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Translational approaches to restoring mitochondrial function in Parkinson's disease

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Abstract:

There is strong evidence of a key role for mitochondrial dysfunction in both sporadic and all forms of familial Parkinson's disease (PD). However, none of the clinical trials carried out with putative mitochondrial rescue agents has been successful. Firm establishment of a wet biomarker or a reliable readout from imaging studies detecting mitochondrial dysfunction and reflecting disease progression is also awaited. We will provide an overview of our current knowledge about mitochondrial dysfunction in PD and related drug screens. We will also summarize previously undertaken mitochondrial wet biomarker studies and relevant imaging studies with particular focus on 31P-MRI Spectroscopy. We will conclude with an overview of clinical trials which tested putative mitochondrial rescue agents in PD patients.

Parkinson's disease is a common, relentlessly progressive neurodegenerative disorder. The pathological hallmark is loss of dopaminergic neurons in the substantia nigra. The resulting motor presentation includes rest tremor, bradykinesia and rigidity but the importance of non-motor symptoms such as cognitive impairment and depression is increasingly recognized, too. Currently available dopaminergic treatment often only addresses the motor impairment partially. This review will summarize our current knowledge about mitochondrial dysfunction as a key target for disease-modifying treatment for PD. We will also provide an update on mitochondrial readouts in PD patients, namely imaging and putative mitochondrial biomarkers, which may become highly relevant in the context of future drug trials.

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Experimental evidence for mitochondrial dysfunction in PD

The mitochondria are an organelle central to cellular energy production through the generation of ATP through oxidative phosphorylation. They are also involved in other functions such as; apoptosis, scavenging of free radicals, and calcium homeostasis [1]. Mitochondria were first implicated in the pathogenesis of Parkinson's disease (PD) when it was discovered that exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which metabolises to the complex I inhibitor MPP+, induces a Parkinsonian phenotype and loss of dopaminergic neurons in the substantia nigra (SN) [2].

Neurons are particularly susceptible to mitochondrial dysfunction, largely due to their extremely high metabolic requirements [3]. Nigral neurons - the cell type predominantly affected in PD – have a higher rate of basal oxidative phosphorylation compared to ventral tegmental area neurons, which are unaffected in PD [4]. This may lead to higher levels of oxidative stress, due to the production of reactive oxygen species (ROS) by the respiratory chain complexes [4]. These neurons also have a higher density of axonal mitochondria which may confer a further increase in risk [5].

ATP production and oxidative phosphorylation complex dysfunction: Reduced complex I activity was first observed in sporadic PD post-mortem brain tissue [6], and platelets [7]. As well as in sporadic cases, mitochondrial dysfunction has been implicated in both autosomal recessive forms of PD (with mutations in *parkin*/PARK2, *PINK1*/PARK6, or *DJ-1*/PARK7), and autosomal dominant forms of PD (with mutations in *alpha-synuclein*/PARK1/4 and

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LRRK2/PARK8, see Figure 1) [8]. For example, fibroblasts derived from patients with *parkin* mutations show a distinct complex I deficiency, resulting in reduced ATP levels [9]. Oxygen consumption is also reduced in *parkin* mutant Drosophila larvae [10], *parkin* knockdown zebrafish embryos also have lowered complex I activity [11]. Interestingly, many of the genes associated with familial PD are involved in maintaining normal mitochondrial function. For example, PINK1 and PARKIN are important for mitophagy [12], Dj-1 is thought to be a redox sensor and can protect cells from oxidative stress [13], and LRRK2 is thought to be involved in regulating mitochondrial fission [14].

Autosomal dominantly inherited, heterozygous mutations in *LRRK2* are the most common cause of familial PD with G2019S being the most frequent mutation [15,16]. In $LRRK2^{G2019S}$ mutant fibroblasts, Mortiboys et al. 2015 described a reduction in oxygen consumption. In contrast, Papkovskaia et al. 2012 found an increase in oxygen consumption. This may be due to the fibroblasts being grown in different media types, galactose containing, and glucose containing, respectively [17,18]. It has also been proposed that uncoupling proteins (UCPs) could be a biomarker for familial PD, as $LRRK2^{G2019S}$ mutant fibroblasts have increased UCP2 mRNA expression [18,19]. UCPs allow the passage of hydrogen ions from the intermembrane space to the matrix independent from complex V [20]. It is thought that UCP2 is protective against mitochondrial ROS levels by reducing the mitochondrial proton gradient, this may therefore be a compensatory mechanism against oxidative stress [19]. $LRRK2^{G2019S}$ mutant fibroblasts have reduced complex III and IV activity [17,21]. In contrast, heterozygous knock-in $LRRK2^{G2019S}$ mice show increases in complex V subunit ATP5A, and complex III subunit UQCR2 protein expression, whilst homozygous $LRRK2^{G2019S}$ knock-in mice have reduced complex I subunit NDUFB8 expression [22].

