypes. There are 2 reports of DaTSCAN in Gaucher disease patients with PD or PD patients with heterozygous GBA mutations (Sunwoo et al, 2011; Kono et al, 2007). Though not formally quantified, inspection of the published images reveals obvious asymmetry of radioligand uptake affecting the caudate and striatum. Here we confirm asymmetric loss of dopaminergic neurons in sporadic PD associated with heterozygous GBA mutations. Multiple studies of Parkin mutation associated PD using both DAT-SPECT and 18-F-DOPA-PET clearly describe a symmetrical reduction in striatal radioligand uptake (Varrone et al, 2004; Ribeiro MJ et al, 2009; Khan et al, 2002). The DAT-SPECT features of PD patients with bi-allelic Parkin mutations reported herein is entirely in keeping with this. DAT-SPECT in PINK1 linked PD is usually described as symmetrical but some reports state that it is asymmetrical in a pattern reminiscent of sporadic PD (Samaranch et al, 2010). As previously described we found that the LRRK2 mutation carriers with PD had asymmetric loss of radioligand uptake on DAT-SPECT (Isaias et al, 2006). The SNCA mutation PD cases reported here had symmetric reduction of ligand binding. This is in keeping with reports of 18F-DOPA in PD with the A53T mutation which demonstrated relatively symmetrical loss of radioligand uptake, however, reports of SNCA multiplication and A30P mutation associated PD describe an asymmetrical pattern of reduced tracer uptake (Kruger et al, 2001; Ahn et al, 2008; Nishioka et al, 2002).

The currently available data on the pathological mechanisms underlying monogenetic PD help to explain the differences in symmetry of radioligand uptake between genotypes (Schapira and Tolosa, 2010). PINK1 and Parkin mutations are generally associated with early onset, relatively
symmetrical parkinsonism (reviewed in Lees et al, 2009). Both *PINK1* and *Parkin* proteins are proposed to play a role in clearing damaged mitochondria, and there is evidence that loss of function of these proteins is associated with mitochondrial dysfunction (de Vries et al, 2012; Abramov et al, 2012). The diffuse and symmetrical loss of dopaminergic neurons reported by us, and many others, may reflect widespread dysfunction and degeneration of striatal dopaminergic neurons due to a general predisposition to neuronal mitochondrial dysfunction in *PINK1* and *Parkin* mutation associated PD. *SNCA* mutations are proposed to act chiefly by facilitating Lewy body formation (Ahmad et al, 2012). The symmetrical loss of dopaminergic neurons on DAT-SPECT reported herein may thus reflect a generalised, homogeneous increase in Lewy body formation in the striatum since the mutation is present in every cell. By contrast, *GBA* and *LRRK2* mutations are most commonly associated with a phenotype which closely resembles late onset sporadic PD. This suggests that these mutant proteins may interact with additional pathological process, either genetic or environmental, predisposing to age related PD and explaining the low penetrance of these genotypes. For example in PD with mono- or bi-allelic *GBA* mutations there is reduction or loss of glucocerebrosidase enzyme activity in the brain, associated with elevated alpha-synuclein deposition as measured by Western blot (Gegg et al, 2012; Mazzulli et al, 2011). Both cell biology and mouse studies indicate that inhibition of glucocerebrosidase activity is associated with elevated alpha-synuclein accumulation. The asymmetric loss of dopaminergic neurons observed in PD with *GBA* mutations may thus reflect a stochastic element whereby there is a focal loss of glucocerebrosidase activity below a critical level eventually resulting in an initially focal accumulation of alpha-synuclein and Lewy body formation. There is evidence from human brain and cell models that alpha-synuclein accumulation results in inhibition of glucocerebrosidase, thus causing a “feed forward” mechanism which could contribute to the spread of pathology and neurodegeneration (Gegg et al, 2012). Recently it has been shown that loss of glucocerebrosidase activity associated with alpha-synuclein accumulation in brain tissue from PD without *GBA* mutations (Gegg et al, 2012). This suggests that a similar mechanism may operate in sporadic PD and may account
partly for the similarities in dopaminergic neuronal imaging and clinical phenotype between sporadic PD and PD associated with *GBA* mutations.

Studies have shown that dopaminergic neuronal imaging improves accuracy of diagnosis in sporadic PD. Here we provide data suggesting that DAT-SPECT and 18-F-DOPA PET also have potential clinical utility in genetic PD. Recessive forms of genetic PD (*Parkin, PINK1*) generally demonstrate relatively symmetric loss of radioligand uptake in the striatum. This symmetrical pattern is also evident in reports of multisystemic neurogenetic syndromes in which parkinsonism is found alongside other signs (e.g. Kufor-Rakeb syndrome). PD associated with heterozygous mutations in *GBA* or *LRRK2 G2019S*, which can present with dominant or apparently sporadic PD, produces an asymmetric pattern of loss of radioligand uptake. Here we found that the *SNCA G209A* mutation is associated with symmetrical loss of dopaminergic neurons on DAT-SPECT, but others have reported asymmetric loss of uptake especially with *SNCA* multiplication. In clinical practice asymmetric loss of radioligand binding should not exclude a genetic aetiology for PD while relatively symmetric decrease should raise suspicion of a monogenetic form of PD. The asymmetric loss of dopaminergic neurons in *GBA* and *LRRK2* associated PD supports the hypothesis of secondary interactions with genetic or environmental factors leading to neurodegeneration in these genotypes.
Concluding remarks

Here, the presence of clinical markers of prodromal Parkinson's disease in a cohort of Type I GD patients and heterozygous carriers of GBA mutations is described (McNeill et al., 2012a). Hyposmia, cognitive impairment and parkinsonian motor signs were more common in these individuals than in controls. There was a statistical correlation between the severity of each of these clinical markers of prodromal Parkinson's disease. This is expected since patients with Parkinson's disease manifest a range of motor and non-motor problems and individuals in the prodromal phase are likely to have these to a lesser degree than in clinically manifest disease (Schapira and Tolosa, 2010). Additionally, the frequency and severity of the scores on the clinical ratings scales used for these prodromal markers worsened with increasing age in the GD and carrier groups, but not in controls. This fits with the proposal that they reflect underlying neurodegeneration since Parkinson's disease is strongly age related. The thickness of the retinal ganglion cell complex and retinal nerve fiber layer, measured by OCT, was thinner in GD patients with prodromal markers of Parkinson's disease than in controls but not in GD patients without prodromal markers. This provides objective evidence of neurodegeneration in this cohort suggesting that the prodromal markers we identified are indeed associated with the prodromal phase of Parkinson's disease.

It is clear that only a minority of GD patients and heterozygous GBA mutation carriers develop PD. The reasons for this are unclear; but may relate to genetic or environmental factors. Co-inheritance of mutations in other PD causing genes seems unlikely given the rarity of LRRK2 and VPS35 in sporadic PD. All of our PD-GBA cases had sequencing of LRRK2 G2019S performed by a previous research fellow, no cases had a pathogenic GBA mutation with LRRK2 G2019S as well. This is in agreement with a previous study (Eblan et al., 2006). In addition the phenotype of PD-GBA with severe hyposmia and non-motor symptoms is distinct from that of Parkin mutation associated PD (Kagi et al., 2010b). It is very unlikely that mutations in rare PD genes such as PINK1, ATP13A2, DJ-1 would explain the occurrence of PD in GD and heterozygous GBA mutation carriers. Several environmental factors such as smoking and coffee consumption
are known to protect against PD (Lees et al, 2009). Given the weak associations between PD and environmental factors it seems less likely that one of these would explain why a subset of GD/mutation carriers develop PD. Our study was not designed or powered to detect associations between environmental factors and risk of PD in GD patients and mutation carriers. This is however an important issue which would require a multi-centre international collaboration to address.

**Bibliography**


Chapter III.

ENDOPLASMIC RETICULUM RETENTION OF GLUCOSYLCERAMIDASE IN FIBROBLASTS FROM GAUCHER DISEASE PATIENTS AND PARKINSON’S DISEASE PATIENTS WITH HETEROZYGOUS *GLUCOCEREBROSIDASE* MUTATIONS
IIIa. Introduction

The biosynthetic pathway of glycosylceramidase

The glucosylceramidase (GCase) protein is synthesised as a 56kDa polypeptide on endoplasmic reticulum (ER) bound polyribosomes and translocated across the ER membrane with cleavage of the leader sequence and glycosylation on 4 asparagine residues (Ron and Horowitz, 2005). GCase then passes through the golgi body and through the endosomal pathway to reach the lysosome via a mannose-6-phosphate receptor-independent pathway, where it expresses its enzymatic function as a 59-63 kDa mature glycoprotein. As GCase passes through the golgi, Golgi mannosidase II removes 2 mannose residues from GCase to form the mature glycoprotein. The steps in GCase biosynthesis are discussed in more detail below.

As GCase passes through the ER it interacts with a variety of protein chaperones and undergoes folding into its active structure. Depletion of ER calcium levels in GD has been associated with reduced folding of the mutant GCase; several studies with calcium channel blockers which raise ER calcium levels have demonstrated that raising ER calcium results in improved GCase protein folding (Ong et al, 2010; Wang et al, 2011). The LIMP-2 (SCARB2) protein is an important chaperone for GCase. It has been shown that LIMP-2 binds to GCase in the ER and ensures it is correctly targeted to the lysosome, at the acid pH of the lysosome LIMP-2 and GCase dissociate (Blanz et al, 2010). The physiological importance of LIMP-2 has been emphasised by the identification of bi-allelic mutations in SCARB2 as causing Action Myoclonus Renal Failure syndrome (AMRFS), a storage disorder in which GCase processing and activity is abnormal (Berkovic et al, 2008). Fibroblasts from these patients have reduced GCase protein levels and enzyme activity with evidence that GCase is aberrantly targeted for extracellular secretion rather than to the lysosome (Blanz et al, 2010). SCARB2 polymorphisms have also been associated with sporadic PD (Michelakakis et al, 2012).

Recently, the phosphatidylinositol kinase pathway has also been demonstrated to be important in targeting GCase to the lysosome (Jovic et al, 2012). Phosphatidylinositol kinase-III beta has been shown to regulate passage of GCase out of the golgi body while Phosphatidylinositol kinase-II alpha was shown to regulate traffic of GCase from endosome to lysosome. Phosphatidylinositol kinase-II alpha inhibition also caused missorting of GCase into the cell
culture medium. Once in the lysosome GCase interacts with its co-factor Saposin-C, which enhances GCase enzyme activity (reviewed in Tamargo et al, 2012). The importance of Saposin-C to GCase function is emphasised by the fact that recessive mutations which affect Saposin-C cause a severe form of GD which has reduced GCase enzyme activity (reviewed in Tamargo et al, 2012). Clearly the GCase enzyme must interact with a variety of chaperones and cellular compartments for it to be correctly targeted to the lysosome and thus there are several steps at which GBA mutations could disrupt GCase enzyme function.

In addition to the lysosomal GCase (also termed GCase-1), 2 additional isoforms of the enzyme exist: GCase-2 and GCase-3 (Aureli et al, 2012). GCase-2 is located chiefly in the plasma membrane (Aureli et al, 2012) and GCase-3 in the cytoplasm (Dekker et al, 2011). GCase-2 can metabolise GCase-1 substrates (e.g. glucosylceramide) while GCase-3 cannot (Dekker et al, 2011). GCase-3 activity is not detectable in fibroblasts and this enzyme will not be considered further here (Aureli et al, 2012). GCase-2 activity is assayed by measuring total cellular GCase activity and subtracting the value for GCase activity measured in the presence of adamantane-pentyl-dNM;N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (DNJ), which is a specific inhibitor of GCase-2 (Aureli et al, 2012). Aureli et al (2012) demonstrated increased GCase-2 activity in fibroblasts from GD patients; with greater elevation of activity in GD Type 1 and 2 than in GD Type 3. This was associated with elevation of GCase-2 transcript levels suggesting that gene induction was responsible for the increased activity of GCase-2 (Aureli et al, 2012). The authors postulate that increased GCase-2 activity is a protective response to compensate for loss of GCase-1 and minimise substrate accumulation. In this thesis GCase is used to mean GCase-1 and the term GCase-2 will be used specifically to refer to this isoform of the enzyme.
The spectrum of GBA mutations and their functional consequences

The GBA mutation spectrum was comprehensively reviewed by Hruska et al (2008). At that time around 250 mutations had been described. Including 203 missense, 18 nonsense, 36 small insertions or deletions, 14 splice site mutations and 15 complex mutations (recombinant mutations or alleles with 2 or more point mutations in cis). Since then several dozen more GBA mutations have been reported in GD, but missense changes remain the most common. A selection of these recently reported mutations will be described here but a more comprehensive list of GBA mutations (>350) can be found at http://www.hgmd.cf.ac.uk/ac/index.php.

Clearly all reported *GBA* mutations act by reducing GCase activity. There are several different predicted/proven mechanisms of action for the different *GBA* mutations. Many of the missense mutations are predicted to cause *GBA* misfolding and subsequent endoplasmic reticulum retention (Ron and Horowitz, 2005). Several missense mutations also affect the active site of GCase and we identified a novel mutation predicted to disrupt the interaction between GCase and saposin-C (Duran *et al*., 2012). Nonsense mutations in GBA are much less frequent and result in a truncated non-functional GCase or unstable mRNA. Most of the recombinant alleles are effectively null alleles (Hruska *et al*., 2008) producing no or unstable mRNA.

Genotype-phenotype correlations in GD are not absolute. But in general GD patients with at least one N370S allele have non-neuronopathic disease and those with other combinations of *GBA* mutations develop Type II or Type III GD. The L444P mutation is generally described as a more severe mutation than N370S since L444P is more commonly associated with neuronopathic GD. Perinatal lethal GD is associated with bi-allelic null alleles, such as recombinant alleles or less commonly a recombinant mutation in heterozygosity with a severe point mutation (Hruska *et al*., 2008).

Even more controversial is the role of the E326K and T369M *GBA* variants. Both have been found to occur more frequently in PD cases than in controls (Lesage *et al*., 2011; Pankratz *et al*., 2012). But whether E326K should be considered a benign polymorphism, a disease modifier or a disease causing mutation in GD is not completely resolved.
Evidence for endoplasmic reticulum associated degradation of mutant glucosylceramidase

As discussed above the majority of *GBA* mutations are missense changes which are predicted to disrupt protein structure (Ron and Horowitz, 2005). Such mutations are known to cause protein misfolding and trigger the ER protein folding quality control mechanism. The ER quality control mechanisms prevent accumulation of misfolded proteins, either by refolding them through the action of molecular chaperones or by directing them to the proteasome for degradation, so preventing the Unfolded Protein Response (UPR) and ultimately cell death by apoptosis (reviewed in Benbrook and Long, 2012). Soluble proteins synthesised on ER bound ribosomes initially bind to chaperones in the ER lumen to aid correct folding. Heat shock protein (HSP) 40 family members, especially BiP/Grp78, HSP 70 and Protein disulfide isomerase play crucial roles in binding unfolded proteins to maintain solubility and permit correct folding (Benbrook and Long, 2012). Nearly all secretory proteins synthesised in the ER undergo N-linked glycosylation, two of the three glucose residues in the protein are trimmed by glucosidase I and II, creating a binding site for the lectins calnexin and calreticulin (Hetz, 2012). These catalyse correct protein folding. The final glucose residue is then removed by glucosidase II, which releases the protein from the lectins. If the protein is still misfolded, it is recognized by the glucosyl transferase (GT), which preferentially recognizes unfolded or molten globule species and reglucosylates the polypeptide (Hetz, 2012). This permits lectin rebinding and prevents the misfolded protein from leaving the ER. If the protein still does not fold correctly then in subsequent cycles of lectin binding and cleavage of glucose residues the binding of calnexin/calreticulin becomes less efficient and the protein is passed to another lectin binding protein: ER degradation enhancing α-mannosidase-like protein. This protein then processes the misfolded protein for retrotranslocation into the cytoplasm (Hetz, 2012). In the cytoplasm the misfolded protein undergoes sequential ubiquitination by E1, E2 and then E3 ubiquitin ligases. The ubiquitinated protein is then delivered to the 26S proteasome for degradation.
GD fibroblasts have been shown to have reduced GCase protein levels (Ron and Horowitz, 2005; Lu et al, 2011) In pulse-chase radiolabelling experiments in transfected HeLa cells Lu et al (2011) demonstrated that initial levels of GCase protein coded for by N370S or L444P GBA mutations are the same as wild type but that there is a rapid decrease in cellular GCase protein levels. Ron and Horowitz (2005) demonstrated that ERAD is responsible for the loss of GCase protein in GD. Using immunofluorescence they demonstrated loss of lysosomal GCase staining and co-localisation of GCase with ER markers in fibroblasts from Type I, II and III GD patients. They also demonstrated increased endo-H sensitivity of the mutant glucosylceramidase in GD cells; suggesting that this endo-H sensitive fraction of GCase had not reached the mid-golgi and was ER retained. These authors correlated higher levels of the endo-H sensitive fraction of GCase with the severity of the GD phenotype. They went on to demonstrate using co-immunoprecipitation that mutant GCase interacts with the ER chaperone calnexin, suggesting a physiological attempt to chaperone/fold the misfolded GCase. Treatment of the cell lines with proteasomal inhibitors resulted in an increase in GCase protein levels, suggesting the proteasome was responsible for degrading mutant GCase. In pulse chase experiments the same authors treated L444P/L444P fibroblasts with cyclohexamide (to stop protein synthesis) and a proteasome inhibitor and demonstrated that in the presence of a proteasome inhibitor the half life of mutant GCase is prolonged. Despite the detailed characterisation performed by these authors it is worth noting that there was no detailed clinical phenotyping of the patients whom fibroblasts were generated from and in 3 of the fibroblast cell lines the genotype was unknown. Also there have been no modern PCR based studies of the effects of GBA mutations on mRNA stability. Further study of the role of ERAD in fibroblasts from well phenotyped and genotyped GD patients, and, in particular, heterozygous carriers of GBA mutations with and without PD, will provide further valuable information on the cellular processing of mutant GCase protein.
The role of Ubiquitin ligases and Parkin protein in glucosylceramidase metabolism

Given the evidence that the proteasome plays a key role in degrading mutant GCase it is clear that the ubiquitin ligase system, which targets proteins to the proteasome, will play a role in GD pathogenesis. By studying fibroblasts derived from L444P/L444P GD patients Ron and Horowitz (2011) demonstrated that after proteasomal inhibition, immunoprecipitated GCase was found to be ubiquitinated. This provides evidence that the ubiquitin ligase system plays a role in the degradation of mutant GCase. Ron et al (2010) investigated whether Parkin protein acts as an E3 ubiquitin ligase of mutant GCase. They demonstrated that in HEK293 cells over expressing the N370S mutant GCase that Parkin and the mutant, but not wild type, GCase protein co-immunoprecipitated. This suggests that Parkin can recognise and bind mutant, misfolded GCase. When increasing amounts of wild type Parkin were transfected into SH-SY5Y cells expressing the N370S GCase mutant it was found that GCase was degraded in a dose dependent manner. Parkin did not degrade wild type GCase. Wild type Parkin was also shown to mediate K48 polyubiquitination of mutant GCase, thus targeting it to the proteasome. The P437L RING finger Parkin mutant was not able to mediate degradation of mutant GCase.

Further evidence of the importance of ubiquitin ligases and the proteasome in GD comes from the study of Lu et al (2010). In cultured fibroblasts from Type I, II and III GD patients they show that GCase enzyme activity is proportional to the amount of GCase protein in the cells and that proteasomal inhibition with lactacystin increases both GCase protein levels and enzyme activity. In a crucial experiment they immunoprecipitate N370S and L444P GCase from GD fibroblasts and show that the GCase has almost normal enzymatic function. Taken with the work of Ron (2005, 2011) this suggests that reduced GCase activity in GD predominantly reflects proteasomal degradation of the protein rather than mutations directly influencing GCase activity by altering the structure of its catalytic site. In addition, Lu et al (2010) show that c-Cbl (an E3 ubiquitin ligase) co-immunoprecipitates with mutant GCase, and that siRNA knockdown of c-Cbl resulted in up regulation of GCase protein expression and enzyme activity in GD fibroblasts. This suggests that c-Cbl plays a role targeting mutant GCase to the proteasome. The ubiquitin-proteasome system thus plays a key role in degrading mutant, misfolded GCase.
Endoplasmic reticulum stress responses in Gaucher disease

When the rate of accumulation of misfolded proteins overwhelms the ability of basal levels of chaperones to correctly fold them the Unfolded Protein Response (UPR) is induced (Hetz, 2012). The UPR consists of 3 elements (Benbrook and Long, 2012): 1. Inhibition of protein synthesis, 2. Induction of chaperone expression and 3. Upregulation of ERAD activity. Upon accumulation of unfolded proteins in the ER BiP/Grp 78 dissociates from the ER stress transducers PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). BiP/Grp78 then binds to unfolded and misfolded proteins. Upon activation, IRE1 splices the mRNA of XBP1, and produces an active transcription factor named spliced XBP1 (XBP1-S), which upregulates ER chaperones and proteins implicated in ERAD (Benbrook and Long, 2012). In addition, IRE1 recruits TRAF2 and ASK1, resulting in JNK activation. The activation of PERK increases phosphorylation of eIF2α, leading to a global reduction of protein synthesis and a concomitant increase in ATF4 translation. In turn, ATF4 induces CHOP, a proapoptotic transcription factor. After the dissociation of Bip, ATF6 translocates to Golgi apparatus, where it is activated by proteolysis. Activated ATF6 transcriptionally induces ERAD genes and upregulates CHOP expression (Hetz, 2012).