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Interestingly, not only have individual complex dysfunctions been implicated in PD but also the supercomplexes that these form. Supercomplexes are thought to increase the efficiency of electron carrier transport and the oxidation of multiple substrates, decrease ROS production, and are essential in complex I stability [23,24]. The disorganisation of mitochondrial supercomplexes has been observed in *PINK1* mutant human fibroblasts and *Pink1* mutant mouse primary neurons, as well as in *Dj1* mutant mouse primary neurons and brain tissue [25]. A *pink* mutant zebrafish line also has specific decrease in complex I and III activity [26]. Inactivation of the mitochondrial calcium uniporter rescues this complex I deficiency in *pink1* mutant zebrafish, suggesting that mitochondrial calcium homeostasis is crucial for maintaining normal function of the mitochondrial respiratory chain [27].

Mitochondrial DNA and PD: mtDNA may be more susceptible than nuclear DNA to damage due to its higher exposure to ROS generated by oxidative phosphorylation [28]. Selective mtDNA damage has been found in nigral, but not cortical neurons, in PD post-mortem brain tissue [29]. There is also an increase in mtDNA somatic point mutations, especially in genes encoding mitochondrial complex IV [30]. Additionally, there are higher levels of mtDNA deletions in PD compared with age matched controls [31], which is associated with decreased complex IV activity. mtDNA deletions also occur during normal ageing, however the mtDNA copy number increases to compensate for this in healthy controls [32]. This upregulation of copy number does not to occur in PD, indicating that mtDNA homeostasis is dysregulated [32].

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Morphology: The regulation of mitochondrial morphology is a tightly controlled process. Optic atrophy protein 1 (OPA1), mitofusin 1 (MFN1), and MFN2 are involved in mitochondrial fusion, whilst dynamin-related protein (DRP1) regulates mitochondrial fission [33]. Mitochondria in post-mortem biopsy samples of the caudate nucleus and skeletal muscle of sporadic PD patients are more variable in size and shape [34,35]. However, morphology changes are difficult to assess in detail in post-mortem PD brain tissue due to the limited preservation of mitochondrial ultrastructural details [8]. Interestingly, increased mitochondrial branching has been described in *parkin* mutant human fibroblasts [9]. In addition, morphological abnormalities, such as swollen mitochondrial cristae and a decrease in density of normal mitochondria, have been found in induced pluripotent stem cell-derived *parkin* mutant neurons [36].

Changes in mitochondrial morphology have also been observed in various PD animal models. For example, an early study looking at mitochondrial abnormalities in *Parkin* mutants, observed that in *Parkin* null Drosophila, mitochondria are swollen and have severely disintegrated cristae [37]. Drosophila primary neurons treated with MPP+ also show a reduction in size, number, and an increase in fragmentation prior to cell death [38]. *LRRK2*^{G2019S} knock-in mouse models show altered mitochondrial morphology within the striatum including; a beads-on-a-string like appearance, condensation, reduction in number through autophagic degradation, and an approximately 10% increase in mitochondria length [22,39,40]. Interestingly, LRRK2 phosphorylates DRP1, mediating mitochondrial fission. Disruptions to DRP1-mediated fission via LRRK2 mutations could lead to changes in mitochondrial dynamics [14,41,42].

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Mitophagy: The removal of typically dysfunctional mitochondria, mitophagy, is a highly important cellular process, as the accumulation of dysfunctional mitochondria can lead to cellular damage. *PINK1* and *parkin*, two genes which have been associated with autosomal recessively inherited familial PD, encode proteins which are essential in mitophagy [12]. PINK1 acts upstream of Parkin [43,44]. PINK1 phosphorylates both ubiquitin and Parkin at its ubiquitin-like domain, to activate parkin as an E3 ligase [45]. This allows the ubiquitination of substrates such as DRP1 and Miro [46,47].

Miro is a protein found on the outer membrane of the mitochondria and connects them to dynein and kinesin motors, enabling microtubule-based transport [48]. The PINK1/Parkin pathway targets Miro for proteasomal degradation prior to mitophagy [47]. Interestingly, LRRK2 is also involved in Miro degradation. The disruption of both Parkin and LRRK2 recruitment to damaged mitochondria coincides with the accumulation of Miro, and leads to a delay in mitophagy in fibroblasts from sporadic PD patients [49]. This could be a potential pathway where both familial and sporadic PD converge.

As well as impaired clearance of damaged mitochondria, the generation of new mitochondria may also be impaired in PD. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) regulates mitochondria biogenesis by inducing the expression of several key biogenesis proteins [50]. PGC1- α knockdown increases α-synuclein aggregation in human neuronal cells, and overexpressing PGC1- α reduces the mitochondrial deficiencies seen in MPP+ treated SH-SY5Y cells, such as reduced ATP levels [51,52]. The role of mitophagy in PD is a major area of intense investigation and discussing it

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fully is beyond the scope of this review. Some recent reviews have covered this area in greater detail [53–56].

Transport: It is important that mitochondria can be transported effectively along the extended length of neurons and meeting the high energy demands of synapses at the axon terminals [57]. *LRRK2* mutations in the Roc-COR domain, $LRRK2^{R1441C}$ and $LRRK2^{Y1699C}$, inhibit bidirectional axonal transport of mitochondria in both Drosophila larvae motor neurons, and rat cortical neuron cultures. However, this effect is not observed in $LRRK2^{G2019S}$ mutant Drosophila [58], suggesting that mutations in the different LRRK2 domains may result in distinct phenotypes. These models use ectopically expressed *LRRK2* mutations, therefore it would be important to also examine mitochondrial transport when these *LRRK2* mutants are expressed at the endogenous locus. This could be achieved using techniques such as CRISPR/Cas9.

To summarize, a wide range of different mitochondrial abnormalities has been reported in PD, including lowered ATP production, oxidative phosphorylation and mitochondrial respiratory chain complex function, abnormal morphology, disturbed transport and impaired mitophagy.

Targeting mitochondria for neuroprotection in Parkinson's disease

As outlined above a large body of evidence exists for the presence of mitochondrial dysfunction in most forms of PD; however there are a limited number of actual drug/compound screens which have been reported investigating mitochondrial protective compounds. A robust, reproducible and biologically meaningful assay is required which is

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amenable to high through put screening. These screens could be target based or phenotypic in nature; however the same strict criteria of assay robustness and signal to noise window must be adhered to.

Instead of large compound screens, much research has been undertaken investigating mitochondrial protective compounds on a smaller scale. Cybrid cells generated by using mitochondria from sporadic PD patients fused with a parental osteosarcoma cell line create cells with a control nuclear background and the mitochondria from sporadic PD patients with altered mitochondrial cellular distribution and reduced mitochondrial membrane potential [59]. These abnormalities, in addition to autophagy/lysosome abnormalities were restored after treatment with NAP (davunetide), an eight amino acid peptide which associates with tubulin and enhances correct microtubule assembly. Other work investigating mitochondrial abnormalities in PD patient cells has shown a protective effect of resveratrol. Resveratrol is a natural polyphenolic compound which activates PGC1-alpha, a master regulator of mitochondrial biogenesis. *parkin* mutant patient fibroblasts showed reduced oxygen consumption, complex I deficiencies and pAMPK/AMPK ratio levels which all improved upon treatment with resveratrol [60]. In addition this study showed there had been a switch to glycolysis that in untreated *parkin* mutant fibroblasts, resveratrol treatment caused a switch back to oxidative phosphorylation [60]. Human pluripotent stem cell derived dopaminergic neurons with MPTP or rotenone induced toxicity have been used for small scale screens [61]. Dopaminergic neurons were differentiated from stem cells and mitochondrial dysfunction induced using either MPTP or rotenone treatment; this enabled not only mitochondrial phenotypes to be assessed but also the number of dopaminergic neurons surviving after compound treatment. The primary screen assessed the effect of 44 compounds with three compounds subsequently chosen for further validation with known

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mechanism of actions including an antioxidant, an anti-inflammatory drug and an mono-aminooxidase A inhibitor. The protective effect of these compounds was validated in the rotenone induced toxicity dopaminergic neuron model [61]. Interestingly this study also identified again resveratrol as a positive hit compound.