If there is ongoing accumulation of misfolded proteins then the UPR may be overwhelmed leading to activation of apoptotic pathways and cell death (Benbrook and Long, 2012). The major pathway of UPR induced apoptosis is CHOP and IRE1 acting directly and indirectly to reduce expression of anti-apoptotic factors such as BCL and upregulating pro-apoptotic factors such as BAX (Hetz, 2012). ER resident caspase-12 can also trigger apoptotic pathways in response to ER stress (Benbrook and Long, 2012). Given the evidence of ER retention of mutant GCase proteins in GD the ER stress response may play a role in cell death in GD. However, there are few studies of ER stress markers in GD and none provide convincing evidence of ER stress induction.
Cha et al (2011) describe reduced expression of the ER stress marker BiP/Grp78 following treatment of fibroblasts from Type II GD patients with erythropoietin. They interpret this as evidence of ER stress in Type II GD. However, they did not compare BiP/Grp78 expression levels between GD and control fibroblasts and so this does not provide evidence of increased ER stress in GD compared to controls. The same group (Lee et al, 2011) report that catechin reduces ER stress in Type I GD fibroblasts. They propose that catechin reduces oxidative stress thereby increasing protein folding. They demonstrate that catechin reduces BiP/Grp78 protein levels on Western blot and immunofluorescence in Type I GD fibroblasts; however they did not demonstrate increased BiP/Grp78 expression in Type I GD fibroblasts compared to cell lines generated from healthy individuals. These studies can therefore not be taken as evidence of ER stress in GD as there was no adequate comparison with controls.

Wei et al (2007) demonstrated increased expression of mRNA and protein for the ER stress markers calnexin, XBP1 and BiP/Grp78 in GD. However, they studied just a single Type I GD and a single Type II GD fibroblast line. Given the intrinsic variability of both fibroblast cell lines and the GD phenotype this paper cannot be taken as definitive proof of ER stress in Type I GD. In contrast Farfel-Becker et al (2009) did not find any evidence of ER stress in various models of neuronopathic GD. Treatment of cultured neuronal and astrocytic cell lines with the GCase inhibitor CBE did not result in upregulation of ER stress markers (XBP1, BiP/Grp78, CHOP) nor was there evidence of ER stress marker upregulation in the brain tissue of GBA null mice. This is probably explained by the fact that there is no misfolded/mutated GCase in these models present to induce ER stress. However, the same authors went on to demonstrate that there was no evidence of ER stress marker upregulation (BiP/Grp78, CHOP, XBP1) in brain tissue of aged (3 months old) L444P/L444P mice or cultured embryonic neurons from these mice. Thus the evidence for ER stress induction in Type I GD is not convincing and further studies are required. Given the consistent evidence of proteasomal degradation of mutant GCase in GD cells it could be argued that this removes misfolded GCase protein and protects against ER stress in this disease.
Agents which can increase glucosylceramidase protein levels and activity in GD

Based upon our understanding of the mechanisms by which GBA mutations lead to GCase protein depletion and dysfunction several agents have been studied for their ability to improve GCase activity in cell culture models. These include anti-oxidants, pharmacological chaperones and histone-deacetylase inhibitors. The questionable value of erythropoeitin and catechin has been discussed above and will not be covered further here.

Lu et al (2011) studied the effects of the histone deacetylase inhibitor (HDACi) suberoylanilide hydroxamic acid (vorinostat) on L444P/L444P and N370S/N370S fibroblasts and demonstrated that it enhances both GCase protein levels and enzyme activity. They also demonstrate that mutant GCase has reduced binding to HSP70 and TCP1 (which are chaperones which promote normal protein folding) and is more likely to be bound to HSP90 and ubiquitinated for degradation. Treatment with the HDACi was shown to increase binding to HSP70 and thus facilitate normal GCase folding, consistent with this the HDACi resulted in reduced GCase ubiquitination. Importantly the HDACi improved protein levels and activity for both N370S and L444P mutant GCase.

Maegawa et al (2009) identified ambroxol hydrochloride, an expectorant, as an agent which enhances the enzymatic activity of GCase by acting as a pharmacological chaperone by screening the National Institutes of Health (NINDS) drug repurposing library of 1 040 drugs previously used in routine clinical practice in man. The initial screen sought to identify compounds which stabilised GCase against thermal denaturation and ambroxol was one of the agents identified in this initial screen. The authors subsequently show that ambroxol increased both the GCase protein levels and enzymatic activity in fibroblasts from patients with genotypes N370S/N370S and F213I/L444P but not L444P/L444P. After removal of ambroxol from culture media, the enhanced GCase activity returned to baseline at 6 days. Lysosomal fractions from treated cells were shown to be specifically enriched in GCase protein levels and activity after ambroxol treatment. This thorough characterisation of ambroxol provides strong evidence that it stabilises GCase protein and enhances its delivery to the lysosome and its enzymatic activity.
Other groups have confirmed the value of ambroxol in improving cellular GCase function. Ron and Horowitz (2011) demonstrated that ambroxol can reduce the endo-H sensitive fraction of GCase and increase its enzymatic activity in fibroblasts from Type II GD. Luan et al (2012) confirm that ambroxol can increase GCase enzymatic activity in GD fibroblasts with a range of genotypes and showed that ambroxol given orally to mice in water is non-toxic, enters the brain and improves cerebellar GCase enzyme activity. In general these studies demonstrate that ambroxol is not effective at enhancing GCase activity in L444P/L444P GD, which is a major drawback to the potential clinical use of this agent. There are still several unanswered questions relating to ambroxol's activity: it needs to be determined if ambroxol induces GCase transcription, what the effects of ambroxol on other lysosomal enzymes is and if ambroxol might show some efficacy in heterozygous cell lines. Investigating these issues were thus major goals of our cell biology study of patient derived fibroblasts.
Studies of autophagy markers in Gaucher disease

The cell physiology of autophagy is discussed in the general introduction, here, papers describing autophagy in GD are discussed. There are no systematic studies of autophagy in Type I GD due to \textit{GBA} mutations. However, Vaccaro 	extit{et al} (2010) studied autophagy in fibroblasts from GD patients with bi-allelic saposin-C mutations. In these cells GCase protein levels are comparable to that of control fibroblasts, but the protein is mislocalised with less of it reaching the lysosome and causing a reduced level of GCase activity. These cells had evidence of glucosylceramide and lipid storage in lysosomes. This group found evidence of enhanced autophagy in Saposin C mutation associated GD: levels of LC3-II (a marker of mature autophagosome) rose to greater levels in disease cells than control cells after starvation suggesting enhanced induction of autophagy. Increased formation of autophagic vesicles upon starvation was confirmed with electron microscopy. This same group subsequently found that decreased degradation of autophagosomes was responsible for increased autophagosome accumulation (Tatti 	extit{et al}, 2012) in these cells. In the same paper Tatti 	extit{et al} (2012) demonstrate normal macroautophagy in 4 GD fibroblast lines. Pacheco 	extit{et al} (2007) describe normal autophagic function in a single fibroblast cell line from a Type I GD patient. Sun 	extit{et al} (2010) utilised immunohistochemistry to describe p62 accumulation in neurons and glia in a mouse model of neuronopathic GD, and suggested this might reflect impaired autophagy. However, this mouse was a cross between a saposin C null mouse and a V394L/V394L homozygous mouse. This is a distinct molecular mechanism from human GD and so the relevance of this result to human pathology is debatable. Of course the different results in fibroblasts and mouse brain tissue could reflect a cell specific effect on autophagy of \textit{GBA} mutations.
Alterations in Lysosomal biochemistry described in Gaucher disease fibroblasts.

Several studies of tissue from GD patients indicate that in addition to deficient GCase activity there is secondary alteration of other lysosomal enzyme activity and that this pattern differs between brain and peripheral tissue (Moffitt et al, 1978). In spleen from both non-neuronopathic and neuronopathic GD, activities of most lysosomal enzymes are not altered (Glucuronidase, sphingomyelinase, arylsulphatase A, beta-galactosidase) while total-beta-hexosaminidase activity was elevated 5 fold and galactocerebrosidase 10 fold (Moffitt et al, 1978). This same study found a different pattern in neuronopathic GD brain in which galactocerebrosidase activity was twice the control value and total-hexosaminidase was only slightly elevated (around 30%) (Moffitt et al, 1978). Natowicz et al (1991) demonstrated a doubling of total-betahexosaminidase activity in serum from adult GD patients. Other groups report similar findings in Type II GD (Chidayat et al, 1987). Recently a large increase in beta-hexosaminidase activity localised to the plasma membrane of GD fibroblasts has been described accompanied by increased GCas-2 activity (Aureli et al, 2012).

The cause and consequences of the secondary alterations in lysosomal hydrolases in GD are unclear. It is plausible that they are compensatory mechanisms. For example elevation of galactocerebrosidase could result in increased metabolism of galactosylceramide to ceramide, to compensate for reduced conversion of glucosylceramide to glucose and ceramide. It is likely that increased activity of beta-hexosaminidase, which produces GM3-ganglioside, will account, at least partly, for the elevated levels of GM3 in GD brain and peripheral tissues (Moffit et al, 1978). Increased GCase-2 activity is likely to be a compensatory mechanism since GCase-2 can metabolise GCase-1 substrates (Aureli et al, 2012).

Fibroblasts from GD with bi-allelic GBA mutations store no or minimal glucosylceramide, in contrast to fibroblasts from GD patients with homozygous saposin-C mutations (see below). Saito and Rosenberg (1984) demonstrated that Type II GD fibroblasts accumulate GM3-ganglioside (around 30% elevation) but not glucosylceramide (Saito and Rosenberg, 1984). However with long term culture the elevation of GM3-ganglioside disappeared. Studying
fibroblasts is therefore unlikely to give insight into the pathophysiological consequences of substrate accumulation.
IIIb. Materials and methods

Generation of patient derived fibroblast cell lines
Skin biopsy was performed after informed written consent was obtained. Biopsy was performed on the non-dominant forearm. Local anaesthesia with lignocaine and an aseptic technique was used. A 6mm Stiefel biopsy punch was used in all cases with wound closure with steristrips. The subcutaneous fat and skin layer were then dissected apart and cultured separately in standard fibroblast growth media (DMEM with 4.5 g/L glucose and Glutamax I, Penicillin and Streptomycin, 10% Fetal calf serum and 1mM of pyruvate). Fibroblasts were grown in 5cm plates until confluent, trypsinized and split. At an appropriate passage fibroblasts were harvested and frozen in liquid nitrogen in media containing 10% DMSO.

Details of alpha-synuclein over expressing cell lines
SH-SY5Y cells (neuroblastoma cells) contain endogenous alpha-synuclein levels that are essentially undetectable by western blot (Alvarez-Erviti et al., 2010). Therefore, to investigate factors modulating cellular alpha-synuclein levels, an SH-SY5Y stable cell line expressing high levels of exogenous wild-type alpha-synuclein were used (gift of Dr J Cooper, Department of Clinical Neurosciences). The alpha-synuclein protein levels in these lines were approximately 10-fold higher than wild type SH-SY5Y lines (Alvarez-Erviti et al., 2010). Alpha-synuclein was cloned in to pcDNA3.1, transfected in to SH-SY5Y cells, and positive clones selected using the antibiotic G418 (20 ug/ml). Exogenously expressed alpha-synuclein contains a hemagglutinin tag (HA) at the C-terminal. All SH-SY5Y cell lines were cultured in 1:1 DMEM/F12 (Invitrogen) supplemented with 10% FCS, non essential amino acids, 1 mM sodium pyruvate and penicillin-streptomycin.

Extraction of DNA and GBA sequencing
DNA was extracted from cultured fibroblasts using the standard QiaAMP DNA mini-kit (www.qiagen.com). Briefly, fibroblasts were trypsinized and re-suspended in PBS then treated with proteinase K and extraction buffer. DNA was then precipitated with ethanol and purified through a column based system. The extracted DNA was then used in the sequencing reaction described above in Chapter 1 to confirm presence of the GBA mutation detected in the patient’s leukocytes in the patient’s fibroblasts.
**Determination of glucocerebrosidase enzyme activity levels**

The enzymatic assay was performed on fibroblasts harvested from a confluent 10cm plate of fibroblasts using a fluorometric assay with the widely used synthetic substrate 4-methylumbelliferyl-β-D-glucopyranoside. Cells were trypsinized, resuspended in media and then washed in PBS. This assay was developed and validated by Dr Matthew Gegg, Department of Clinical Neurosciences UCL Institute of Neurology.

The cell pellet was then resuspended in dH2O and sonicated for 1 minute. Enzyme activity was measured in duplicate under 3 conditions: 1. With McIlvaine citrate-phosphate ph 5.4, 2. With McIlvaine citrate-phosphate pH 5.4 and sodium taurocholate (149mM, to inhibit GCase-2) and 3. With McIlvaine citrate-phosphate pH 5.4 and DNJ (to inhibit GCase-2). Ten µL of cell sonicate was then added to 40µL of solution 1, 2 and 3. Ten µL of dH2O was added to 40µL of 1, 2 and 3 to make blanks. Fifty µL of substrate solution (10mM 4-methylumbelliferyl-β-D-glucopyranoside) was added to each tube at timed intervals and the tubes incubated at 37°C for 60 minutes. At timed intervals the reaction was then stopped with 0.25M glycine buffer at pH10.4. Fluorescence was then read with excitation of 365nm and emission 450nm. All enzymatic assays were quantified on a Bio Tek Synergy HT plate reader.

GCase-2 activity was determined from the formula: GCase-2 activity=total GCase activity – Gcase activity measured in the presence of DNJ. An initial control reaction was performed with conduritol-b-epoxide (a selective GCase-1 inhibitor) to ensure that the measured activity was indeed GCase-1. The GCase-1 activity quoted in this study was that measured in the presence of sodium taurocholate, since this activates wild type GCase-1 and inhibits mutant GCase-1, permitting distinction of wild type and pathogenic enzyme activity.

**Assay of total-beta hexosaminidase and beta-galactosidase enzymatic activity**

Total-beta hexosaminidase and beta-galactosidase activity levels were measured on fibroblasts harvested from a single well of a 6 well plate. For total-beta hexosaminidase fibroblasts were lysed in dH2O and 10 uL of 0.2mg/mL cell lysate was used for the reaction. Each sample was assayed in triplicate in a 96 well format. Each reaction was started by adding 30 uL of reaction
mix to 10 μL of cell lysate per well. The reaction mix was 20μL of buffer (MVA citrate buffer at pH 4.2: citrate 0.1M and Na2HPO42H2O 0.2M) and 10 uL of 8mM substrate solution (2.71 mg of 4-methylumbelliferyl-2-acetoamido-2-deoxy-6-sulfo-b-D-glucopyranoside/1 mL dH2O). The reaction was incubated at 37οC for 30 minutes and stopped by adding 200uL of 0.2M glycine buffer (pH 10.4). Blanks were reaction mix plus water and the standard was 200 uL of the glucosylceramidase standard plus 1mL of stop buffer. Fluorescence was then read with excitation of 365nm and emission 450nm.

Beta-galactosidase was assayed in a 96 well format. Fibroblasts were resuspended in dH2O and sonicated for 1 minute. Ten microlitres of lysate was added to a 96 well plate in triplicate for each cell line and the reaction started by adding 30μL of reaction buffer. The reaction was incubated for 30 minutes at 37οC and stopped by adding 200uL of 0.2M glycine stop buffer. Reaction mix consisted of 1 mg of substrate (4-methylumbelliferyl-B-D-galactopyranoside) dissolved in 3mL of assay buffer (MVA citrate buffer at pH 4.2: citrate 0.1M and Na2HPO42H2O 0.2M). Blanks were reaction mix plus water and the standard was 200 uL of the glucosylceramidase standard plus 1mL of stop buffer. Fluorescence was then read with excitation of 365nm and emission 450nm. Protein in each sample was measured using the BCA kit described above and enzyme activity normalised to protein concentration in each sample.

**Western blot analysis of glucocerebrosidase protein levels**

Fibroblasts were trypsinised and re-suspended in 1 mL PBS. Each sample was then treated with 100 μL lysis buffer with protease inhibitors (1% Triton X-100 with 1/1000 leupeptin, PMSF, pepstatin, NaV) and left on ice for 15 minutes. Cell lysates were then centrifuged at full speed for 10 minutes and the supernatant transferred to fresh tubes. Protein levels in the lysates were assayed with a BCA kit (Pierce product number 23228).

Lysates were diluted so each lane was loaded with 30μg of protein. Lysates were mixed with loading dye and reducing agents and heated at 70 degrees for 10 minutes. Lysates were then electrophoresed at 200V for 35 minutes on a NuPage gel. Proteins were transferred to a PVDF
membrane, blocked in non-fat milk (5%), treated with primary antibody for 1 hour, washed and incubated with HRP labelled secondary antibody. Antibody binding was then detected using an ECL Chemiluminescence kit (Pierce ECL Western blot substrate, product number 32106). Primary antibodies used are described in table 10.

Band intensity was quantified using Image-J (National Institutes of Health, Bethesda, http://rsbweb.nih.gov/ij/). The intensity of the band for the protein of interest for each cell line was divided by the intensity of the band for actin (loading control) in the same lane to quantify the amount of the protein of interest in that cell line.

**Table 11. Primary antibodies used for Western blotting**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Company</th>
<th>Dilution, incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocerebrosidase</td>
<td>Abcam, ab55080, mouse</td>
<td>1: 1000, 1 hour</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Abcam, ab40697, mouse</td>
<td>1: 1000, 1 hour</td>
</tr>
<tr>
<td>Actin</td>
<td>Abcam, AC15, mouse</td>
<td>1: 50 000, 1 hour</td>
</tr>
<tr>
<td>BiP/Grp78</td>
<td>Abcam, ab21685, rabbit</td>
<td>1: 1000, 1 hour</td>
</tr>
<tr>
<td>Calnexin</td>
<td>Abcam, ab22595, rabbit</td>
<td>1: 1000, 1 hour</td>
</tr>
<tr>
<td>LIMP-2</td>
<td>Abcam, ab106519, rabbit</td>
<td>1: 1000, 1 hour</td>
</tr>
<tr>
<td>P62</td>
<td>Abcam, ab56416, mouse</td>
<td>1: 1000, overnight</td>
</tr>
<tr>
<td>LC3</td>
<td>Cell signalling, rabbit</td>
<td>1: 1000, overnight</td>
</tr>
<tr>
<td>LAMP1</td>
<td>Abcam, mouse</td>
<td>1: 1000, overnight</td>
</tr>
<tr>
<td>Anti-haemagluttin</td>
<td>Covance, HA.11, mouse</td>
<td>1: 5000, 1 hour</td>
</tr>
</tbody>
</table>

**Endoglycosidase- H assay for endoplasmic reticulum retention of GCase**

To determine endoglycosidase-H sensitivity of GCase 20 ug of protein was mixed with a 1:10 dilution of glycoprotein denaturing buffer (New England Biolabs P0702S kit) and heated at 100 °C for 10 minutes. For each cell line a positive sample and a negative reaction mix was made; in the positive reaction mix there was endoglycosidase-H enzyme (2 uL of 500 000 U/mL enzyme stock with 2 uL of reaction buffer and 6 uL of dH2O) while the negative reaction mix had dH2O instead of endoglycosidase-H. Both reaction mixtures were incubated at 37°C for 12 hours and the reaction terminated by adding 6 uL SDS sample buffer and 1 uL of reducing agent. Western blotting using the anti-GCase antibody was then performed using the protocol above. The endoglycosidase-H sensitive fraction (representing the ER retained fraction) of GCase was calculated by dividing the intensity of the endo-glycosidase-H sensitive GCase band (the band of lowest
molecular weight which was not seen in untreated lanes, figure 11a) on Western blotting by the intensity of all the detected GCase in the lane.

**Proteasomal inhibition**
Proteasome inhibition was performed using a published protocol (Ron et al, 2005). Ten centimetre petri dishes of control and disease fibroblasts were incubated with ALLN (26 mM) and MG132 (15 uM) for 20 hours. For each cell line a petri dish treated with MG132 and ALLN was compared to a control petri dish treated with DMSO only.

**Immunofluorescence localisation of glucocerebrosidase protein in fibroblasts**
The subcellular localisation of the glucocerebrosidase protein in fibroblasts was studied using antibodies raised against human glucocerebrosidase (Abcam, ab55080, 1:50) and calnexin (an endoplasmic reticulum marker, Abcam, ab22595, 1:500). Fibroblasts were grown on 22mm coverslips overnight, fixed in 4% paraformaldehyde for 20 minutes and permeablised in ice cold methanol for 15 minutes. Non-specific staining was blocked with 5% goat serum. Fibroblasts were then incubated with the primary antibody for 1 hour at room temperature and washed before incubation with secondary antibody (Alexafluor-488 rabbit anti-mouse or Alexafluor-568 goat anti-rabbit, 1:500) and counterstained with DAPI. Antibodies were made up in PBS. Cells were photographed using a fluorescence microscope equipped with an ApoTome and AxioVision software (all Zeiss, Jena, Germany).

**Quantitative PCR**
RNA was extracted from cultured cells (< 10^6 cells) using the RNeasy kit (Qiagen) and converted to cDNA with QuantiTect reverse transcription kit (Qiagen). Relative expression of GCase, TFEB and β-actin mRNA was measured with Power SYBRgreen kit (Applied Biosystems) using a STEP One PCR machine (Applied Biosystems). β-actin mRNA levels were used to normalise data. Primers are listed in Supplementary Table 1. Relative expression was calculated using the ΔC_T method.
Table 12. Primer targets and sequences for quantitative PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5’-TCT ACA ATG AGC TGC GTG TG-3’&lt;br&gt;5’-GGT GAG GAT CTT CAT GAG GT-3’</td>
<td>58</td>
</tr>
<tr>
<td>TFEB</td>
<td>5’- CCAGAAGCGAGAGCTCACAGAT-3’&lt;br&gt;5’-TGTGATTGTCTTTCTTCTGCGG-3’</td>
<td>58</td>
</tr>
<tr>
<td>GCase</td>
<td>5’-TGC TGC TCT CAA CAT CCT TGC C-3’&lt;br&gt;5’-TAG GTG CGG ATG GAG AAG TCA A-3’</td>
<td>58</td>
</tr>
</tbody>
</table>

Treatment of fibroblasts with ambroxol hydrochloride

A titration was performed to determine the optimum concentration of ambroxol hydrochloride to treat fibroblasts with. A single control line and a single GD fibroblast cell line were treated with 10uM, 30uM and 60uM of ambroxol dissolved in DMSO (final concentration of DMSO 60 uL in 10 mL culture media) for 5 days. Fibroblasts were grown on 10 cm petri dishes and treated with 60uM of ambroxol hydrochloride dissolved in DMSO and then harvested for assessment of GCase protein and activity levels after treatment. For each line fibroblasts harvested from a 10 cm plate of untreated cells was used as a negative control and treated with DMSO only.

Cholesterol assay

Total cholesterol was measured with the Amplex red cholesterol kit (www.invitrogen.com). Fibroblasts were lysed in sucrose buffer (100uL). Twenty microlitres of cell lysate was added to 30uL of reaction buffer (supplied in kit). Cell lysates were assayed in duplicate and in the presence of cholesterol esterase in order to calculate total cholesterol. Fifty microlitres of reaction mix was added to each well to start the reaction and the reaction mixture was incubated for 15 minutes at 37oC protected from light. The reaction mixture for one plate of 16 wells (ie 8 samples done in duplicate) consisted of 15 uL of amplex red reagent, 10 uL of horse radish peroxidise, 10 uL of cholesterol oxidase and 1 ul of cholesterol esterase made up to final volume with 960uL of reaction buffer (supplied in kit). After 15 minutes the reaction was quantified on a Bio Tek Synergy HT plate reader with excitation 570 and emission 585 nm.
**Statistical analysis**

All analysis was performed using PASW version 20.1 (IBM). Non-parametric variables (i.e. percentages) were compared with a Mann-Whitney U-test. Parametric variables (i.e. rates of enzyme activity) were compared with a t-test.
## IIIc. Results

**Details of patient derived fibroblast cell lines**

Table 12 summarises the details of the patient derived fibroblast cell lines. The 2 carriers who underwent skin biopsy were specially selected to have no clinical markers of prodromal PD, no family history of PD and to be of advanced age so as to minimise the chances that they would develop PD in future (i.e. that they are truly non-manifesting carriers, NMC). DNA was extracted from each cell line and carriage of $GBA$ mutations confirmed by direct sequencing of the entire $GBA$ gene (performed by Dr R Duran, Department of Molecular Neuroscience). The mean age of the disease group (GD, PD and NMC combined) did not differ from that of the control group (55.9 years versus 69, $p=0.13$). They were also matched for sex (disease group 8/12 male versus control group 2/3 male, $p=0.14$). Fibroblast lines from 3 $GBA$ mutation negative PD cases were used as a disease control (2/3 female, mean age 77±4 years). In addition 4 fibroblast lines from patients with recessive early onset PD with bi-allelic $Parkin$ mutations were provided by Dr J Taanman (Department of Clinical Neurosciences, UCL Institute of Neurology). The $Parkin$ cells had the following mutations: 1. exon 3 deletion/exon 3 duplication, 2. exon 2-3 deletion/exon 2-3 deletion, 3. exon 3 deletion/P275W, 4. exon 4-5 deletion/exon 4-5 deletion.
Table 13. Clinical and demographic characteristics of patients fibroblast cell lines were generated from

<table>
<thead>
<tr>
<th>Patient identifier</th>
<th>Genotype</th>
<th>Prodromal markers of PD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gaucher disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GD01, M/50</td>
<td>N370S/c1263del55</td>
<td>Nil</td>
</tr>
<tr>
<td>GD02, M/70</td>
<td>N370S/N370S</td>
<td>Nil</td>
</tr>
<tr>
<td>GD03, M/40</td>
<td>N370S/L444P</td>
<td>Nil</td>
</tr>
<tr>
<td>GD04, M/61</td>
<td>N370S/203insC</td>
<td>Hyposmia, MoCA score 18/30</td>
</tr>
<tr>
<td>GD05, M/40</td>
<td>N370S/L444P</td>
<td>Isolated motor sign</td>
</tr>
<tr>
<td><strong>Carrier</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C39, F/80</td>
<td>N370S/Wt</td>
<td>Nil</td>
</tr>
<tr>
<td>C41, M/62</td>
<td>L444P/Wt</td>
<td>Nil</td>
</tr>
<tr>
<td><strong>PD-GBA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD01, F/53</td>
<td>N370S/Wt</td>
<td>Clinical PD</td>
</tr>
<tr>
<td>PD02, M/45</td>
<td>N370S/Wt</td>
<td>Clinical PD</td>
</tr>
<tr>
<td>PD03, M/72</td>
<td>L444P/Wt</td>
<td>Clinical PD</td>
</tr>
<tr>
<td>PD04, F/61</td>
<td>RecNcil/Wt</td>
<td>Clinical PD</td>
</tr>
<tr>
<td>PD05, E326K/E326K</td>
<td>E326K/E326k</td>
<td>Clinical PD, no overt GD</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN01, M/78</td>
<td>Wt/Wt</td>
<td>Nil</td>
</tr>
<tr>
<td>CN02, M/81</td>
<td>Wt/Wt</td>
<td>Nil</td>
</tr>
<tr>
<td>CN03, F/50</td>
<td>Wt/Wt</td>
<td>Nil</td>
</tr>
</tbody>
</table>

**GCase protein levels are reduced in GD, PD-GBA, carrier and control fibroblast lines**

Western blotting revealed a significant reduction in GCase protein levels in all GD (median GCase levels 48 % [IQR 25-55%] of control, P<0.001), PD-GBA (median 60 % [IQR 55-79%] of control p<0.001), E326K/E326K [median 80% of control [IQR 75-80%], p=0.003) and NMC cell lines (median 85 % of control [IQR 80-100%], p=0.043).

**GCase enzyme activity in GD, PD-GBA, carrier and control fibroblast lines**

GCase activity (measured in the presence of sodium taurocholate) was reduced by 99% compared to untreated cells in control fibroblasts treated with the GCase-1 inhibitor conduritol-b-epoxide (40uM for 12 hours), confirming that the activity we were measuring is that of GCase-1. Results of GCase Western blotting and activity are summarised in figure 10. GCase activity
(measured in the presence of sodium taurocholate) in GD fibroblasts was significantly lower than controls (mean 15 +/- 14 nmol/hour/mg protein compared to 300 +/- 100 nmol/hour/mg protein, p<0.001). The GCase activity of the GD group was 4% (IQR 2-5.6%) of the control group. GCase activity in PD-GBA (246 +/- 173 compared to 300 +/- 100, p=0.49) and NMC (300 +/- 121, p=0.99) was not significantly lower than controls. GCase activity in fibroblasts from the E326K homozygous PD case was significantly reduced (mean activity 99 +/- 8 nmol/hour/mg protein, p=0.001) compared to control, but to a lesser degree than seen in GD. GCase activity was not deficient in fibroblasts from GBA mutation negative PD (mean 250 nmol/hour/mg protein, p=0.5).

**Correlation between GCase protein levels and GCase enzyme activity**

When GCase protein levels and enzyme activity were correlated a significant, positive correlation was observed (Spearman's rho=0.79, p=0.001) such that increasing levels of GCase protein measured on Western blot were associated with increasing levels of GCase enzyme activity. This suggests that there is a relative loss and not an absolute deficiency of GCase activity in PD-GBA and NMC with loss of enzyme activity in proportion to reduction of GCase protein levels.

**Assessment of LIMP-2 protein levels in patient derived fibroblasts**

To ensure that GCase depletion was not secondary to loss of LIMP-2 expression Western blot measurement of LIMP-2 levels was performed. LIMP-2 levels did not differ from controls in either GD (median 93% of control [IQR 90-105%], p=0.33), PD-GBA (median 93% of control [IQR 90-100%], p=0.12) or NMC (median 95% [IQR 90-105%], p=0.1) fibroblasts.
Figure 11. Assessment of GCase protein and activity levels.

11a. Western blot of GCase protein levels. Note reduction of GCase protein levels in GD, PD-GBA and NMC compared to control lines. G = GBA, A = actin.

11b. Graph summarising GCase protein levels quantified from Western blot in GD, PD-GBA, E326K/E326K and NMC compared to control lines. Results are mean of 3 experiments +/- 1 standard deviation.

11c. Graph summarising GCase activity levels in GD, PD-GBA, E326K/E326K and NMC compared to control lines. Results are mean of 3 experiments for each cell line in each group +/- standard deviation.

11d. Graph summarising GCase mRNA levels in GD and PD-GBA cell lines. There was no reduction of transcript levels in N370S/N370S, E326K/E326K or N370S/Wt fibroblasts.
qPCR assessment of GCase mRNA levels in GD and PD-GBA

*GBA* transcript levels were reduced in GD lines with N370S/recombinant mutation or N370S/L444P and in PD-GBA with L444P or RecNeil mutations, but not in N370S homozygous GD, E326K homozygous or PD-GBA fibroblasts with N370S alleles (figure 10d).

**Immunofluorescence of GCase localisation in patient derived fibroblasts**

Indirect immunofluorescence using antibodies to GCase protein demonstrated that in control cells GCase accumulated in punctate vesicular structures at the cell periphery, in a pattern resembling that of LAMP1 immunostaining (figure 11d). In PD-GBA and NMC cells the pattern of GCase staining resembled control. However, in GD lines there was a severe reduction in GCase staining, with few or no punctate vesicular structures seen. This suggests that even though total cellular GCase (assessed by Western blotting) was reduced in PD-GBA and NMC, cellular compensatory mechanisms exist to increase lysosomal delivery of GCase and preserve enzyme activity.

**Assessing evidence of ER retention of GCase: Endoglycosidase-H digestion of GCase**

ER retained forms of GCase carry carbohydrates which are sensitive to cleavage by endoglycosidase-H (figure 11a, 11b). The endoglycosidase-H sensitive fraction of GCase was significantly higher in GD fibroblasts (mean 12.2% +/- 4 compared to mean 0.76 %, p<0.001), E326K/E326K (mean 15% +/-5%, p<0.001), and PD-GBA fibroblasts (mean 3.5 +/- 3.5%, p=0.026), indicating ER retention (figure 11b). As expected, cell line PD4 did not show an elevated endoglycosidase-H sensitive fraction, in keeping with its mutant allele being a recombinant allele and producing no/very truncated protein.

**Assessing evidence of ER retention of GCase: calnexin immunofluorescence**

To confirm ER retention of GCase indirect immunofluorescence using antibodies to GCase and calnexin (an ER marker) was performed. In GD lines there was evidence of co-localisation of GCase and calnexin in the peri-nuclear region where the ER is located (figure 11d). Clear evidence of ER retention in the PD-GBA lines could not be demonstrated. This is likely due to rapid degradation of the ER retained mutant GCase in these lines resulting in quantities of ER retained GCase too small to be detected by immunofluorescence.
**Figure 12. Evidence of endoplasmic reticulum retention in Gaucher disease.**

12a. Representative western blots of cell lysate treated with endoglycosidase-H for 12 hours. Note appearance of low molecular weight band in GD samples (arrow), which represents endoplasmic reticulum retained GCase protein. “+” lanes were treated with endoglycosidase-H.

12b. Bar chart summarising percentage of endoglycosidase-H sensitive GCase in GD, PD-GBA and NMC compared to controls. Results are mean of 3 experiments.


12d. Top panel: immunofluorescence of control cells showing vesicular staining of GCase (green) at the periphery of the cell and perinuclear, reticular staining of calnexin (red, ER marker). Bottom panel: colocalisation of GCase and calnexin represented by yellow perinuclear staining with minimal green vesicular staining pattern in 2 GD cell lines. 1
**Effect of proteasomal inhibition on GCase metabolism in GD and PD-GBA**

Given the reduced GCase protein levels and evidence of ER retention of GCase in the disease fibroblasts we hypothesized that reduced GCase levels may result from proteasomal degradation. Disease and control fibroblasts were treated with MG132 and ALLN for 20 hours and then harvested. Proteasome inhibition resulted in elevated GCase protein levels, as assessed by Western blotting, within GD fibroblasts (mean 49 +/- 43% increase, p=0.029) and PD-GBA fibroblasts (mean increase 21 +/- 17 %, p=0.043) to a greater degree than in controls (mean 2% increase). As expected, cell line PD4 did not show an elevated GCase protein level after proteasome inhibition, in keeping with its mutant allele being a recombinant allele and producing no/very truncated protein.

**Assessment of ER stress markers in GD and PD-GBA**

Given the evidence of ER retention of GCase we assessed the cell lines for evidence of ER stress by Western blotting (figure 11c). There was BiP/Grp78 elevation on Western blotting of GD (median 60% increase compared to controls [IQR 20-100% increase], p=0.0016) and PD-GBA cells (median 15% increase [IQR 0-50%], p= 0.0023). Calnexin levels were significantly elevated in GD fibroblasts (median 50 % increase [IQR 50-100%], p=0.003) and PD-GBA fibroblasts (median 32 % increase [IQR 12-50%], p=0.019). It should be noted that ER stress markers were not elevated in PD04 (RecNcil/Wt).

**Assessment of GCase metabolism in fibroblasts with bi-allelic Parkin mutations**

*Parkin* protein may act as a ubiquitin ligase to facilitate degradation of mutant GCase. We therefore examined metabolism of GCase in fibroblasts from 4 patients with bi-allelic Parkin mutations. There was no alteration in GCase protein levels as assessed by Western blotting (*Parkin* median 102% [IQR 90-110%] of control mean, p=0.69), nor was there alteration in the endoglycosidase-H sensitive fraction of GCase (no endoglycosidase-H sensitive GCase is present in Parkin cells). To simulate the effects of protein misfolding induced by GCase mutations, we treated Parkin and control fibroblasts with tunicamycin (5 µg/mL of media for 24 hours), which inhibits glycosylation and proper protein folding. Induction of the unfolded protein response was
confirmed by elevation of Bip/Grp78 on Western blotting (greater than 2-fold increase in Bip/Grp78 levels in treated compared to untreated cells, data not shown). After treatment with tunicamycin the levels of GCase within Parkin and control cells decreased by a similar amount (control median reduction 32 % [IQR 25-35%] vs disease median reduction 28.7% [IQR 25-30%], p =0.58), presumably reflecting degradation of abnormally folded protein. This does not support the contention that Parkin protein is an E3-ubiquitin ligase for GCase.

Assessment of lysosomal mass in GD, PD-GBA, carrier and control fibroblast lines
To ensure that the reduction in GCase protein levels in the fibroblast lines was not due to generalised depletion of lysosomes a study of other lysosomal markers was performed. LAMP-1 Western blotting demonstrated no difference in the levels of this endo-lysosomal marker between GD (median 95 % of control [IQR 90-105%], p>0.05) and PD-GBA (median 98% of control [IQR 95-110%]) or control (figure 12c). In keeping with this, activity levels of beta-galactosidase did not differ between GD (mean 600 +/-153 nmol/hour/mg, p=0.3), PD-GBA (mean 549 +/-200 nmol/hour/mg, p=0.9) and controls (mean 563 +/-119 nmol/hour/mg). On Western blotting, the amount of Cathepsin D was significantly increased in GD (median 55% increase [IQR 32-100%], p<0.0001), PD-GBA (median 33% increase [IQR 20-40%], p=0.002) and NMC (median 27% increase [IQR 20-40%], p=0.003) fibroblasts (figure 11c). Total beta-hexosaminidase activity was elevated in GD (mean 12 800 +/-3 200 nmol/min/mg, p=0.008), PD-GBA (12 400 +/-5 700 nmol/min/mg, p=0.004) and NMC (mean 12 750 +/-1800 nmol/min/mg, p=0.001) compared to controls (mean 5200 +/-1000 nmol/min/mg). Enzymatic activity of GCase-2 (defined as total GCase activity – GCase activity measured in the presence of DNJ [a specific inhibitor of Gcase-2]) was significantly higher in GD (mean 7.5 +/-3.9 nmol/hour/mg, p<0.001), PD-GBA (mean 6.3 +/-3.1 nmol/hour/mg, p=0.008) and NMC than controls (mean 7.3 +/-0.9 nmol/hour/mg, p=0.023). Taken together, these data suggest that there are secondary changes in Cathepsin D, GCase-2 and total beta-hexosaminidase metabolism which are not due to an overall expansion or contraction of the lysosomal compartment in GD, PD-GBA and NMC.
Macroautophagy is normal in GD and PD-GBA fibroblasts

Given the evidence of subtle lysosomal dysfunction we investigated macroautophagy and cholesterol metabolism. At baseline (i.e. without starvation) there was no elevation of p62 in GD (median 101% [IQR 90-110%] of control, p=0.9), PD-GBA fibroblasts (median 100% [IQR 95-105%] of control, p=0.8) or NMC (median 99% of control, p=0.8)(figure 12a). At baseline (i.e. without starvation) there was no alteration of LC3-II in GD (median 82% [IQR 75-100%] of control, p=0.062) or PD-GBA (median 88% [IQR 80-100%] of control, p=0.13) or NMC (median 99% of control)(figure 12a). Starvation increased LC3-II expression in control (median increase 20% [IQR 10-20%]) and GD (median increase 100% [IQR 40-200%], p=0.95) indicating intact induction of autophagy. This suggests that macroautophagy is not abnormal. Total cholesterol levels were not altered in GD fibroblasts (mean 142+/−9.7 ug cholesterol/mg protein, p=0.096) or PD-GBA (mean 147+/−27 ug cholesterol/mg protein, p=0.238) compared to controls (mean 178+/−17 ug cholesterol/mg protein).
Figure 13. Assessment of autophagy and lysosomal metabolism in GD and PD-GBA.

13a. Western blots of LC3 and p62. There is no alteration in levels of LC3-II or p62 in GD or PD-GBA compared to control cell lines.

13b. Graph of total beta-hexosaminidase activity in GD, PD-GBA and NMC compared to control. Results are mean of 3 experiments for each cell line in each group +/- 1 standard deviation.

13c. Western blot demonstrating increased cathepsin D (CTSD) levels in GD and PD-GBA compared to control (top panel), and unaltered LAMP1 levels in GD and PD-GBA (bottom panel) with large increases in expression of LAMP1 after ambroxol treatment (lanes marked +).