A limited number of larger compound screens have been undertaken and are detailed below. We have previously undertaken the first drug screen in PD patient tissue using fibroblasts from *parkin* mutant patients [62]. In this study we used mitochondrial membrane potential as the screening assay; a parameter which we had previously identified as being reduced in these *parkin* mutant fibroblasts [9]. We screened 2000 compounds for their rescue effect on mitochondrial membrane potential in fibroblasts from two *parkin* mutant patients. We furthermore undertook secondary screening utilising cellular ATP levels (also reduced in untreated *parkin* mutant fibroblasts versus controls) over a range of concentrations. We subsequently undertook measurement of the individual respiratory chain complexes with the 'top hit' compound (ursocholanic acid, UCA). 24 hour treatment with UCA increased the activity of complexes I, II, III and IV in both control and *parkin* mutant fibroblasts. Based upon the structure of UCA we identified another compound which was not included in the original screening library, ursodeoxycholic acid (UDCA) which is already licensed for use in man for the treatment of primary biliary cirrhosis. We subsequently observed a mitochondrial rescue effect of UDCA in *parkin* mutant fibroblasts as well as *LRRK2*^{G2019S} mutant fibroblasts from manifesting and non-manifesting carriers [17,62]. The beneficial effect of UDCA was confirmed in *parkin* deficient mouse primary cortical neurons and a *LRRK2*^{G2019S} transgenic *Drosophila* model [57].

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UDCA also has a protective effect in several other models of PD. Treatment with UDCA attenuates the toxic effects of sodium nitroprusside in a SHSY5Y model of PD. SHSY5Y are a human, neuroblastoma derived cell line. Specifically, the levels of reactive oxygen species were normalised, mitochondrial membrane potential was maintained and cellular glutathione levels restored [63]. Furthermore several markers of apoptosis were attenuated including caspase 3 activation and nuclear fragmentation. Other, subsequent studies using TUDCA, the taurine conjugate of UDCA have shown very similar effects in the SHSY5Y model after CCCP treatment [64]; mitochondrial membrane potential and reactive oxygen species were again normalised after TUDCA treatment. Mitochondrial dysfunction has been noted as a major phenotype in several *C. elegans* genetic models of PD induced by expressing or deleting *alpha synuclein*, *parkin* or *DJ1* [65]. Treating these mutant *c.elegans* strains with TUDCA gave full protection against further toxic insult. TUDCA also recovers a mitochondrial and endoplasmic reticulum (ER) phenotype observed in *Drosphila* models; ablation of *Marf1* (a mitochondrial assembly regulatory factor) in *Drosophila* induces a combined phenotype of mitochondrial and ER stress which is ameliorated by treatment with TUDCA [66]. In a rotenone induced rat model of PD UDCA treatment normalised ATP levels, increased dopamine content, reduced (and therefore returned to normal) expression of NFkb, Bcl-2, Bax and caspase 9, reduced caspase 8, 3 and 9 activation and dampened the inflammatory response of TNFa and interleukins [67]. UDCA also improved mitochondrial morphology by EM and increased striatal dopamine (DA) levels 3 fold. TUDCA treatment was also effective in protecting against MPTP induced Parkinsonism in rodents; this effect was mediated by reducing reactive oxygen species and activating Akt with subsequent activation of Akt-mediated, downstream pro-survival pathways [68].

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Other *in vitro* compound screens to date have utilised alpha-synculein over-expression induced toxicity identifying AGK2, a Sirt-2 inhibitor, as a potential candidate compound [69]. A compound screen utilising rotenone induced toxicity in SHSY5Y cells identified carnosic acid as a suppressor of toxicity [70]. Yeast systems have been utilised by several groups to perform compound screens investigating mitochondrial protection. Su et al undertook a screen of 115,000 compounds in yeast with validation of the top hits in an alpha synuclein-transgenic *C. elegans* model with downregulated transcriptional profile of mitochondrial genes, abnormal mitochondrial morphology and increased ROS production. Initial compounds were selected based upon their ability to restore growth and prevent cell death; subsequently these compounds were found to partially reverse the mitochondrial transcriptional changes as well as functionally restoring mitochondrial morphology and reducing ROS production [71]. Taken together, our studies and the work of others have identified a number of promising mitochondrial protective compounds, some of which warrant further investigation for their efficacy in clinical trials.