13d. Graph summarising densitometric analysis of cathepsin D levels in GD and PD-GBA.
Effect of ambroxol hydrochloride on GCase metabolism

To determine the optimal dose of ambroxol hydrochloride, a titration of 10uM, 30uM and 60uM treatment was performed. The titration was performed in a single GD cell line compared to control. As assessed by Western blotting an increase of 100%, 100% and 400% was noted with 10uM, 30uM and 60uM of ambroxol respectively in the GD cell line and 5%, 15% and 20% in the control line. Treatment at 60uM produced the greatest increase in GCase protein expression, it was therefore decided to use this dose to evaluate the effects of ambroxol.

Ambroxol treatment resulted in a significant elevation of GCase protein levels in GD (median increase 100% of untreated cells [IQR 87-200% increase], p=0.004) and PD-GBA (median increase 50 % of untreated cells value [IQR 50-130%], p=0.04) compared to control fibroblasts (median 10 % increase of untreated [IQR 10-20%], p>0.05) on Western blotting (figure13a). Ambroxol treatment also resulted in a significant elevation of GCase activity levels (figure 13c). In GD fibroblasts there was a median 400% increase (IQR 300-500%, p=0.004) in GCase activity (measured in the presence of sodium taurocholate) compared to untreated cells. After ambroxol treatment the GCase activity of GD fibroblasts remained significantly below mean control cell activity (median 13.7% [IQR 7.1-18%] of control group activity, p<0.001). In PD-GBA fibroblasts there was a median 375% (IQR 275-500%) increase in enzyme activity (p=0.009). In control cells GCase activity increased a median of 40% (IQR 30-40%). Enzyme activity in PD04 increased by a median of 30 %, being no different from controls (p>0.05). Treatment with ambroxol resulted in a complete disappearance of the endoglycosidase-H sensitive fraction from both GD and PD-GBA fibroblasts (figure 14b). This suggests a reduction in the proportion of GCase retained in the endoplasmic reticulum after ambroxol treatment.

Ambroxol hydrochloride induces GBA gene transcription

GBA mRNA levels increased significantly in GD (median 100% increase [IQR 87-150%], p<0.05), PD-GBA (median 200% [IQR 150-200%], p<0.05) and control cell lines (median 100% [IQR 100-150%], p<0.05): suggesting induction of transcription (figure 13d). This was associated with upregulation of TFEB transcript levels in GD (median 152% [IQR 100-150%], p<0.05), PD-GBA (median 25% [IQR 15-25%], p<0.05) and control (median 100% increase, p<0.05).
Effect of ambroxol hydrochloride on lysosomal metabolism

Ambroxol treatment resulted in elevation of LAMP-1 protein levels, total cholesterol and beta-galactosidase activity accompanied by significant reductions in cathepsin D levels and total beta-hexosaminidase activity. LAMP-1 protein levels increased by a median of 100% (IQR 100-200%) in GD, 100% (IQR 100-200%) in PD-GBA and 100% (IQR 100-200%) in controls. Total cholesterol increased by a median of 200% (IQR 100-600%) in GD, 300% (IQR 150-650%) in PD-GBA and 300% (IQR 100-300%) in controls. Beta-galactosidase activity increased to the same extent in GD (median 50% increase, IQR 45-100%, p=0.54), PD-GBA (median 70%, IQR 36-100%, p=0.90) and control (median 60%, IQR 50-60%). In GD fibroblasts Cathepsin D levels were reduced by a median of 35% [IQR 25-50%] compared to untreated cell lines (p<0.001). In PD-GBA cell lines Cathepsin D levels were reduced by a median of 22% [IQR 15-40%] compared to untreated cells (p=0.0002). Cathepsin D levels did not change in ambroxol treated controls. Total beta-hexosaminidase activity fell by a median of 36.5% (IQR 26.2-50% fall, p=0.012) in GD and 30% (IQR 20-50%, p=0.019) in PD-GBA with a median increase in activity of 8% in controls. GCase-2 activity was significantly reduced in both GD (mean 1.6+/-.0.3 after treatment compared to mean 7.5+/-.3 nmol/hour/mg before, p=0.012) and PD-GBA (mean 0.5+/-.0.3 after treatment compared to mean 6.3+/-.1 nmol/hour/mg before, p=0.001) after ambroxol treatment, but there was no change in control cell GCase-2 activity (figure 14a). This suggests an increase in lysosomal mass with reduction in cathepsin D and beta-hexosaminidase in response to restored GCase activity in GD and PD-GBA.

Ambroxol hydrochloride treatment increases ER stress markers but not cell death

After ambroxol treatment BiP levels rose in GD (median 33% increase [IQR 24-40%], p=0.001) but not PD-GBA (median increase 1%, IQR -3% to +4%, p=0.3). Calnexin increased in GD (median 52% increase, IQR 33-100%, p<0.001) but not PD-GBA (median increase 3%, IQR 0-9%, p=0.065). There was no evidence of cell death after ambroxol treatment of control or GD fibroblasts (median decrease of 15% fluorescent units/5 000 cells treated for GD cells versus median decrease of 11% of FU/5 000 cells for control treated, p=0.96).
Figure 14. Effect of ambroxol treatment on GCase in GD and PD-GBA fibroblasts.
14a. Western blot demonstrating significant increase in GCase protein levels (top row) after ambroxol treatment of GD and PD-GBA fibroblasts. Middle row shows decrease in cathepsin D after ambroxol treatment. Lanes marked (+) were treated with ambroxol.
14b. Graph summarising results of densitometry of GCase protein levels on Western blot after ambroxol treatment demonstrating significant increase in GD and PD-GBA cell lines. Results are mean of 3 experiments +/- 1 standard deviation.
14c. Graph demonstrating absolute increase in GCase activity after ambroxol treatment. Results are mean of 3 experiments +/- 1 standard deviation. Even though there was a significant increase in activity after ambroxol treatment, activity in GD cells did not approach the GCase activity range of control fibroblasts.
14d. Graph demonstrating the increase in GCase transcript levels, expressed as a percentage of untreated controls. Results are mean of 3 experiments +/- 1 standard deviation.
Figure 15. Effect of ambroxol treatment on lysosomal markers.

15a. Graph demonstrating a significant decrease in absolute GCase-2 activity levels after ambroxol treatment. Results represent mean of 3 experiments +/- 1 standard error. Control represents 3 control lines measured in triplicate.

15b. Western blot demonstrating a decrease in the endo-glycosidase H sensitive fraction of GCase in ambroxol treated GD cells. This implies a reduction in endoplasmic reticulum trapping of GCase. Lanes marked “-“ are without ambroxol treatment and “+” are after ambroxol treatment. All lanes were treated with endo-glycosidase H. Arrows point to endo-H sensitive
Ambroxol hydrochloride decreases alpha-synuclein levels in alpha-synuclein over expressing neuroblastoma cell lines

After 5 days of ambroxol hydrochloride treatment (60 uM) levels of HA-tagged alpha-synuclein in the over expressing neuroblastoma cell line decreased significantly (median fall 15% [IQR 15-25%], p=0.0034)(figure 15a-c). This was accompanied by an increase in GCase (median 30% increase [IQR 15-40%], p=0.011) and LAMP-1 protein levels (median 140% increase [IQR 125-155%], p=0.031). This suggests that ambroxol is acting to increase lysosomal mass and thus degrade alpha-synuclein.

Figure 16. Effect of ambroxol treatment on alpha-synuclein over expressing neuroblastoma cells.

16a. LAMP-1 Western blot demonstrating increased LAMP-1 expression after ambroxol treatment.
16b. GCase Western blot demonstrating increased GCase expression after ambroxol treatment.
16c. Western blot demonstrating decreased HA tagged alpha-synuclein in ambroxol treated neuroblastoma cells.
IIIId. Discussion.

**Mechanisms by which GBA mutations cause loss of GCase activity**

Here we report a detailed characterisation of the cellular processing of mutant GCase protein in a panel of fibroblasts from GD patients (n=5), PD-GBA patients (n=5) and non-manifesting carriers (n=2). The GD fibroblasts express a reduced amount of GCase protein when compared to control fibroblasts (median GCase levels 48 % [IQR 25-55%] of control, P<0.001). This is entirely in keeping with the results of Ron *et al* (2005) who demonstrated GCase levels of 30-70% of normal in Type I GD fibroblasts. The GCase protein levels in PD-GBA (median 60 % [IQR 55-79%] of control p<0.001, p=0.003) and NMC cell lines (median 85 % of control [IQR 80-100%], p=0.043) were reduced compared to control fibroblasts but not to the same extent as in Type I GD fibroblasts. This is in keeping with the results of Gegg *et al* (2012) who demonstrated reduced GCase protein levels on Western blotting of substantia nigra from PD brain from patients with heterozygous *GBA* mutations. These results clearly reflect the presence of 1 wild type allele in the heterozygous fibroblasts.

The mean GCase activity level for the Type I GD cells as a group was 5% of control values. This is similar to the values reported by Ron *et al* (2005) for Type I GD. The GCase activity levels of the PD-GBA and NMC fibroblasts were not significantly lower than that of the control cells. However, when the GCase protein levels for each cell line were correlated with the GCase enzyme activity was proportional to the amount of GCase protein. Thus while there was not an absolute deficiency of GCase activity in the heterozygous cells there was evidence of a relative reduction of GCase activity in proportion to GCase protein depletion. This is in keeping with the work of Lu *et al* (2011) who demonstrated that N370S or L444P mutant GCase extracted from GD fibroblasts had near normal enzymatic activity and that it was the reduction in GCase protein levels in these cells which accounted for depletion of GCase activity.
In the cell lines studied here fibroblasts carrying N370S and E326K mutations had normal levels of GBA transcripts. The L444P, recombinant (RecNcil, Rec[c.1263del55] (Sarria et al, 1999) and 203insC mutations (which is predicted to cause a frameshift and premature stop codon, with a truncated transcript that is likely to decay (Frischmeyer and Dietz, 1999)) were associated with reduced mRNA levels. Previous studies had demonstrated normal levels of L444P and N370S mRNA with reductions of A120G and P415R mutation bearing mRNA (Ohashi et al, 1990) in transfected NIH 3T3 cells. Tayebi et al (1998) demonstrated reduced mRNA levels for a patient with homozygous GBA null mutations. However, the mRNA in these studies was quantified with Northern blotting, which is less sensitive for detection of mRNA levels than the qPCR technique utilised in the current study. Thus the reduction in transcript levels for certain GBA mutations we identified is likely to reflect the greater sensitivity of our technique. Moreover, Germain et al (2001) demonstrated severe reduction in GBA mRNA levels caused by the g.5255del T GBA mutation, predicted to cause a frameshift and stop codon. This demonstrates that qPCR can detect reductions in GBA transcript levels due to GBA mutations. It seems reasonable to suggest that the RecNcil and Rec[c.1263del55] mutations will produce an unstable mRNA or reduce transcription given the structural re-arrangement of the GBA gene on these alleles. It is unclear why the L444P mutation should cause a reduction in GBA transcripts since the mutation is not predicted to cause mRNA instability (Hruska et al, 2008). It might be that as the misfolded L444P protein induces the unfolded protein response there is a reduction in L444P transcription as part of this. Given that the N370S and E326K variants are broadly accepted as less severe mutations it might be that the unfolded protein response is induced to a lesser extent than for L444P with no accompanying reduction in GBA transcription. Nonsense mediated decay is unlikely to play a role given that the L444P mutation is not predicted to form a premature stop codon (Hruska et al, 2008).
However, given that all cell lines had reduced GCase protein and activity levels a post-translational process must also play a role in interefering with GCase activity in these cells. Previous work has demonstrated evidence of ER retention and proteasomal degradation in GD (Ron and Horowitz, 2005). Here we confirm that this process occurs in fibroblasts from Type I GD patients and PD-GBA. The endoglycosidase-H sensitive fraction of GCase was increased in all cells (apart from the PD line RecNcil/Wt), and in GD fibroblasts GCase protein and the ER marker calnexin co-localised on immunofluorescence. Treatment with proteasome inhibitors increased GCase levels suggesting that ER retained GCase is degraded in the proteasome. Thus GBA mutations reduce GCase activity by at least 2, non-mutually exclusive mechanisms: 1. reducing GBA translation or causing transcript instability and 2. triggering ER retention and proteasomal degradation. Both of these processes represent valid therapeutic targets.

Alternative explanations for the depletion of GCase from the fibroblasts include loss of a cofactor for GCase synthesis or a generalised loss of lysosomes. As discussed in the introduction LIMP-2 is vital for GCase trafficking to the lysosome and genetic variation at SCARB-2, the LIMP-2 gene, is associated with sporadic PD (Michelakakis et al, 2012). It was therefore conceivable that alterations in LIMP-2 expression might occur in response to decreased GCase activity (i.e. increased expression of LIMP-2 to compensate for reduced GCase expression) or that genetic variation in SCARB2 associated with PD might result in reduced LIMP-2 expression and secondary depletion of GCase. Our Western blotting study clearly shows that LIMP-2 protein levels are not depleted in GD or PD-GBA/NMC and thus this does not contribute to loss of cellular GCase in association with GBA mutations. To assess for a generalised decrease in lysosomal mass in GD we performed Western blotting for LAMP1 expression, demonstrating no difference between disease and control cells, suggesting no general loss of lysosomes. Likewise there is no evidence of loss of lysosomes in PD-GBA brain tissue (Gegg et al, 2012).

We recently described loss of GCase protein and enzyme activity in the substantia nigra of PD-GBA and PD without GBA mutations (Gegg et al, 2012), and cell biology studies suggest that alpha-synuclein accumulation depletes GCase (Mazzulli et al, 2011). The reduction in GCase activity and protein in heterozygous GBA mutation carriers, as evident in NMC fibroblasts, may thus predispose to age related loss of neuronal GCase activity secondary to age related alpha-
synuclein accumulation. Several studies indicate that alpha-synuclein accumulates in brain tissue from aged humans without PD providing support for this mechanism (Mazzulli et al, 2011). Once this reduction in GCase activity crosses a hypothetical critical threshold it will result in a “feed-forward” mechanism as lysosomal dysfunction leads to alpha-synuclein accumulation, which in turn exacerbates loss of GCase. Since all GBA mutations are predicted (or proven) to cause ER retention or reduced GBA transcription/mRNA stability this hypothesis explains how both recombinant alleles and point mutations in the GBA gene can predispose to PD.

How alpha-synuclein inhibits GCase activity is unknown. Alpha synuclein accumulation in cells has been shown to alter endoplasmic reticulum calcium efflux and enhance autophagic activity (Cali et al, 2012), this could in theory deplete cellular GCase and also cause endoplasmic reticulum retention of GCase as the protein requires calcium ions to aid folding. Colla et al (2012) demonstrate that alpha-synuclein oligomers accumulate in the endoplasmic reticulum. It has been shown that alpha-synuclein and GCase can physically interact, suggesting that alpha-synuclein oligomers could physically trap GCase in the endoplasmic reticulum. We could not examine the interaction between GCase activity and alpha-synuclein in our cell model since fibroblasts do not express alpha-synuclein. But clearly a cell with compromised GCase activity and upregulated unfolded protein response will be vulnerable to depletion of GCase secondary to alpha-synuclein accumulation.
The role of Parkin protein in GCase metabolism

There is evidence that Parkin protein acts as a ubiquitin ligase to facilitate the degradation of mutant GCase protein (Ron et al, 2010). However, this evidence comes from cell lines over expressing Parkin and GCase proteins. Here we assessed the metabolism of GCase in fibroblasts from patients with bi-allelic Parkin mutations. We hypothesised that mutant Parkin proteins would not be able to ubiquitinate GCase and target it for degradation. Neither GCase protein levels, enzyme activity nor the endoglycosidase-H GCase sensitive fraction were altered in Parkin cells. This is in keeping with the study of Ron et al (2010) which demonstrated that Parkin only bound to and ubiquitinated mutant, and presumably misfolded, GCase protein. We then treated cell lines with tunicamycin, which inhibits protein glycosylation and induced the Unfolded Protein Response, in order to simulate GCase misfolding due to GBA mutations. This caused degradation of GCase protein in both control cells and Parkin mutation carrying cells to the same degree. Tunicamycin induces severe protein misfolding and triggers the Unfolded Protein Response, clearly causing degradation of GCase. If Parkin protein played an important role in targeting mutant, misfolded GCase to the proteasome then GCase degradation should have been inhibited in these cell lines. It might be that Parkin protein only plays a role in “neuronal” SHSY-5Y cell lines utilised by Ron et al (2010) or that Parkin and GCase only interact when the proteins are over expressed and not at endogenous levels. Clearly further studies into the interaction between Parkin and GCase are required to define the importance of Parkin as an E3 ubiquitin ligase for GCase.
The role of ER stress in GD and PD-GBA pathogenesis

Induction of the Unfolded Protein Response and ER stress are well described in both PD and Lysosomal storage disorders. Wei et al (2007) demonstrated upregulation of calnexin, Grp78 and XBP1 in fibroblasts from a range of neuronopathic (neuronal ceroid lipofuscinosis) and non-neuronopathic (Type I GD, GM1-gangliosidosis, Tay-Sachs disease) Lysosomal Storage Disorders. Gegg et al (2012) showed upregulation of ER stress markers in substantia nigra from PD-GBA. There are several post-mortem studies demonstrating increased expression of endoplasmic reticulum stress markers in PD brain (see general introduction). Several cell biology studies indicate that alpha-synuclein accumulation can directly induce endoplasmic reticulum stress suggesting that this pathway is of central importance in dopaminergic cell death (Colla et al, 2012). The expression of ER stress markers in Lysosomal Storage Disorders chiefly relates to ER retention of mutant lysosomal enzymes. There is also evidence that lysosomal inhibition with ammonium chloride can induce ER stress, presumably secondary to inhibition of trafficking of proteins from ER to lysosome. Both GD and PD-GBA fibroblasts had elevated BiP/Grp78 and calnexin levels, suggesting ER stress. That ER stress markers are upregulated in fibroblasts from NMC suggests that ER stress is an important early mechanism in the pathogenesis of PD-GBA. Moreover, as fibroblasts express only very low levels of SNCA it is clear that ER stress is not related solely to Lewy body accumulation and is therefore an early process in PD-GBA.
Autophagy and lysosomal biochemistry in GD and PD-GBA

There were no gross alterations in lysosomal metabolism in the GD or PD-GBA fibroblasts. Lysosomal mass was not altered. This is in keeping with data from GD fibroblasts, spleen, liver and bone marrow mesenchymal cells (Moffitt et al., 1978; Campeau et al., 2009). Macroautophagy was not altered in GD or PD-GBA fibroblasts. At baseline neither p62 nor LC3-II levels were different to controls, and LC3-II was induced by starvation to a similar extent in GD and controls. These results are similar to that of Tatti et al. (2012) who demonstrated normal macroautophagy in N370S/N370S fibroblasts. In contrast the same group report impaired autophagy in fibroblasts from homozygous saposin C mutation associated GD (discussed below). Though there was no clear increase in lysosomal mass in GD or PD-GBA cathepsin D protein levels, GCase-2 activity and total-beta hexosaminidase activity were increased. Cathepsin D mRNA levels have been reported to be elevated in brain tissue from a mouse model of neuronopathic GD (Vitner et al., 2010) and Cathepsin B, K and S activities are elevated in GD plasma (Moran et al., 2000). Cathepsin D can proteolytically activate saposin C by cleaving it from pro-saposin and thus Cathepsin D upregulation may be a homeostatic response to decreased GCase activity in GD fibroblasts. Multiple studies report elevation of total-beta-hexosaminidase activity in GD fibroblasts, spleen and blood with some also identifying elevated activity in the blood of carriers (Moffitt et al., 1978). GCase-2 can metabolise GCase-1 substrates and clearly upregulation of GCase-2 could be a compensatory mechanism for reduction of GCase-1 activity (Aureli et al., 2012).

It is noteworthy that several studies indicate that the alterations in lysosomal biochemistry in peripheral tissues and human brain differ; for example the elevation in beta-hexosaminidase activity is not reported in post-mortem brain from GD patients. Thus the absence of defective macroautophagy in the fibroblasts studied here should not be taken as evidence that defective autophagy does not occur in the brain in patients with GBA mutations. Studies of mouse models of neuronopathic GD have demonstrated accumulation of p62 histologically (Sun et al., 2010), suggesting that defective autophagy may occur in the central nervous system in GD. This issue remains to be conclusively addressed in mouse models and neuronal culture systems.
It is interesting to contrast the reported alterations in macroautophagy in GD with homozygous saposin C mutations (Tatti et al, 2012) compared to our GD fibroblasts with GBA mutations. In saposin C deficient cells there is failure of autophagosome degradation due to cathepsin B and D downregulation. Transfection of cathepsin B and D restored autophagy in these cells. In GD there is no clear evidence of defective autophagy and cathepsin D levels are upregulated in fibroblasts and cathepsin B reported to be upregulated in blood (Moran et al, 2000). In saposin C deficient cells GCase is potentially active (ie in vitro assays using synthetic substrates show normal activity) but in vivo activity is severely deficient as GCase is not trafficked properly to the lysosome resulting in glucosylceramide and ceramide accumulation. In contrast GD fibroblasts with GBA mutations have relatively more residual GCase activity and store little or no substrate. It might be this substrate accumulation which alters autophagy in GD with saposin C mutations. Thus the organs which accumulate substrate in GD, such as the brain, may manifest altered autophagy.

The mechanisms by which alpha-synuclein are degraded are thought to involve both the proteasome and the lysosome. Some argue that proteasomal degradation is more important under physiological conditions and that lysosomal degradation via chaperone mediated autophagy (CMA) is more significant under pathological conditions. Alvarez et al (2010) demonstrate that Hsc70 binds to alpha-synuclein and directs it to the lysosome by docking with LAMP2. The identity of the proteases involved in alpha-synuclein degradation remain unclear. One candidate is cathepsin D, overexpression of this enzyme results in reduced cellular alpha-synuclein levels and cathepsin D knock out mice have evidence of neuronal alpha-synuclein accumulation (Cullen et al, 2009). The upregulation of cathepsin D reported by us and others, suggests that it is not alteration of this enzyme's activity which leads to alpha-synuclein accumulation in association with GBA mutations. Interestingly, depletion of GM1-ganglioside has been postulated to disrupt alpha-synuclein interactions with membranes and possibly lead to its pathological accumulation (Martinez et al, 2007). Increased activity of beta-hexosaminidase could result in depletion of GM1-ganglioside and consequent perturbation of alpha-synuclein interactions with membranes. The upregulation of beta-hexosaminidase activity is relevant in this regard as a possible mechanism by which secondary changes induced by GBA mutations could result in alpha-synuclein accumulation. Against this is the fact that the product of this
enzyme, GM3-ganglioside, has been shown to bind alpha-synuclein and inhibit aggregation (Di Pasquale et al, 2010). An alternative mechanism is that altered lysosomal biochemistry in GD and PD-GBA may inhibit CMA. This has been demonstrated in mucolipidosis type IV fibroblasts (Venugopal et al, 2009) which displayed reduced degradation of CMA targets upon serum withdrawal. However, in this disease the mutated protein TRPML1 directly interacts with Hsc 70 giving clearly explaining why CMA should be abnormal. There is no rationale to support the hypothesis that CMA will be abnormal in GD and it seems less likely that this is the mechanism by which GBA mutations lead to alpha-synuclein accumulation. However, given that alpha-synuclein is not expressed in fibroblasts, neuronal culture systems should be used to examine the mechanisms by which altered lysosomal biochemistry caused by GBA mutations leads to Lewy body formation.

Reductions in total cholesterol including low density and high density lipoprotein have been consistently reported in blood from GD patients (Stein et al, 2011) and heterozygous carriers (Pocovi et al, 1998). This abnormal lipid profile is reversed by enzyme replacement therapy and is attributed to increased catabolism of apolipoproteins B100 and A1. We could not identify any alteration of total cholesterol levels in GD or PD-GBA fibroblasts. Some authors have reported that GD fibroblasts have elevated cholesterol levels (Salvioli et al, 2005). However, this report used Filipin staining, which is not a quantitative technique. Ron and Horowitz (2007) describe elevated intracellular cholesterol in dermal fibroblasts from a severely affected GD patient and postulate that elevated intracellular cholesterol is associated with more severe phenotypes in GD. However, this patient has been shown to carry a heterozygous SCARB2 mutation, this gene has been implicated in cholesterol flux and this might explain why this cell line had elevated intracellular cholesterol (Velayati et al, 2011). The role of alterations in intracellular cholesterol metabolism in GD remains unclear, but of potential relevance since lysosomal cholesterol can inhibit GCase (Salvioli et al, 2005) and disruptions in membrane lipids could potentially result in alpha-synuclein accumulation.
The cellular pathophysiology of the E326K GBA mutation

It is unclear if the E326K variant should be considered a benign polymorphism, a pathogenic mutation or a genetic modifier of GD (Horowitz et al, 2011). In the context of PD it is clear that E326K is found more frequently in PD than in controls (Lesange et al, 2011; Pankratz et al, 2012; Ron and Horowitz, 2010), both in the heterozygous and homozygous state, and can be considered a genetic risk factor for PD. No GD patients with homozygous E326K variants have been reported, but there is evidence that it acts as a genetic modifier since when it is present in GD patients with homozygous or compound heterozygous GBA mutations it is associated with a more severe phenotype (Horowitz et al, 2011). However, given the high frequency of clinically silent GD with homozygous N370S mutations it is plausible that E326K homozygosity could cause very mild, subclinical GD which is not clinically recognised (Balwani et al, 2010). To resolve this issue will require comprehensive phenotyping of E326K homozygotes, for example with nuclear medicine bone scans and bone marrow histological examination.

Thus, it is not clear if the E326K homozygous PD patient should be classified as having PD as a clinical manifestation of GD or PD related to GBA mutations. That the E326K fibroblasts had GCase protein levels and activity intermediate between controls and GD lines fits with reports of GCase activity in transfected cell systems where E326K activity was compared to that of N370S and L444P (Horowitz et al, 2011). Given the intrinsic variability of fibroblasts, the GCase activity obtained from a single cell line does not permit us to conclusively state that E326K homozygosity will not cause enzyme deficiency sufficient to cause GD. However, it seems that the E326K variant has a less severe molecular and cellular effect on GCase than the GD associated mutations and this might explain its reduced pathogenicity.
Ambroxol hydrochloride as a possible enzyme enhancing strategy for GD and PD-GBA

There is strong evidence from several research groups that ambroxol hydrochloride improves GCase protein levels and enzymatic activity; probably by acting as a chaperone and increasing mutant GCase protein folding and lysosomal delivery (Maegawa et al, 2009; Luan et al, 2012). Here we confirm these findings by demonstrating increased GCase protein levels, on Western blot, and increased enzymatic activity in fibroblasts from Type I GD patients treated with ambroxol hydrochloride. We also show that ambroxol hydrochloride enhances the GCase protein levels and enzyme activity in PD-GBA fibroblasts. In GD, PD-GBA and control fibroblasts treatment with ambroxol hydrochloride there was a robust and significant elevation of GBA mRNA levels. This suggests that ambroxol can induce GBA transcription. Control, GD and PD-GBA fibroblasts treated with ambroxol demonstrated increased transcription factor EB (TFEB) mRNA levels. This demonstrates that ambroxol upregulates GBA mRNA levels via a pathway involving TFEB. However, the fact that ambroxol treatment caused a significant reduction in the endoglycosidase-H sensitive fraction of GCase (i.e. the endoplasmic reticulum retained portion of the protein, figure 14b) suggests that it also acts as a chaperone.

Despite the significant increase in GCase protein levels and activity in GD fibroblasts treated with ambroxol, the absolute GCase activity in these cells remained very low compared to control fibroblasts (see figure 13c). It is unclear if this increase in GCase enzyme activity would be clinically relevant. However, the fact that it is associated with reductions in the elevation of cathepsin D and GCase-2 and total-betahexosaminidase activity suggests that it is having some beneficial effect on cellular biochemistry. Importantly, our study of ambroxol treatment of alpha-synuclein over expressing neuroblastoma cells demonstrated a reduction in alpha-synuclein levels. This was accompanied by increases in GCase and LAMP-1 protein. Alpha-synuclein is degraded by both macroautophagy and chaperone mediated autophagy, which are dependent upon the lysosome (Alverez et al, 2010). Thus it seems reasonable to suggest that increased lysosomal mass associated with TFEB induction by ambroxol treatment might result in increased alpha-synuclein turnover. It is also possible that ambroxol treatment could result in decreased alpha-synuclein synthesis. Whilst we cannot exclude this, it seems less likely given that TFEB induction has not been reported as a mechanism of transcriptional repression (Palmieri et al, 2011). It is also not clear from our study whether the increase in GCase activity would result in
reduction of substrate accumulation since fibroblasts accumulate minimal, if any, substrate in GD.

In keeping with ambroxol hydrochloride inducing TFEB expression, there was a general increase in lysosomal mass in treated cells. In control fibroblasts LAMP-1, beta-galactosidase and total cholesterol increase significantly with minor increases in cathepsin D and GCase protein levels and total-beta hexosaminidase activity. The increase in cholesterol may reflect increased synthesis of lysosomal membranes. However, it has been reported that TFEB can induce PRKAG2, a member of the AMPK (MAP kinase) gamma subunit family which stimulates cholesterol biosynthesis, which may explain the large increase in total cholesterol after ambroxol treatment (Palmieri et al, 2011). The pattern in GD and PD-GBA fibroblasts was similar but with significant decreases in cathepsin D protein levels, GCase-2 activity and total beta-hexosaminidase activity. This suggests that ambroxol is triggering a general increase in lysosomal mass by inducing TFEB, but that in GD and PD-GBA cells the accompanying improvement of GCase deficiency is reversing the postulated compensatory mechanisms of cathepsin D, GCase-2 and beta-hexosaminidase over expression. In support of the contention that reduced cathepsin D expression reflects normalisation of lysosomal function is the observation that enzyme replacement therapy reduces cathepsin B, K and S activity in the plasma of GD patients (Moran et al, 2000).

Transcription factor EB (TFEB), a member of the basic helix-loop-helix leucine-zipper family of transcription factors is a “master regulator” of lysosomal function (Palmieri et al, 2011). TFEB induces lysosomal gene expression by binding to E-boxes in their promoters. This genetic network has been collectively termed CLEAR (Coordinated Lysosomal Expression and Regulation) and consists of at least 471 target genes (Palmieri et al, 2011). Cells overexpressing TFEB have increased lysosomal number/mass and increased ability to degrade lysosomal substrates. Most TFEB targets are lysosomal proteins including enzymes (e.g. GCase, cathepsins, hexosaminidase), lysosomal membrane proteins (e.g. LAMP1), proteins essential for lysosomal acidification, lysosomal biogenesis and autophagy (e.g. beclin-1)(Palmieri et al, 2011). TFEB targets also include endosomal, mitochondrial and endoplasmic reticulum proteins. TFEB itself is induced by peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A) binding to its promoter (Tsunemi et al, 2012). An additional layer of regulation
comes from mammalian target of rapamycin (mTOR) complex 1 (mTORC1), which controls phosphorylation and nuclear translocation of TFEB (Settembre et al, 2012).

TFEB induction in a murine model of Huntington disease was associated with clearance of Huntington aggregates (Tsunemi et al, 2012). Inhibition of glycogen synthase kinase 3 resulted in clearance of amyloid precursor protein in cultured neuronal cells via upregulation of TFEB and increased autophagy (Parr et al, 2012). This suggests that TFEB upregulation represents a potential therapeutic target for a broad range of neurodegenerative diseases associated with protein aggregates, such as PD, and also for enzyme enhancement in lysosomal storage disorders. Ambroxol hydrochloride is a small molecule which can cross the blood brain barrier and has been trialled successfully for non-neuropathic manifestations of Type I GD (Zimran et al, 2012). Our cell biology study has shown that amboxol hydrochloride can upregulate GBA and normalise certain aspects of lysosomal biochemistry. This suggests that ambroxol hydrochloride is an excellent candidate drug to trial as a neuroprotective agent both for carriers of heterozygous GBA mutations and GD patients.
Summary and concluding remarks

Here we confirm that fibroblasts from GD patients are deficient in GCase activity and show that there is a relative reduction in GCase enzyme activity in fibroblasts from PD-GBA and NMC in proportion to the reduction in GCase protein levels in these cell lines. This reduction in GCase levels was due to a combination of reduced \textit{GBA} transcript levels - for RecNcil and L444P mutations - and endoplasmic reticulum retention of GCase protein. Reduced GCase protein levels will clearly predispose to further loss of GCase activity triggered, for example, by age related alpha-synuclein accumulation (Gegg \textit{et al}, 2012). This mechanism explains why almost all \textit{GBA} mutations associated with GD have been linked to increased PD risk, since all of them are predicted (or proven) to cause reduced transcript levels or cause protein misfolding and endoplasmic reticulum retention leading to reduced GCase protein levels.

The reduction in GCase activity did not induce defects in macroautophagy in any of the cell lines. This likely reflects the fact that there is some residual GCase activity and no storage of substrate, in contrast to saposin C deficiency in which there is absolute loss of GCase activity and substrate accumulation. However, elevated cathepsin D protein levels and GCase-2 and beta-hexosaminidase activity in GD and PD-GBA suggests subtle lysosomal dysfunction in these cell lines. It is possible that autophagy is dysfunctional in neuronal lines, which accumulate substrate, and this is suggested by the accumulation of p62 in brain tissue from mouse models of GD.

Ambroxol hydrochloride increases GCase protein levels and activity in both GD and PD-GBA cell lines via induction of TFEB and increased \textit{GBA} transcription. The reduction in the endoglycosidase-H sensitive (i.e. ER retained) fraction of GCase suggests that ambroxol has an additional activity as a pharmacological chaperone increasing GCase folding and delivery to the lysosome. Ambroxol hydrochloride should be further investigated as an enzyme enhancing strategy for lysosomal storage disorders and PD-GBA.
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CHAPTER IV.
ANALYSIS OF MITOCHONDRIAL FUNCTION AND MARKERS OF OXIDATIVE STRESS IN FIBROBLASTS FROM GAUCHER DISEASE PATIENTS AND PARKINSON’S DISEASE PATIENTS WITH HETEROZYGOUS GLUCOCEREBROSIDASE MUTATIONS
IVa. Introduction

General aspects of mitochondrial physiology

Mitochondria are membrane enclosed organelles ranging from 0.5-1 μM in diameter (reviewed in Vafai and Mootha, 2012). Mitochondria are the major source of cellular adenosine triphosphate (ATP). Mitochondria are composed of an outer membrane, an inner membrane, the intermembrane space, cristae and the matrix (Otera et al, 2013).

The outer membrane encloses the entire mitochondrion and contains large numbers of integral proteins called porins, which allow molecules of 5 000 Daltons or less to diffuse freely (Vafai and Mootha, 2012). Larger proteins can enter the mitochondrion if they have a targeting sequence which binds to the mitochondrial translocase on the outer membrane (Fox, 2012). The intermembrane space has the same ion concentration as the cytosol but the protein composition is different; for example cytochrome C is localised to the intermembrane space. The mitochondrial inner membrane is impermeable, partly because of its high cardiolipin content (Yurkova et al, 2011), and almost all molecules require special transport proteins to cross this membrane (e.g. proteins utilise the translocase of the inner membrane). The inner membrane has a crucial physiological function as it contains the enzymes of the electron transport chain (Vafai and Mootha, 2012). The inner membrane is folded into numerous cristae which increases its surface area considerably in order to maximise membrane area for ATP synthesis. The matrix is the space enclosed by the inner membrane and contains the mitochondrial genome and enzymes of the citric acid cycle and enzymes for oxidation of pyruvate and fatty acids (Stobbe et al, 2012).

Most ATP is generated by oxidative phosphorylation carried out by the electron transport chain (reviewed in Vafai and Mootha, 2012; Schapira, 2012). At complex I electrons are passed to the electron transport chain from NADH, generated by the citric acid cycle, and at complex II electrons are donated from succinate. Electrons flow down the electron transport chain from complex I-IV before being accepted by oxygen to form water. A small proportion of electrons escape and react directly with oxygen to form the reactive oxygen species superoxide. Passage of electrons down the electron transport chain generates energy which pumps protons into the intermembrane space. Complex I is the major site of electron leak and thus of superoxide production (Gandi and Abramov, 2012). Leakage of electrons can also occur from complex III
under conditions where there is a high mitochondrial membrane potential. Complexes I, II and III act as proton pumps to establish the proton gradient. Protons flow back across the inner mitochondrial membrane through the Fo subunit of Complex V (ATP synthase) releasing energy for the F1 subunit to phosphorylate ADP to ATP (Abramov et al, 2012). Coupling of establishment of a proton gradient across the inner mitochondrial membrane with transfer of electrons down the electron transport chain to synthesis of ATP is vital. However, the energy created by the proton gradient can be “uncoupled” from ATP synthesis by Uncoupling Proteins (UCPs) which offer an alternate route for protons to cross the inner mitochondrial membrane and enter the matrix (Divakaruni and Brand, 2011). Energy dissipated in this way generates heat in brown adipose tissue (Divakaruni and Brand, 2011).

The components of mitochondria are encoded in both the nuclear and mitochondrial genome (mtDNA)(reviewed in Vafai and Mootha, 2012). The mitochondrial DNA encodes 13 peptides which contribute to the following components of the respiratory chain: Complex I (NADH dehydrogenase), Complex III (coenzyme Q-cytochrome c reductase), Complex IV (cytochrome c oxidase) and Complex V (ATP synthase). Mitochondrial rRNA and tRNA are also encoded in the mtDNA. Forty-five genes encode the subunits of Complex I, only 7 of which are mitochondrial with the rest being encoded by nuclear DNA. Complex II (succinate dehydrogenase) is entirely encoded by nuclear genes. Complex III-V are encoded by a mixture of nuclear and mitochondrial genes. A complete list of genes encoding the mitochondrial respiratory chains can be found at the HUGO Gene Nomenclature Committee website (http://www.genenames.org/genefamilies/mitocomplex).

Thus mutations in both nuclear and mitochondrial DNA can give rise to mitochondrial DNA diseases (reviewed in Schapira, 2012). For example, mutations in the nuclear or mitochondrial components of Complex I or II can cause Leigh’s syndrome (infancy/childhood onset dystonia and seizures with basal ganglia cavitation on brain imaging). While mutation of the mitochondrial components of Complex I cause Leber’s hereditary optic neuropathy (LHON). Some mitochondrial disorders are due to deletions of mtDNA resulting from faulty repair and replication – for example polymerase gamma mutations.
Figure 17. Schematic representation of electron transport chain.
The role of mitochondrial dysfunction in Parkinson’s disease

The role of mitochondrial dysfunction in PD is discussed in more detail in Chapter I: General Introduction. Multiple studies have demonstrated the importance of mitochondrial dysfunction in PD. Loss of mitochondrial complex I subunits has been demonstrated by immunohistochemistry in substantia nigra from PD patients (reviewed in Schapira, 2012), and reduced complex I activity demonstrated in PD brain tissue (substantia nigra and frontal cortex) (Schapira et al., 1990), PD platelets (Krige et al., 1992) and fibroblasts (Grunewald et al., 2010). Moreover, primary mitochondrial disorders can present with parkinsonism, especially Polymerase-gamma (POLG) mutations (Davidzon et al., 2006). The cause of mitochondrial dysfunction in sporadic PD is unclear. However, studies of substantia nigra from aged brain and PD brain demonstrated clonally expanded mitochondrial DNA (mtDNA) deletions, with associated loss of respiratory chain subunits and mitochondrial dysfunction (Reeve et al., 2008).