Mitochondrial dysfunction and the search for Parkinson's disease biomarkers

The development of robust biomarkers in PD has long been a priority for clinicians and researchers alike. Biomarkers that are sensitive, specific and predictive of disease course, or able to monitor response to treatment would drastically change clinical practice. PD remains a clinical diagnosis in practice, but a biomarker exhibiting these qualities would potentially allow earlier detection of the disease, differentiation between PD and other extrapyramidal disorders such as multisystem atrophy (MSA) or even the diagnosis of PD in prodromal phases. For example, the association between REM sleep behaviour disorder (RBD) and PD is well developed now, and a biomarker able to determine those with RBD who will

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progress to PD would be a huge step forward in preventing progression of the disease once effective disease modifying therapies are available. Even without disease modifying therapies, the development of biomarkers to aid in diagnosis and prognosis would be of benefit to clinician and patient. Having objective evidence of a patient's disease state may allow clinicians to deliver a more accurate prognosis in terms of cognitive involvement, motor symptoms or potential response to specific therapies. Furthermore, having biomarkers that could monitor response to therapy would give objective guidance in tailoring and optimising the management of individual PD patients.

As described above, mitochondrial dysfunction and oxidative stress are intrinsically related, key players in the pathogenesis of PD and a common pathological step in seemingly all forms of idiopoathic and familial PD, regardless of the many genetic factors involved. Thus, searching for biomarkers based upon this critical pathological process seems an ideal approach. Here we will discuss the previously researched biomarkers and those that are becoming increasingly more relevant.

8-hydroxyguanosine and 8-hydroxy-2-deoxyguanosine: 8-hydroxyguanosine (8-OHG) and 8-hydroxy-2-deoxyguanosine (8-OHdG) are produced when reactive oxygen species (ROS) hydroxylate the C-8 position of deoxyguanosine or guanosine residues in DNA and RNA. Post mortem midbrain sections of the substantia nigra (SN) in 6 PD patients show clear evidence of cytoplasmic nucleic acid damage with significantly elevated levels of 8-OHG in patients compared to 8 controls, but also compared to 4 Dementia with Lewy bodies (DLB) cases and 4 multiple system atrophy with parkinsonian symptoms cases (MSA-P) [72].

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Although less invasive tests are generally preferable both serum and urinary 8-OHG/8-OHdG will still be confounded by other systemic processes. CSF analysis may therefore allow the quantification of 8-OHG in relation to CNS oxidative stress more accurately CSF 8-OHG was significantly raised in 24 PD patients with a negative correlation with disease duration, and no clear correlation with serum levels [73]. These results were replicated in a study of 101 PD patients using ELISA to examine 8-OHG in the CSF [74]. A study of 20 patients found significantly greater levels of 8-OHG in PD patients using HPLC and correlated with disease duration [75]. However, although 8-OHG does seem clearly elevated in the CSF of PD patients, similar changes were also observed in the CSF of MSA patients (with no significant difference between MSA and PD) and Alzheimer's disease [76,77]. Thus, 8-OHG appears to be a nonspecific marker, reflecting cell stress/DNA damage across different neurodegenerative disorders.

Assessment of serum levels of 8-OHG in a small study of 24 untreated PD patients found no difference between PD compared to controls using gradient reversed-phase HPLC [73]. However, a slightly larger study of 48 PD patients and 22 age matched controls did find a significant difference in total serum levels between these groups using an ELISA method. There was no correlation between serum levels and age, age of onset or disease duration and no significant differences when comparing groups by PD medication use. Of note, the 8-OHG levels were higher in the serum of female PD patients compared to male [76]. A larger study of 211 PD patients examining leucocyte derived 8-OHG (thus trying to directly measure intracellular 8-OHG) using HPLC again detected higher levels when comparing PD to control subjects and this difference increased with disease severity independent of levodopa treatment [78]. This larger study also seemed to better control for confounding factors (such as smoking and diabetes).