Mitochondrial dysfunction has also been reported in several types of monogenetic PD. Fibroblasts from LRRK2 G2019S mutation associated PD demonstrate reduced ATP synthesis and increased mitochondrial proton leak due to altered expression of uncoupling proteins (Papkovskaia et al., 2012). Both fibroblasts carrying bi-allelic ATP13A2 mutations and neuronal cell lines with ATP13A2 knock down demonstrate altered mitochondrial physiology compatible with defective mitophagy, such as increased maximal oxygen consumption rate after treatment with mitochondrial depolariser and altered mitochondrial morphology (Grunewald et al., 2012). It is postulated that loss of ATP13A2 function causes lysosomal dysfunction and accumulation of dysfunctional mitochondria (Grunewald et al., 2012). Mitochondrial dysfunction has also been reported in models of Parkin and PINK1 associated PD. Grunewald et al. (2010) demonstrated reduced ATP synthesis and increased mitochondrial mass in fibroblasts from PD patients with bi-allelic Parkin mutations. Abramov et al. (2012) demonstrate a number of mitochondrial abnormalities in fibroblasts from PINK1 patients including reduced mitochondrial membrane potential with the mitochondria consuming ATP via the F1F0-ATPase in order to maintain mitochondrial membrane potential. Thus mitochondrial dysfunction plays a role in the pathogenesis of both sporadic and monogenetic PD.
The role of mitochondrial dysfunction in lysosomal storage disorders

Though there is no primary defect of mitochondrial function in lysosomal storage disorders, several studies point to a secondary accumulation of defective mitochondria due to altered mitophagy. In muscle from Pompe disease patients there is evidence of accumulation of enlarged, morphologically abnormal mitochondria (Raben et al, 2012). Similar findings were observed in a mouse model of Multiple Sulfatase deficiency (a recessive lysosomal storage disorder caused by mutations in the \textit{sulfatase modifying factor 1} gene) (de Pablo-Latorre et al, 2012). Mitochondria from livers of these mice displayed reduced membrane potential and ATP content. These mitochondrial changes were associated with release of cytochrome-C and cell death. This was associated with impaired Parkin mediated ubiquitination of mitochondria and defective mitophagy. Other lysosomal storage disorders show similar defects. In fibroblasts from mucolipidosis type II and III there is impaired degradation of autophagosomes and mitochondrial fragmentation with impaired mitochondrial function (Otomo et al, 2009). Takamura et al (2008) demonstrated enhanced autophagy in a knock-out mouse model of GM1-gangliosidosis (recessive mutations of beta-galactosidase) accompanied by evidence of mitochondrial fragmentation and reduced mitochondrial membrane potential. In contrast Sansanwal et al (2010) demonstrated increased mitophagy associated with impaired mitochondrial function in nephropathic cystinosis. Accumulation of defective mitochondria, secondary to impairment of mitophagy, may be a common pathological mechanism in many types of lysosomal storage disorders.
Evidence of mitochondrial dysfunction in Gaucher disease

There are no studies reporting on mitochondrial function in GD models, either cellular or animal. Cleeter et al (2012) demonstrated a reduction of ADP phosphorylation and a reduction in mitochondrial membrane potential in neuroblastoma cell lines treated with the GCase inhibitor conduritol-b-epoxide. However, this results in a very severe inhibition (99%) of GCase activity which is much greater than that seen in humans with bi-allelic GBA mutations. They excluded a non-specific toxic effect of conduritol-b-epoxide by recapitulating the findings of mitochondrial abnormalities in GCase knock downs in neuroblastoma lines. Functional imaging (31-P magnetic resonanse spectroscopy) studies of PD patients with heterozygous GBA mutations did not demonstrate any evidence of mitochondrial dysfunction (Brockmann et al, 2012). However, clearly functional neuroimaging may not detect subtle mitochondrial dysfunction and biochemical studies of fibroblasts from PD-GBA patients are required to address the issue of mitochondrial function as a predisposing factor to the development of PD in GBA mutation carriers.

Reactive oxygen species and antioxidant defences

Reactive oxygen species are chemically reactive molecules containing oxygen. Atomic oxygen has two unpaired electrons in separate orbits in its outer electron shell, and is thus susceptible to free radical formation (Gandi and Abramov, 2012). Sequential reduction of oxygen by addition of electrons leads to the formation of several ROS: superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion and nitric oxide. There are several sources of intracellular reactive oxygen species generation, as discussed in Chapter I General Introduction, but of particular importance is incomplete reduction of oxygen by the mitochondrial electron transport chain. The constant generation of reactive oxygen species by aerobic cells means that cells must possess a robust antioxidant defence system to prevent damage. When this defence system is overwhelmed by increased production of reactive oxygen species oxidative damage to proteins, lipids and DNA occurs.
Antioxidants are species which can donate electrons to molecular centres which are vulnerable to loss of electrons and therefore to generation of free radicals and initiation of oxidative chain reactions (reviewed in Dinkova-Kostova and Talalay, 2008). The antioxidant defence system of cells consists of a range of enzymes (e.g. superoxide dismutase, catalase) which play a role in defences against free radical mediated damage. Superoxide dismutase (SOD) catalyzes the conversion of two superoxide anions into a molecule of hydrogen peroxide (H2O2) and oxygen (O2). In peroxisomes, the enzyme catalase converts H2O2 to water and oxygen, and thus completes the detoxification initiated by superoxide dismutase. In addition there are 2 types of small molecule antioxidant: 1. direct antioxidants (such as glutathione, ascorbic acid, vitamin K) which scavenge oxidation products directly and 2. indirect antioxidants which are substances which can induce the so called phase 2 cytoprotective response to oxidative stress by upregulating a variety of antioxidant genes. The genes induced by the indirect antioxidants include antioxidant enzymes (e.g. Superoxide dismutase, catalase), enzymes which synthesise glutathione, direct antioxidants such as bilirubin and enzymes that detoxify electrophiles such NAD(P)(H):(quinone acceptor)oxidoreductase 1(NQO1). Many of these are regulated by the KEAP1-NRF2 pathway (reviewed in Dinkova-Kostova and Talalay, 2010).
Evidence of oxidative stress in Parkinson’s disease

Several post-mortem studies of PD brain have demonstrated accumulation of products of oxidative damage to membranes (reduced polyunsaturated fatty acid levels, elevated 4-hydroxy nonenal) (Cheng et al, 2011), proteins (protein carbonyls) and DNA (8-hydroxy guanine)(Alzam et al, 1997) in the neurons of the substantia nigra along with loss of the antioxidant reduced glutathione. A high prevalence of mitochondrial DNA deletions in substantia nigra neurons has also been described – which is thought to be due to oxidative damage (Bender et al, 2006).

Elevated levels of oxidative stress markers have also been found in blood (8-OHG) and cerebrospinal fluid (4-HNE, 8-OHG) samples from PD patients (Abe et al, 2003). There are several possible sources for ROS production in PD. Complex I inhibition with MPTP or rotenone is associated with increased superoxide radical formation (Smith and Bennett, 1997). Mutations in a number of PD genes cause mitochondrial dysfunction associated with oxidative stress. The cellular pathophysiology of these genes is discussed in Chapter I: General Introduction. Alpha-synuclein can also interact with mitochondria and induce oxidative stress in some models (Gandi and Abramov, 2012). Increased ROS production by NADPH oxidase occurs in microglia in rotenone and MPTP treatment models (Gao et al, 2003), while PINK1 deficiency results in NADPH activation and ROS production (Gandhi et al, 2009).

Dopaminergic neurons are also exposed to additional oxidative stress produced by the metabolism of dopamine by monoamine oxidase (which generates hydrogen peroxide) and the auto-oxidation of dopamine (which generates superoxide). This may partly explain the selective vulnerability of dopaminergic substantia nigra neurons (Gandi and Abramov, 2012). Thus there is much evidence that oxidative stress is a key mediator of neuronal injury and death in PD.
Evidence of oxidative stress in lysosomal storage disease

There is strong evidence of oxidative stress in the majority of lysosomal storage disorders studied. In brain tissue from a mouse model of Niemann-Pick Type C disease there was elevation of nitrosylated proteins. Vazquez et al (2011) reported elevation of transcript levels for antioxidant enzymes (e.g. hem-oxygenase 1) in cerebella from NPC null mice, consistent with oxidative stress. Fu et al (2010) studied serum from 37 Niemann-Pick Type C patients, demonstrating a significant reduction in coenzyme Q10 (CoQ10) and trolox equivalent antioxidant capacity (TEAC), consistent with oxidative stress. Reddy et al (2006) demonstrated upregulation of multiple genes involved in regulating oxidative stress in fibroblasts derived from Niemann-Pick Type C patients. Accumulation of dysfunctional mitochondria has been suggested as a cause of oxidative stress in this disorder (reviewed in Vazquez et al, 2011). An alternative mechanism is loss of activity of peroxisomal catalase, which may relate to c-able/p73 being translocated to the nucleus as part of an apoptotic programme and not phosphorylating catalase, thus leading to degradation of catalase (reviewed in Vazquez et al, 2011).

Fabry disease is an X-linked lysosomal storage disorder due to mutations of α-galactosidase, leading to accumulation of globotriaosylceramide (Gb3). Biancini et al (2012) demonstrated markers of lipid oxidation (malondialdehyde) in blood from Fabry patients, indicating oxidative damage. Shen et al (2008) show that adding Gb3 to cultured Fabry disease vascular endothelial cells results in increased intracellular generation of free radicals, suggesting that Gb3 accumulation could trigger oxidative stress.

There is strong evidence of oxidative stress in Batten disease (neuronal ceroid lipofuscinosis). Wei et al (2008) studied fibroblasts from NCL Types 1, 3, 6 and 8 demonstrating upregulation of oxidative stress genes catalase and superoxide dismutase-2. In 2007 Benedict et al reported accumulation of carbonylated proteins and upregulation of superoxide dismutase-2 in brain tissue from CLN3 null mice. Histopathological studies of brain tissue from patients with late infantile neuronal ceroid lipofuscinosis demonstrated 4-hydroxynonenal in neurons and upregulation of pro-apoptotic proteins (Hachiya et al, 2006).
Filippon et al (2011) demonstrate increased malondialdehyde levels and carbonylated protein levels in blood from patients with mucopolysaccharidosis type II (MPS II), indicative of oxidative stress. Arifi et al (2011) demonstrated upregulation of transcript levels for several antioxidant genes in the brain of a mouse model of Sanfilippo disease (MPS IIIA). In a mouse model of MPS Type II (Hunter syndrome) there was evidence of upregulated superoxide dismutase and catalase activity, suggestive of oxidative stress (Reolon et al, 2009). Pereira et al (2008) demonstrated reduced catalase and upregulated superoxide dismutase activity in blood from MPS Type I patients, taken as evidence of oxidative stress and providing evidence of the clinical relevance of these findings. The cause of oxidative stress in the MPS is unclear. In one study of an MPS III mouse, upregulation of phagocyte NAD(P)H oxidase mRNA was found, implication inflammatory mechanisms, at least in part, in oxidative stress in this group of lysosomal storage disorders (Reolon et al, 2009).

There is also evidence of oxidative stress in GM1-gangliosidosis. There was upregulation of transcripts for catalase and superoxide dismutase-2 in fibroblasts from a GM1-gangliosidosis patient (Wei et al, 2008). Takamura et al (2008) demonstrated increased sensitivity of neurons from a beta-galactosidase knockout mouse model to oxidative stress, possibly related to abnormal mitochondrial function.

It is clear that oxidative stress represents a pathologic mechanism which is likely to be common to many, if not all, of the lysosomal storage disorders. Given the diverse range of genes involved in this group of diseases it is unlikely that a single mechanism accounts for oxidative stress in all of them. Possible mechanisms of oxidative stress include generation of free radicals by dysfunctional mitochondria, which accumulate secondary to defective mitophagy, as discussed above. Alternatively the toxic substrates which accumulate in lysosomal storage disorders may generate oxidative stress by activating enzymes which generate free radicals. It is also possible that defects in autophagy may impinge upon the cell's antioxidant defences. For example, defective lysosomal degradation of KEAP1 could inhibit the NRF2 antioxidant pathway and
render cells vulnerable to oxidative damage (Dinkova-Kostova and Talalay, 2010). Iron accumulation has been reported in some lysosomal storage disorders, and it is possible that accumulating unchelated iron could participate in Fenton reactions ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^+ + \text{OH}^-$ or $\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OOH}^+ + \text{H}^+$) and generate oxidative stress (Stein et al, 2010).

Evidence of oxidative stress in Gauchers disease

Several studies, both in patient derived blood samples and in cell models point to an important role for oxidative stress in Gaucher disease. Roversi et al (2006) reported elevated catalase activity and reduced superoxide dismutase activity in blood samples from Gaucher disease patients, with an increase in total glutathione levels after infusion of enzyme replacement therapy. They argue this is consistent with enzyme replacement ameliorating oxidative stress levels. Moraitou et al (2008) report reduced plasmalogen levels in red blood cells from Gaucher patients (peroxisomal activities were otherwise normal), which they interpret as being secondary to oxidative stress. In a neuroblastoma cell line treated with the GCase inhibitor conduritol-b-epoxide Cleeter et al (2012) report that dihydroethidium oxidation rates are increased and that aconitase activity is decreased, consistent with oxidative stress. Degunato et al (2007) report elevated rates of dihydroethidium oxidation in fibroblasts from Type II Gaucher disease patients along with elevated protein carbonyl levels. Thus there is much evidence that oxidative stress is an important pathogenic factor in lysosomal storage disorders and Gaucher disease. Because of this, it is a major goal of this study to characterise oxidative stress and its mechanisms in Type I Gaucher disease fibroblasts, and investigate the potential role of oxidative damage as a pathogenic factor in PD-GBA fibroblasts.
IVb. Methods

Assessment of mitochondrial function in patient derived fibroblasts

ADP phosphorylation assay

Fibroblasts harvested from 1 subconfluent 10cm plate was used for assay of ADP phosphorylation rate in each cell line; each cell line was assayed in biological triplicate. Before assaying ADP phosphorylation in patient cells a titration was performed to determine the optimal volume of 1mg/mL digitonin solution to use with human fibroblasts; 2 uL, 4 uL, 8 uL and 16 uL were used in titration reaction. With 4uL of digitonin the rate of ATP synthesis measured reached a peak and plateaued with 8 – 16 uL of digitonin. Therefore 4uL of digitonin was selected for use in the reaction mixture. Cells were trypsinised, pelleted and washed 3 times in ice cold PBS (5 minutes, 2500 rpm, 4 degrees centigrade). Prior to the last spin cells were counted in a haemocytometer. After washing cells were re-suspended in incubation medium (25mM Tris, 150mM Kcl, 2mM K-EDTA, 10mM K2HPO4 at pH 7.4) to give a concentration of 2.5 x 10^5 cells. Substrate mixes were then prepared immediately before use:

Glutamate/malate (I, III, IV): 20 uL 0.5M glutamate
20 uL 0.5M malate
20 uL 50mg/mL bovine serum albumin
40 uL 25mM ADP
4 uL 10mg/ml digitonin in EtOH

Succinate (II, III, IV): 20 uL 0.5M succinate
10 uL 4mg/10mL rotenone
20 uL 50mg/mL BSA
40 uL 25mM ADP
4 uL 10mg/ml digitonin in EtOH

Ascorbate (IV): 10 uL 0.2M ascorbate
5 uL 10 mM N, N, N, N-tetramethyl-p-phenylenediamine
20 uL 50mg/mL BSA
40 uL 25mM ADP
4 uL 10mg/ml digitonin in EtOH
Each substrate solution was made up to 1mL with incubation buffer. Two control reactions were also set up: A. cells + substrates + acid (acid kills the cells therefore this measures the amount of ATP present in the cells before the reaction and controls for any contamination of the ADP with ATP) and B. cells + BSA (calculates background rate of ATP synthesis when no ATP present). Each cell line was measured in duplicate for each substrate and both disease cells and control cells were measured in the same experiment. One hundred uL of substrate or control mix was added to a 1.5mL eppendorf, at timed intervals 100 uL of cell suspension was added to the tubes. The tubes were placed in a rotating incubator for 30 minutes (37 degrees centigrade, 120 rpm). A final cell count for each cell suspension was then made (mean of 3 measurements). At timed intervals the reaction was stopped by adding 11.5 uL of 70 % perchloric acid to each tube. Mixes were then incubated on ice for 30 minutes, centrifuged at 13 000 rpm for 10 minutes and the supernatants aliquoted into fresh tubes. Each reaction mix was then neutralised with 20 uL of neutralisation buffer (3M K2CO3 in 0.5M triethanolamine), pH was checked with pH paper. Samples were incubated on ice for 20 minutes and then centrifuged at 13 000 rpm for 10 minutes. Supernatants were then aliquoted into fresh eppendorfs.

ATP concentration was assayed with the ATP bioluminescence kit from Roche (High sensitivity II kit). Glutamate/malate and succinate/rotenone reactions were diluted 1/50 in PBS, ascorbate samples diluted 1/5 in PBS. Fifty microlitres of sample were diluted with 50uL of dilution buffer (from Roche kit) then 100uL of luciferase added. Luminescence was measured immediately on a luminometer. For each experiment an ATP luminescence standard curve was drawn by measuring luminescence of ATP standards.

The rate of ATP synthesis was calculated as follows. The ATP standard curve was used to convert the sample luminescence to moles of ATP. This figure was multiplied by 50 as the sample was diluted 1/50 or by 5 if the sample was diluted 1/5. This figure was then multiplied by 4 as only 50 uL of a reaction volume of 200 uL was used. This figure was then normalised to a cell count of 2.5 x 10^4. The result was then divided by 30 to give moles ATP/min/2.5 x 10^4 cells. This figure was multiplied by 4 to give ATP/min/10^5 cells. The value for the 2 controls was then subtracted from the result for the respective substrates. The mean ATP synthesis rate
for each cell line, taken from 3 repeat experiments, was compared to control cell lines using students t-test with statistical significance at the 5 % level

**Seahorse XF-24 bioanalyser**

The Seahorse XF-24 analyses mitochondrial and non-mitochondrial energy metabolism in live cells in a multiwell format in real-time. Oxygen consumption rate is measured in pmol of oxygen consumed per minute and is proportional to ATP synthesis rate in untreated cells (i.e. basal conditions). Measurement of oxygen consumption rate after treatment with un-coupler provides a measure of maximal oxygen consumption, which is proportional to total mitochondrial mass. Proton leak can be assessed by measuring oxygen consumption rate after treatment with oligomycin. Non-mitochondrial respiration is studied by measuring oxygen consumption rate after treatment with an inhibitor of the electron transport chain such as Antimycin A. Validation for this methodology is provided by the manufacturer on their website [http://seahorsebio.com/products/instruments/analyzers.php](http://seahorsebio.com/products/instruments/analyzers.php).

Each well of the multiwell plate (24 wells) is an individual reaction chamber, there is an oxygen consumption electrode (containing an oxygen sensitive fluorophore) for each well on the plate on the seahorse bioanalyser. This electrode forms a transient micro-chamber over the monolayer of cells in each well when it is inserted into the well at 2-5 minute intervals to assay metabolites. Oxygen consumption by the cells is measured in real time by the electrode in each well which assays removal of oxygen from the cell culture media kinetically to generate an oxygen consumption rate for the cell line. The Seahorse XF-24 has several ports which permit sequential injection of drugs to assay aspects of mitochondrial physiology.

Fibroblasts used in this assay were grown on 10 cm plates. These cells were not allowed to reach confluence for at least 3 passages prior to the assay as confluence could in theory affect mitochondrial metabolism. Cells were then trypsinised and resuspended in culture media, cells were then counted and diluted in 300uL of media per well so that the desired density of cells per well was obtained. An initial optimisation was performed to determine which density of cells should be used for the experiments. Optimisation of oligomycin, FCCP and Actimycin A dose is described in the results section. Before the cells were analysed in the Seahorse XF-24 the culture
media was aspirated off and phenol red free assay media added to each well. The Seahorse XF-24 analysis was performed by George Chennell (UCL Scientific Support Services). The data from each experimental run was analysed by Alisdair McNeill with advice from George Chennell.

Figure 18. Example of Seahorse “mito stress test profile” from Seahorse XF-24 provided by Seahorse bioscience.

Basal respiration (blue portion of profile) is proportional to ATP synthesis rate. The addition of oligomycin inhibits ATP synthase and OCR measured after this treatment gives the rate of activity of this enzyme and can be used to calculate proton leak. FCCP dissipates the mitochondrial membrane potential (ie uncouples mitochondria) and the OCR with this treatment gives a measure of maximal respiratory capacity of the cells. Antimycin D and rotenone are added to inhibit the respiratory chain, which can give a measure of spare respiratory capacity and non-mitochondrial respiration.
**Citrate Synthase activity assay**

Fibroblasts were grown to subconfluence on 10cm plates, trypsinised, washed once in PBS and made into a pellet. Cells were then lysed with 50 uL of 0.25% Triton X 100, kept on ice for 15 minutes and then centrifuged at 15 000 xg for 10 minutes. The supernatant was then aliquoted. For the assay 10 uL of cell supernatant was mixed with 20 uL of oxaloacetic acid and 970 uL of reaction mix (2.25 mL Tris buffer, 90uL of acetyl coenzyme A, 90 uL of DTNB, 45 uL of 10% triton X 100, 1.89 mL dH2O). This assay has been validated by Dr David Chau, UCL Department of Clinical Neurosciences.

The assay was performed on a 96 well plate using a Bio Tek Synergy HT plate reader. The plate reader was preheated to 30 degrees centigrade. Two hundred and fifty microlitres of the reaction mixture was added to each well, with each cell being assayed in triplicate. Blanks consisting of reaction mix without cell lysates were also assayed in triplicate. The protein concentration for each cell line was measured using the BCA kit described in the methods section of Chapter III. Citrate synthase activity was calculated as nmol fluorescent product/mg protein/minute.

**Single cell analysis of dihydroethidium and monochlorobimane**

Cytosolic ROS production was monitored by single cell analysis of dihydroethidium (DHE) oxidation rate. For each cell line fibroblasts were imaged with 2 uM of DHE in HBSS (Hepes buffered salt solution, 156 NaCl, 3 KCl, 2 MgSO4, 1.25 KH2PO4, 2 CaCl2, 10 glucose, and 10 HEPES, pH adjusted to 7.35 with NaOH). No preincubation (“loading”) was performed to minimise intracellular accumulation of the oxidised product. Fluorescence measurements were obtained using a Nikon (Tokyo, Japan) epifluorescence inverted microscope equipped with a cooled CCD camera. DHE was excited at 490nm and images were collected at intervals of 10 s, digitized, and stored for off-line analysis using software from Andor (Belfast, UK). Each cell line was imaged in biological triplicate. The rate of DHE oxidation and the rate of change of the ratio of oxidised to non-oxidised DHE was calculated. Fibroblast lines were assayed in triplicate with 3 control lines compared to 4 GD, 4 PD-GBA and 2 NMC fibroblast lines.
Cellular glutathione levels were assayed with monochlorobimane (MCB) dye. MCB fluoresces after being conjugated to glutathione by glutathione s-transferase, and so its fluorescence is proportional to cellular glutathione content.”. Fibroblasts were grown on coverslips and incubated with MCB dissolved in HBSS for 30 minutes before imaging. MCB was imaged with excitation of 394nm and emission of 490 nm. Fibroblast lines were assayed in triplicate with 3 control, 3 GD and 2 PD-GBA lines compared.

**Assessment of mitochondrial form factor**
Mitochondria were immunostained using an antibody to TOM20 (Santa-Cruz Biotechnology, FL145, 1: 250, incubated for 1 hour) on fixed cells, using the technique outlined in Chapter III. Cells were photographed using a fluorescence microscope equipped with an ApoTome and AxioVision software (all Zeiss, Jena, Germany). Mitochondrial morphology was assessed using the Form Factor (FF). By means of ImageJ (NIH, Bethesda), raw images were binarized, mitochondrial area and outline were measured and the form factor was calculated (defined as \( \frac{P_m^2}{4\pi A_m} \)), where \( P_m \) is the length of the mitochondrial outline and \( A_m \) is the area of the mitochondrion.

**OxyBlot Kit (western blot quantification of protein carbonyl levels)**
Oxidative damage to proteins introduces carbonyl groups (aldehydes and ketones) at lysine, arginine, proline or threonine residues. The OxyBlot kit (Chemicon International) utilises an immunoblotting technique to detect carbonylated proteins at concentrations as low as 5 femtomoles. The kit is based on the principle that carbonylated residues in proteins react with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone (DNP-hydrazone), which can be detected with an antibody in an immunoblot. Cell lysates are made with 50mM DTT added to the lysis solution to prevent protein oxidation being induced by cell lysis. For each cell line five microlitres of lysate was then denatured by addition of 5 microlitres of 12% SDS (giving a final concentration of 6% SDS), with a duplicate made for each sample to act as a negative control. Ten microlitres of 2,4-dinitrophenylhydrazine (DNPH) was then added to each
sample and 10 microlitres of control solution (provided in the kit) to the respective negative controls. DNPH reacts with carbonylated residues to form 2,4-dinitrophenylhydrazone (DNP-hydrazone). After 15 minutes incubation 7.5 microlitres of neutralization buffer (provided in the kit) was added to each sample. Lysates were then loaded onto a 4-12% NuPage gel and electrophoresed at 200v before transfer to a PVDF membrane. Membranes were blocked in 1% bovine serum albumin in PBS-T, then incubated with the kit’s primary antibody to DNP. Membranes were then incubated with the kit’s secondary antibody and developed with ECL chemiluminescence solution. An antibody to beta-actin was used as a loading control and band intensity quantified with Image-J. All cell lines were assayed in triplicate.
IVc. Results

Patients and fibroblast lines studied

Fibroblasts were generated from skin biopsies from 5 Type I GD patients, 4 heterozygous carriers with PD (PD-GBA), 1 PD patient with homozygous E326K GBA mutations and 2 carriers without PD (non-manifesting carriers, NMC). For comparison fibroblasts were generated from 3 PD patients without GBA mutations and 3 controls without neurological disease or GBA mutations. The clinical characteristics and mutation spectrum of the patient cohort are given in Table x. Details of the general cell culture conditions are given in Chapter III.

<table>
<thead>
<tr>
<th>Table. Characteristics of patient derived fibroblasts</th>
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<td>GD02, M/70</td>
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<td>Control</td>
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<td>CN02, M/81</td>
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<td>CN03, F/50</td>
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Optimisation of ADP phosphorylation assay

Before assaying ADP phosphorylation in patient cells a titration was performed to determine the optimal volume of 1mg/mL digitonin solution to use with human fibroblasts; 2 uL, 4 uL, 8 uL and 16 uL were used in a titration reaction. With 4uL of digitonin the rate of ATP synthesis measured reached a peak and plateaued with 8 – 16 uL of digitonin. Therefore 4uL of digitonin solution (1mg/mL) was selected for use in the reaction mixture.
**Optimisation of Seahorse XF-24**

The number of fibroblasts to use per well was optimised on a single plate with 10 000, 20 000, 30 000 and 50 000 cells per well in each row. The cell number giving the greatest oxygen consumption rate was selected. Fifty thousand cells per well gave the greatest oxygen consumption rate and this was selected as the concentration of cells to use per well for the experiments. A titration of FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone) was performed using 250 nM, 500nM, 750 nM and 1 000nM; 750nM gave the greatest increase in oxygen consumption rate with a plateau reached at higher concentrations. This was therefore selected as the concentration of FCCP to use to assay maximal oxygen consumption rate. The dosage of oligomycin (2 uM) and antimycin A (3 uM) were selected according to standard Seahorse protocols for working with human dermal fibroblasts.

**No alteration of ADP-phosphorylation rate or oxygen consumption rate in Gaucher disease or PD-GBA**

There was no significant difference in ADP phosphorylation rates for complex I-IV between Gaucher disease (550+/−190 pmolesATP/min/10⁵ cells, p=0.5) and control cell lines (600+/−150 pmolesATP/min/10⁵ cells). There was no difference for complex II-IV between Gaucher disease (360+/−170 pmolesATP/min/10⁵ cells, p=0.5) and control cell lines (420+/−160 pmolesATP/min/10⁵ cells), or complex IV GD (65+/−20 pmolesATP/min/10⁵ cells, p=0.5) and control cell lines (60+/−30 pmolesATP/min/10⁵ cells). There were no differences between oxygen consumption rates (OCR) as assessed by the Seahorse XF-24 between Gaucher disease (143+/−25 pmol O2/ug/min, p=0.7), PD-GBA (135+/−40 pmol O2/ug/min, p=0.5) and control lines (137+/−30 pmol o2/ug/min).

After treatment with the uncoupling agent oligomycin there was no difference in oxygen consumption rates between control (13+/−5 pmol O2/min/mg) and Gaucher disease (20.9+/−8 pmol O2/min/mg, p=0.185) or control (19+/−2 pmol O2/min/mg) and PD-GBA (28+/−7 pmol O2/min/mg, p=0.1). Thus there is no evidence for increased mitochondrial proton leak (mitochondrial uncoupling) in Gaucher disease or PD-GBA. Proton leak was calculated as oxygen consumption rate after oligomycin treatment – oxygen consumption rate after antimycin A treatment.
There was no difference in non-mitochondrial respiration rate between control (mean 40 +/- 5 pmol O2/min/mg) and Gaucher disease (mean 32 +/- pmol O2/min/mg, p=0.17) or control (mean 34 +/- 10 pmol O2/min/mg) and PD-GBA (mean 36 +/- 9 pmol O2/min/mg, p=0.79). Non-mitochondrial respiration was measured from the oxygen consumption rate after treatment with Antimycin A.

**No alteration in mitochondrial mass in Gaucher disease or PD-GBA**

After treatment with FCCP the maximal OCRs did not differ between Gaucher disease (341 +/- 89 pmol O2/ug/min, p=0.5), PD-GBA (395 +/- 143 pmol O2/ug/min, p=0.92) and control lines (365 +/- 54 pmol O2/ug/min). Enzymatic activity of citrate synthase did not differ between control (mean 191 +/- 21 nmol/min/mg), Gaucher disease (mean activity rate 183 +/- 30 nmol/min/mg, p=0.63) or PD-GBA (mean 196 +/- 56 nmol/min/mg, p=0.80). This suggests there is no difference in mitochondrial mass and implies that mitophagy is not deficient.

**No alteration in mitochondrial morphology in Gaucher disease or PD-GBA**

The value for form factor obtained by analysing 6 cells each from 2 control lines stained with TOM20 antibody was set at 100% as the reference value, and the form factors of the Gaucher disease and PD-GBA fibroblasts were expressed as a percentage of this. There was no difference in mitochondrial morphology (form factor) between GD (p=0.4), PD-GBA (p=0.5) and control cells. This provides further evidence that mitophagy is not abnormal since defective mitophagy would be associated with accumulation of dysmorphic mitochondria.
Figure 19. Analysis of mitochondrial function.
19a. Basal oxygen consumption rate (pmol o2/min/mg) does not differ between GD, PD-GBA or controls.
19b. Maximal oxygen consumption rate (pmol o2/min/mg) after FCCP treatment (0.75 uM) does not differ between GD, PD-GBA and controls.
19c. Form factor does not differ between GD, PD-GBA and controls (results are normalised to controls which have form factor set to 100).
19d. Immunofluorescent staining of mitochondrial network with Tom20 for calculation of form factor. Top panel: representative control cells, bottom panel: representative GD cells.
The rate of DHE oxidation is elevated in GD and PD-GBA fibroblasts
The rate of DHE oxidation was significantly elevated in Gaucher Disease (median 40 % increase, IQR 15-70%, p<0.001), PD-GBA (median 55% increase, IQR 45-70%, p<0.001), NMC (median 70% increase, IQR 65-75%, p=0.002) and the E326K homozygous (median 50% increase, IQR 10-60%, p=0.012) fibroblast lines.

Elevated levels of carbonylated proteins occur in Gaucher disease and PD-GBA
In addition there were elevated levels of carbonylated proteins in Gaucher disease (median 50% increase, IQR 25-75%, p<0.001) and PD-GBA cell lines (median 0% increase, IQR 0-75%, p=0.03). It should be noted that the increase in carbonylated proteins in PD-GBA was entirely due to line PD03 (L444P/Wt).

Reduction of glutathione levels in Gaucher disease and PD-GBA fibroblasts
The intensity of staining of Gaucher disease (median 77 % of control values [IQR 50-90%], p=0.001) and PD-GBA fibroblasts (median 72% [IQR 50-92%] of control, p=0.001) with MCB was significantly less than that of control fibroblasts. This suggests a reduction in cellular glutathione levels, possibly due to depletion due to oxidative stress.
Figure 20. Oxidative stress assays in Gaucher and Parkinson's disease fibroblasts.

20a. Graph summarising significant increases in rates of DHE oxidation for GD, PD-GBA and NMC compared to control cells.
20b. Representative oxyblots demonstrating increased carbonylated proteins in GD and increased carbonylated proteins in PD03 line only.
IVd. Discussion

**Mitochondrial function is not altered in Gaucher disease and PD-GBA fibroblasts**

None of the mitochondrial biochemical parameters examined were altered in Gaucher disease or PD-GBA fibroblasts compared to control cell lines. ADP-phosphorylation rate and basal oxygen consumption rate, which is proportional to ATP synthesis rate, did not differ in Gaucher disease or PD-GBA fibroblasts compared to control cells. This suggests that there is no defect of mitochondrial ATP synthesis such as occurs with mutations of the respiratory chain complex enzymes. Thus there is no evidence from the study of Gaucher disease and PD-GBA fibroblasts that a deficiency of Complex I activity, which commonly occurs in PD (Schapira, 2012), plays a role in the mechanisms by which GBA mutations predispose to PD. Moreover, there was no alteration in oxygen consumption rates in Gaucher disease fibroblasts or PD-GBA fibroblasts compared to control fibroblasts after treatment with Antimycin A. This demonstrates that there is no difference in non-mitochondrial respiration between Gaucher disease and PD-GBA fibroblasts and control fibroblasts. If there was mitochondrial impairment then a compensatory increase in non-mitochondrial respiration would be expected to occur. Oxidative damage usually causes some degree of mitochondrial dysfunction (Gandhi and Abramov, 2012), and it is somewhat unexpected that the cell lines used in this study had oxidative stress with normal mitochondrial function. It is possible that subtle mitochondrial dysfunction is present, but which would only be detected by alterations in mitochondrial membrane potential. But, due to time constraints it was not possible to perform single cell analysis of mitochondrial membrane potential with dyes such as Tetramethylrhodamine (TMRM) so we cannot exclude this possibility. Alternatively it might be that the oxidative stress in the fibroblasts is of a relatively low severity and that antioxidant defences are buffering the free radical production, manifested by depletion of glutathione in the cells, and protecting the mitochondria.
No evidence for mitochondrial uncoupling in Gaucher disease or PD-GBA fibroblasts

The oxygen consumption rate after treatment with oligomycin was not different in Gaucher disease or PD-GBA fibroblasts compared with control fibroblast lines. This suggests that there is no increased proton leak across the mitochondrial membrane. Basal proton leak is unregulated (i.e. occurs directly through the phospholipid inner mitochondrial membrane) and relates to metabolic rate, while induced proton leak is controlled by the adenine nucleotide translocase (ANT) and the uncoupling proteins (UCP) (Divakaruni and Brand, 2011). The physiological role of mitochondrial uncoupling, associated with activation of uncoupling protein-1, is to generate heat in brown fat (Divakaruni and Brand, 2011). Increased proton leak has been demonstrated in fibroblasts from PD patients carrying the LRRK2 G2019S mutations (Papkovskaia et al, 2012), associated with increased basal oxygen consumption rate and reduced ADP-phosphorylation and mitochondrial membrane potential. This mechanism clearly does not operate in Gaucher disease or PD-GBA since basal oxygen consumption was not altered and there was no difference compared to controls in oxygen consumption rate after oligomycin treatment. This suggests that the mechanisms by which GBA and LRRK2 G2019S mutations cause PD are different.

No evidence for defective mitophagy in Gaucher disease or PD-GBA fibroblasts

Cellular mitochondrial quality control is an important process to prevent accumulation of dysfunctional mitochondria and cellular toxicity. Two mitochondrial quality control mechanisms exist: 1. fusion of mitochondrial and 2. mitophagy (degradation of dysfunctional mitochondria by the lysosome) (Sun et al, 2012). As discussed above altered mitophagy has been reported in several lysosomal storage disorders, and is associated with decreased Parkin mediated ubiquitination of mitochondria in multiple-sulfatase deficiency. In mitophagy Parkin protein is thought to be recruited to damaged mitochondria, which have reduced membrane potential, by PINK1 protein, though the mechanism by which PINK1 translocates Parkin from the cytoplasm to mitochondria is unknown (Sun et al, 2012). Parkin docks with damaged mitochondria by interacting with voltage dependent anion channels (VDACs 1, 2 and 3) on the mitochondrial membrane (Sun et al, 2012). Parkin ubiquitinates mitochondrial membrane proteins such as mitofusin-2 and miro to target the mitochondria for degradation in the lysosome. Ubiquitinated
mitochondria are then recognised by p62 protein and targeted to the lysosome (Huang et al, 2011).

The fact that maximum oxygen consumption rate after treatment with FCCP did not differ between Gaucher disease, PD-GBA and control fibroblasts suggests that mitophagy is not altered. Treatment with FCCP is known to dissipate the mitochondrial membrane potential and induce uncoupling so that the oxygen consumption rate becomes proportional to the mitochondrial mass. This also induces mitophagy. If mitophagy was defective in Gaucher disease or PD-GBA fibroblasts then the maximum oxygen consumption rate would be expected to be significantly higher than in control fibroblasts. The fact that it was not suggests that mitophagy is intact.

Further evidence for intact mitophagy comes from the fact that citrate synthase activity was not altered in Gaucher disease fibroblasts or PD-GBA fibroblasts compared to control cells. Citrate synthase activity is proportional to mitochondrial mass (Grunewald et al, 2010). If there was a significant impairment of mitophagy it would be expected that the Gaucher disease cells would have an increased mitochondrial mass. The fact that this was not the case is evidence that mitophagy is not defective.

Analysis of mitochondrial morphology using immunofluorescent analysis of fixed fibroblasts stained with anti-TOM20 antibody did not demonstrate any differences between Gaucher disease, PD-GBA and controls. In cell lines with damaged mitochondria and impairment of mitophagy there is accumulation of mitochondria with abnormal morphology. For example, Grunewald et al (2010) describe reduced branching of mitochondria and increased fragmentation, which manifests as decreased form factor, in fibroblasts from PD patients with bi-allelic Parkin mutations. The morphology of mitochondria in Gaucher disease, PD-GBA and control fibroblasts was not distinguishable and the form factor did not differ significantly. This suggests that mitochondrial morphology is normal in Gaucher disease and PD-GBA, providing more evidence that the mitophagy pathway is likely to be intact.
**Why is mitophagy not defective in Gaucher disease?**

As discussed in the introduction there is evidence that the majority of lysosomal storage disorders are associated with impaired mitophagy. Here we demonstrate that mitophagy appears to be intact in fibroblasts from Gaucher disease patients and PD patients with heterozygous *GBA* mutations.

Why is there this difference between Gaucher disease and other lysosomal storage disorders? One explanation might be the degree of severity of autophagic dysfunction. For example, in multiple-sulfatase deficiency there is dysfunctional mitophagy along with clear evidence of defective macroautophagy (electron microscopy demonstrates accumulation of autophagocytic vesicles with impaired fusion with lysosomes)(de Pablo-Lattore *et al*, 2012). The same is true in Kufor-Rakeb syndrome (*ATP13A2* mutations), in which fibroblasts have increased mitochondrial mass, associated with reductions in mitochondrial ATP production, consistent with defective mitophagy and also clear evidence of defective lysosomal physiology in general (e.g. defective lysosomal acidification and degradation of mitochondria)(Dehay *et al*, 2012).

Thus the fact that mitophagy is not altered in Gaucher disease or PD-GBA may reflect the intact macroautophagy system in these cell lines. In Chapter III we report that there is no elevation of p62 or alteration of LC3-II levels in Gaucher disease or PD-GBA fibroblasts and describe intact induction of autophagy by starvation. Thus it seems that for mitophagy to be deficient there needs to be a relatively severe impairment of the lysosome associated with defective macroautophagy.
**Oxidative stress plays a role in the pathogenesis of Gaucher disease**

The rate of dihydroethidium oxidation was significantly elevated in Gaucher disease fibroblasts. This was accompanied by evidence of elevated accumulation of protein carbonyls as measured by the Oxyblot technique. Similar results were reported by Degunato *et al.* (2007) in fibroblasts from patients with Type II Gaucher Disease. The results of our study of oxidative stress markers in Type I Gaucher disease fibroblasts are also in keeping with work by several groups reporting evidence of elevated oxidative stress markers in blood from Gaucher disease patients (Moraitou *et al.*, 2008). The current study thus demonstrates that oxidative stress operates in both adult non-neuronopathic (Type I) Gaucher disease as well as paediatric neuronopathic (Type II) Gaucher disease.

As noted in the introduction to this chapter there is evidence of oxidative stress in many of the lysosomal storage disorders. In contrast, the genes involved, and substrates which accumulate, differ widely across the spectrum of the 50 or so lysosomal storage disorders. Moreover, disruption of macroautophagy and mitophagy are described in many, but not all, lysosomal storage disorders. A case in point being the normal autophagy and mitophagy pathways in the Gaucher disease and PD-GBA fibroblasts in the current study. It seems reasonable to postulate that oxidative stress may represent a common pathogenetic mechanism in all lysosomal storage disorders. Because of its potential role across the spectrum of lysosomal storage disorders it is clearly an important therapeutic target.

Oxidative stress could potentially contribute to several of the clinical manifestations of Gaucher disease. Oxidative damage to bone marrow stem cells could contribute to the aetiology of myeloma in Type I Gaucher disease by causing DNA damage (Walters *et al.*, 2011). Oxidative damage could obviously also contribute to mutations underlying other malignancies associated with Type I Gaucher disease such as melanoma, and melanocytes have been shown to be sensitive to oxidative stress induced DNA damage (Ibarrola-Villava *et al.*, 2011). Indeed, oxidative stress is associated with secretion of soluble factors by myofibroblasts which promote melanoma invasiveness (Comito *et al.*, 2012). There is also some data associating bone disease
with oxidative stress. Spinal bone mineral density was negatively correlated with rising markers of lipid peroxidation in serum in post-menopausal women (Cervellati et al, 2012) and oxidative stress has also been shown to induce apoptosis of osteoblasts (Li et al, 2012). As discussed in the introduction there is much evidence that oxidative stress plays a major role in PD pathogenesis, and the potential role for oxidative stress in PD-GBA is discussed later in this section.

**Oxidative stress plays a role in the pathogenesis of PD-GBA**

The rate of dihydroethidium oxidation was significantly elevated in PD-GBA fibroblasts compared to control fibroblasts. This was accompanied by evidence of elevated protein carbonyls in the fibroblast line with the L444P/Wt genotype. Evidence of oxidative stress has been reported in studies of fibroblasts from several different genetic subtypes of PD, including Parkin (Grunewald et al, 2010) and Pink1 mutation associated PD (Gegg et al, 2009). It seems probable that oxidative stress will be present, to a greater or lesser degree, in every cell of the body in patients with heterozygous GBA mutations. It is well documented that neurons are especially vulnerable to damage by free radicals, due to their high oxygen consumption (10 fold greater than other cell types) and the prominent role of nitric oxide as a neurotransmiter (nitric oxide can form damaging reactive peroxynitrite species)(Gandhi and Abramov, 2012). Thus it seems reasonable to propose that neurons in individuals with heterozygous GBA mutations will manifest oxidative damage though this remains to be examined experimentally.

Based on the evidence from this study, it is logical to suggest that oxidative stress is an early event in the pathogenesis of PD-GBA. This is because oxidative stress is present in fibroblasts from non-manifesting carriers (elevated dihydroethidium oxidation rate) and thus is occurring before the onset of clinical PD. There are several mechanisms by which oxidative stress could predispose to PD. Oxidative damage to mitochondrial membranes could result in age related mitochondrial dysfunction in brain; examination of mitochondrial function in fibroblasts cannot exclude this. Ongoing oxidative damage could damage lysosomal membranes and potentially interfere with lysosomal function, while oxidative damage to proteins could also increase the
propensity of GCase protein to misfold and be retained in the endoplasmic reticulum. Perhaps most significantly oxidative damage to alpha-synuclein can predispose to aggregation and Lewy body formation (Giasson et al., 2000). Thus, oxidative stress is a common pathological mechanisms across multiple different lysosomal storage disorders and is also a strong candidate as an important mechanism by which GBA mutations predispose to PD.

**Oxidative stress occurs in E326K homozygous fibroblasts from a patient with PD**
As discussed in Chapter III it is unclear if the E326K GBA variant is a disease causing mutation, a disease modifying allele or a benign polymorphisms (Horowitz et al., 2011). Here we describe evidence of oxidative stress in an E326K/E326K fibroblast line from a PD patient who did not have overt evidence of Gaucher disease. No Gaucher disease patients with E326K/E326K genotype have been reported, but in Gaucher disease patients who have E326K on the same allele as a confirmed GBA mutation tend to have a more severe phenotype (Horowitz et al., 2011). The E326K GBA variant is more frequent in sporadic PD cases than in controls (Lesage et al., 2011). It seems more likely that the PD patient with homozygous E326K mutations has PD related to GBA mutations rather than PD as part of Gaucher disease due to E326K/E326K genotype. In the E326K/E326K homozygous fibroblast line it is clear that oxidative stress occurs and likely contributes to the pathogenesis of PD.

**Mechanisms of oxidative stress in Gaucher disease and PD-GBA**
The mechanisms causing oxidative stress in GD and PD-GBA are unclear. In other lysosomal storage disorders, for example Niemann-Pick Type C, accumulation of damaged mitochondria, which have increased production of reactive oxygen species, has been proposed to cause oxidative stress (reviewed in Vazquez et al., 2012). Dysfunctional mitochondria seem less likely to play a role in causing oxidative stress in Gaucher disease or PD-GBA given that mitochondrial biochemistry is normal and that there is no evidence of defective mitophagy. Degunato et al (2007) provide data suggesting that NADPH oxidase is activated by substrate accumulation in Type II GD fibroblasts, but this seems less likely to explain why heterozygous cells have oxidative stress since they are not be expected to accumulate metabolites. Moreover, studies suggest that little, if any, glucosyleceramide accumulates in Gaucher disease fibroblasts (Saito and Rosenberg, 1985).
There are several alternative mechanisms to explain why oxidative stress occurs in Gaucher disease and PD-GBA. Upregulation of beta-hexosaminidase in Gaucher Disease and PD-GBA may predispose to oxidative stress, since this enzyme produces GM3 ganglioside, which can prime cells to produce large amounts of reactive oxygen species in response to glutamate toxicity (Sohn et al, 2006). Dysregulation of the KEAP1-NRF2 pathway could also predispose cells to oxidative stress. NRF2 translocates to the nucleus to upregulate antioxidant genes after oxidative stress causes KEAP1 to dissociated from NRF2, KEAP1 is then directed to the lysosome or proteasome for degradation (Dinkova-Kostova and Talalay, 2010). There is evidence that p62 plays a role in KEAP1 degradation and that lysosomal dysfunction may interfere with KEAP1 degradation. If KEAP1 degradation by the lysosome were dysregulated then it is possible that upregulation of antioxidant pathways could be impaired resulting in vulnerability to oxidative stress. Altered calcium metabolism has been reported in Gaucher disease. Glucosylceramide accumulation enhances calcium release from intracellular stores by increasing the sensitivity of the ryanodine receptor to agonists (Pelled et al, 2005). This could in theory result in elevated cytosolic calcium levels which activate enzymes such as NADPH oxidase to cause oxidative stress.
Summary and concluding remarks
Here we studied fibroblasts from Gaucher disease and PD-GBA patients and demonstrated that mitochondrial biochemistry is not altered. There is no evidence of defective ATP synthesis, nor is there evidence of mitochondrial uncoupling or elevated non-mitochondrial respiration. After treatment with FCCP cellular oxygen consumption rates did not differ between Gaucher disease, PD-GBA and control cell lines. This suggests that mitophagy is not defective in these cell lines. In support of this is the fact that mitochondrial branching was not reduced in Gaucher disease or PD-GBA fibroblasts, since if mitophagy were defective, and abnormal mitochondria were accumulating, one would expect mitochondrial morphology to be altered.

There is evidence of oxidative stress in Gaucher disease and PD-GBA fibroblasts. Oxidative stress may be a common pathogenic mechanism occurring in most or all types of lysosomal storage disorders. Oxidative stress was also present in fibroblasts from non-manifesting carriers of GBA mutations. This suggests that oxidative stress is an early event in PD-GBA pathogenesis and not a secondary consequence of metabolic derangements occurring later in the disease course. Thus oxidative stress may be a major mechanism by which GBA mutations predispose to the development of PD.
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CHAPTER V.
CONCLUDING REMARKS: A LOSS OF FUNCTION MODEL TO EXPLAIN HOW \textit{GLUCOCEREBROSIDASE} MUTATIONS MAY PREDISPOSE TO PARKINSON'S DISEASE
There is much evidence that patients with GD and their heterozygous GBA mutation carrier relatives have a significantly increased risk of developing PD (Bultron et al., 2010; Sidransky et al., 2009; McNeill et al., 2012b). One study estimated the lifetime risk of PD in GD patients as being 21 fold greater than that of controls (Bultron et al., 2010) and heterozygous GBA mutation carriers (parents of Gaucher disease patients) have a 10-15 % risk of developing PD by age 80 years (McNeill et al., 2012). Clearly GD patients and carriers are a group at substantially increased risk of developing PD. We hypothesised that by studying them clinically for markers of prodromal PD we could efficiently identify a cohort of subjects in the prodromal or early stages of PD. To do this we recruited GD patients and their obligate carrier relatives from the NSCAG funded Lysosomal storage disorder clinics at the Royal Free Hospital (London) and the Addenbrookes Hospital (Cambridge). We studied them for prodromal markers of PD such as hyposmia by using validated clinical ratings scales and generated fibroblast cell lines from a selection of study participants.

We can identify GD patients and heterozygous GBA mutation carriers with prodromal signs of PD

As detailed in Chapter II a cohort of 75 Type I GD patients and 41 of their heterozygous GBA mutation carrier relatives underwent clinical phenotyping. As a group, the median Smell Identification Test and Montreal Cognitive Assessment scores were significantly lower in GD patients and heterozygous GBA mutation carriers than in matched controls. In addition parkinsonian motor signs were more common in GD patients and carriers. Clearly there are multiple possible causes for these prodromal markers. Hyposmia could be related to disease of the nose or sinuses or to smoking (Doty, 2012). We minimised this confounder by excluding participants from the smell test if they had an upper respiratory tract infection/pathology or who were current smokers. We also could not exclude other aetiologies for cognitive impairment such as vascular dementia or Alzheimer's disease. The limitations of the motor examination in detecting motor signs of parkinsonism are discussed in chapter 2. To gather more evidence that the prodromal markers we identified in these individuals reflect underlying neurodegeneration we performed optic coherence tomography on a subset of GD patients – demonstrating an association of retinal thinning with prodromal markers in these patients (as described and discussed in chapter 2). This part of our study provides evidence in support of the argument that some of the GD patients and heterozygous mutation carriers expressing prodromal markers such as hyposmia will be in the early stages of PD.
Fibroblasts from participants with prodromal markers are a model to study how *GBA* mutations predispose to PD

Our clinical study provided evidence that we could identify individuals in the early or prodromal stages of PD using clinical markers such as hyposmia. We thus hypothesised that fibroblasts generated from this cohort could be studied to help us understand both the cellular biology of GD (i.e. glucocerebrosidase deficiency) and the mechanisms by which heterozygous carriage of *GBA* mutations predisposes to PD. Fibroblast lines were generated from partial thickness skin biopsies taken from 5 Gaucher disease patients, 2 carriers without PD and 4 carriers with PD.

Fibroblasts from GD patients had a severe deficiency of GCase protein levels and activity, while PD-GBA and NMC fibroblasts had a less marked reduction in GCase protein levels and a decline in enzyme activity proportional to the loss of GCase protein. Loss of GCase protein was due to endoplasmic reticulum retention and proteasomal degradation of GCase protein. This was accompanied by elevation of endoplasmic reticulum stress markers calnexin and BiP/Grp78. Thus these fibroblasts can be used to model the effect of loss of GCase activity and endoplasmic reticulum stress induced by mutant, misfolded GCase.

*GBA* mutations are associated with oxidative stress but not dysfunctional autophagy in fibroblasts

None of the disease fibroblast cell lines assessed had evidence of abnormal autophagy as neither p62 nor LC3-II protein levels were altered (as described in detail in chapter 3). In keeping with this there was no evidence of defective mitophagy, as mitochondrial mass was not greater in the Gaucher disease fibroblasts and maximum oxygen consumption rate after FCCP treatment was not greater in GD or PD-GBA fibroblasts than control lines. Defective mitophagy has been reported in several lysosomal storage disorders (see references from chapter 4). The absence of this mechanism in GD fibroblasts may reflect the relatively mild storage phenotype of GD fibroblasts which accumulate little, if any, substrate and have normal macroautophagy. We did however demonstrate elevation of cathepsin D protein levels, GCase-2 activity and total-beta-hexosaminidase enzyme activity in GD and PD-GBA fibroblasts. This may reflect compensatory lysosomal mechanisms in response to reduction of GCase activity.
There was evidence of oxidative stress in GD, PD-GBA and NMC fibroblasts with elevation of dihydroethidium oxidation rates and accumulation of carbonylated proteins. The mechanism of oxidative stress is unclear; but it seems unlikely to be related to mitochondrial generation of free radicals given the normal mitochondrial functional assays. Oxidative stress is likely to be an early event in the pathogenesis of PD-GBA given that there was evidence of increased free radical production in fibroblasts from NMC. Oxidative stress is well recognised as an important pathogenic process in both lysosomal storage disorders and PD (Schapira & Tolosa, 2010), and could contribute to predisposition to PD in many ways, not least by increasing alpha-synuclein aggregation.

**A loss of function model explaining why GBA mutations predispose to PD**

Recent work from our group (Gegg *et al*, 2012), and others (Mazulli *et al*, 2011), has demonstrated that loss of GCase protein and enzyme activity occurs in the substantia nigra of PD patients with and without *GBA* mutations. This suggests that loss of neuronal GCase activity is a key, nodal point in the pathogenesis of both PD-GBA and sporadic PD.

The current study helps elucidate the mechanisms by which *GBA* mutations lead to loss of cellular GCase activity. In fibroblasts, the L444P and recombinant mutations result in reduction of *GBA* transcript levels (described in detail in chapter 3). This clearly has the potential to contribute to reduced GCase protein expression. In addition, there is evidence of endoplasmic reticulum retention and degradation of GCase protein in GD and PD-GBA fibroblasts. Thus *GBA* mutations can reduced *GBA* transcript levels and trigger endoplasmic reticulum retention of GCase protein.

In fibroblasts with heterozygous *GBA* mutations there is a reduction in GCase protein levels and enzyme activity. Given the results of the study by Gegg *et al* (2012), it is highly probable that neurons in the *substantia nigra* of these individuals will manifest the same enzymatic phenotype. It is logical to suggest that cells with a reduction in GCase protein levels and activity will be vulnerable to further depletion of GCase activity by putative environmental interactions. The only
“environmental” factor currently described which can be associated with reduced GCase expression is elevation of alpha-synuclein levels (Mazulli et al., 2011). In cell lines overexpressing alpha-synuclein there is reduction of GCase protein and activity (Gegg et al., 2012). The mechanisms by which alpha-synuclein inhibits GCase is unknown, and possible pathways are discussed in detail in the introduction to chapter 3. The mechanism accounting for loss of GCase activity in substantia nigra from sporadic PD cases is unclear. It is possible that unrecognised mutations in GBA may play a role – but this seems unlikely to account for the results from 3 separate research groups (Gegg et al., 2012; Mazulli et al., 2011; Kurzawa-Akanbi et al., 2012). It is also plausible that age related accumulation of alpha-synuclein in the substantia nigra may result in secondary depletion of GCase protein and activity.

It seems reasonable to hypothesise that depletion of GCase activity in substantia nigra neurons below a putative critical threshold could then lead to dysfunction of the lysosome or autophagy pathways. This could result in an exacerbation of alpha-synuclein accumulation. In support of this, work in our lab (Cleeter et al., 2012) has demonstrated that inhibition of GCase with conduritol-b-epoxide results in accumulation of alpha-synuclein. We could not examine this hypothesis directly in fibroblasts as they do not express alpha-synuclein.

Oxidative and endoplasmic reticulum stress are alternative pathways by which GBA mutations could contribute to increased risk of PD. As discussed in chapter 4, oxidative stress has a number of damaging effects on the cell. In the context of PD chronic low level oxidative stress in the substantia nigra could contribute to aggregation of alpha-synuclein and Lewy body formation. Endoplasmic reticulum stress can lead to activation of apoptotic pathways and predispose to loss of dopaminergic neurons.

This loss of function hypothesis can explain why both heterozygous GBA mutation carriers and Gaucher disease patients have an increased risk of developing PD. Under this hypothesis it is neuronal loss of GCase activity which is a key nodal point in the development of PD in these patients. Several pathways could lead to this loss of activity; heterozygous or bi-allelic GBA mutations, genetic variation in pathways which interact with GCase (e.g. LIMP-1/SCARB2) or depletion of GCase protein by interaction with an “environmental factor” such as alpha-synuclein. This also explains how such a wide spectrum of GBA mutations - including missense, nonsense and recombinant alleles – can predispose to PD since all of these can reduce GCase expression by translational and/or post-translational mechanisms. Under this model oxidative and endoplasmic
reticulum stress are contributory factors which act as mechanisms of neuronal injury triggered by \textit{GBA} mutations.

A major problem with this hypothesis is that the age related risks of PD in GD and heterozygous \textit{GBA} mutation carriers are very similar. If loss/reduction of neuronal GCase activity is the nodal point in PD pathogenesis in these individuals, then one would expect PD risk to be much higher in GD patients than in heterozygous \textit{GBA} mutation carriers. This is because neuronal GCase activity will be much lower in GD patients, and so pathogenic processes leading to neurodegeneration should theoretically be triggered much earlier than in carriers, in whom a lag time in which neuronal GCase activity is depleted must occur before PD pathogenesis is triggered. A potential explanation to account for this is that only \textit{relatively minor reductions} in GCase activity, as seen in fibroblast cell lines with heterozygous \textit{GBA} mutations, are sufficient to predispose to alpha-synuclein accumulation. Under this model, any further reductions in GCase activity below this putative threshold will not further increase PD risk but lead to lysosomal storage of substrate (i.e. GD). In support of this is the observation that the E326K/E326K genotype is associated with PD, without apparent evidence of GD, and that the E326K/E326K fibroblasts display a partial deficiency of GCase activity compared to GD fibroblasts. Flowchart 1, on the next page, summarises this hypothesis.
Figure 21. Mechanism by which GBA mutations lead to increased Parkinson's disease risk

Bi-allelic \textit{GBA} mutations

- Reduction of GBA mRNA levels
- ER trapping of mutant GCase
- GCase deficiency

Heterozygous \textit{GBA} mutations

- Reduction of GBA mRNA levels
- ER trapping of mutant GCase
- Exacerbation of GCase loss by age related SNCA accumulation

- Reduction of GCase activity below threshold for SNCA degradation

- SNCA accumulation

- Secondary mechanisms of cell injury: Oxidative stress

- Neurodegeneration
Limitations of current study and suggestions for future research
The clinical component of this study is limited by the non-specificity of the prodromal markers of PD studied. As noted previously there are many non-neurological causes of hyposmia and many neurological causes other than PD for hyposmia and cognitive impairment (Doty, 2012). Retinal thinning, as revealed by optical coherence tomography, is also not specific for PD (Hajee et al, 2009). Thus future work needs to examine whether these prodromal markers identified do in fact predict future development of PD. One approach to this is to perform repeated tests of olfaction and cognition and an examination for parkinsonian signs at a period of 12 months or more after the initial examination. This is currently being performed by Dr Michelle Beavan (Clinical Research Associate, UCL Institute of Neurology). An alternative approach is to correlate the clinical markers of prodromal PD with an objective biomarker of PD. This could be achieved by undertaking dopamine transporter imaging in a selection of GD patients and heterozygous GBA mutation carriers with and without prodromal markers of PD. This would however be limited by the fact that dopamine transporter imaging as limited sensitivity in detecting dopaminergic neuronal loss in the earliest stages of PD.

The cell biology arm of the project was limited chiefly by the fact that we were examining a non-neuronal cell line. There are differences in both lysosomal and mitochondrial physiology between fibroblasts and neurons. Thus it is possible that the absence of mitochondrial dysfunction and defective autophagy might reflect cell specific effects rather than a true absence of a role for these mechanisms in the pathogenesis of PD-GBA. Fibroblasts do not express alpha-synuclein, this was a major limitation since it meant we could not assess the impact of GBA mutations on alpha-synuclein metabolism. A further limitation of the cell biology project was that the fibroblasts had a diversity of mutations – despite coming from GD patients with a Type I phenotype – which will account for some of the variability in the variables studied between cell lines. Some of these limitations can be addressed by generating dopaminergic neurons from induced pluripotent stem cells generated from patient derived fibroblasts. These cells will be neuronal and express alpha-synuclein, permitting us to address the crucial question of how GBA mutations influence alpha-synuclein metabolism. I have been awarded an MRC Centenary Award 1 year extension to my Research Training Fellowship in order to begin generating dopaminergic neurons from my panel of patient derived fibroblasts.
Concluding remarks

Heterozygous $GBA$ mutations are numerically the most important risk factor for developing PD. For example, $GBA$ mutations are of far greater prevalence in PD than mutations in other genes such as $LRRK2$, $PINK1$ and $Parkin$. Moreover, the contribution of $GBA$ mutations to PD pathogenesis eclipses the relatively small increased risk conferred by environmental factors such as being a non-smoker. It should be remembered that the role of $GBA$ mutations in PD pathogenesis was discovered by careful clinical observation and simple family history studies of GD families and not through expensive genetic techniques. The story of how the aetiological role of $GBA$ mutations in PD was discovered is a classic example of how studying rare disease can illuminate the pathogenesis of a common disorder.

The high prevalence of $GBA$ mutations in sporadic PD cohorts and the reduction of GCase enzyme activity in post-mortem substantia nigra from PD brain gives important clues as to the pathogenesis of sporadic PD. There are several studies showing alteration of autophagy markers in post-mortem PD brain and the lysosome is a major site for alpha-synuclein degradation. The evidence implicating reduction of GCase activity in PD thus corroborates and confirms the potential importance of autophagy in PD pathogenesis. However, perhaps most importantly, reduction of neuronal GCase activity in sporadic PD represents a novel, tractable therapeutic target for this common neurodegenerative disease. There are a number of small molecules which can enhance GCase enzyme activity and cross the blood brain barrier (e.g. isofagamine, ambroxol), these represent excellent candidates for future drug trials in PD and may pave the way for new classes of disease modifying drug.
Bibliography