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Urinary 8-OHdG was also elevated in 61 PD patients compared to age-matched controls using gas chromatography-mass spectroscopy. Interestingly 8-OHdG was higher in the early stages of the disease before trending down, and there was a significant negative correlation between cumulative levodopa intake (which would increase with length of disease) and urinary 8-OHdG [79]. However this trend of reduction in 8-OHdG with disease duration has not been replicated in another similarly sized study of 72 patients using ELISA technique which found a positive correlation with disease duration [80] . Urinary 8-OHdG can also be elevated in other disease states including malignancy, somewhat hampering the specificity of this method [81].

In conclusion, although it seems well documented that 8-OHG is elevated in CSF (and serum in some studies) of PD patients it seems to lack specificity and poorly correlates to desirable clinical measures. Of note, many of these studies were small with poor correction for confounding factors and ELISA seemed to detect much larger quantities of 8-OHG/8-OHdG compared to HPLC making comparison difficult.

Glutathione and Glutathione-S-transferase: Glutathione is a reducing agent that acts through the catalytic action of glutathione-S-transferase to reduce xenobiotics and electrophiles under oxidative stress. Oxidised glutathione has been measured in CSF as well with one post mortem study of 48 CSF samples finding significantly reduced levels of CSF oxidised glutathione compared to controls [82], however total glutathione levels in the CSF do not seem to differ between PD, MSA, Alzheimer's and age matched controls [83]. Oxidised glutathione was significantly higher in 80 PD patients compared to controls but there was no difference in total or reduced glutathione [84]. Serum glutathione peroxidase (an antioxidant

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enzyme utilising glutathione) had a higher activity in 29 PD patients compared to controls but this result awaits confirmation in a larger study [85]. Glutathione-S-transferase (GST), especially its Pi (GST-Pi) isoform was markedly increased in post mortem frontal cortex samples of PD patients [86]. In contrast, GST-Pi was decreased in the CSF of 43 PD patients [87]. To summarize, there is a paucity of studies examining consistently different measures of glutathione including total glutathione, oxidised glutathione GST-Pi, and glutathione peroxidase. This makes comparison and synthesis of this data difficult.

DJ-1: DJ-1 is the protein encoded by the gene *DJ-1* at the PARK7 locus with a still relatively unclear action. It appears to have a key role in oxidative stress where it stabilises Nrf2, a transcription regulator for antioxidant responses [88]. It also undergoes C-terminal cleavage in mild oxidative stress allowing it to exhibit a protease function that seems cytoprotective against oxidative stress induced apoptosis [89]. Under oxidative stress it also seems neuroprotective upon localizing to mitochondrial membranes and the cell nucleus [90]. Although contentious in its inclusion in this section, DJ-1 seems intimately related to mitochondrial dysfunction and oxidative stress. CSF analysis of DJ-1 has bought about varied results. Total DJ-1 in CSF by immunoblot assay showed a significant upregulation of DJ-1 in 40 PD patients in early stages of the disease compared to both later stages and control groups. However, there was no difference between the late stages of the disease and controls [91]. DJ-1 in CSF analysis using ELISA showed elevated levels in both MSA and PD patients (n=18 and 36 respectively) compared to controls. There was also a difference between MSA and PD, with a mean of 710ng/L in MSA compared to 525ng/L in PD with 418ng/L in controls [92]. Another group used a Luminex assay and mass spectrometry to quantify DJ-1 in CSF of 117 PD and 50 AD cases as well as controls. A lower level of total DJ-1 in CSF was observed in PD compared to both healthy controls and AD once haemoglobin

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contamination had been removed as a confounder. There was no difference between AD and controls [93]. Studies looking at total DJ-1 in serum and plasma were contradictory with the initial study finding an increase in total DJ-1 in 104 PD patients compared to controls [94]. However, a subsequent study did not find any differences between 95 PD cases and controls, both these studies used a similar ELISA techniques [95]. To try and clarify these results a further study used a Luminex assay and separated out all cell components from plasma and controlled for confounders such as platelet contamination and haemolysis given that 95% of DJ-1 in the blood is located in red blood cells. This very thorough approach did not reveal any differences of plasma DJ-1 levels between 126 PD patients, 33 AD patients and 122 controls [96]. Since then DJ-1 has been analysed further in the serum, looking closely at DJ-1 protein and the isoforms generated in post translational modification showed a specific isoform that was significantly decreased in 75 PD patients and 14 AD patients compared to 30 controls and one isoform that was increased in PD and AD, however both of these isoforms lacked specificity for either condition. Additionally assessing for specific post-translational modifications is fraught with difficulty as many of these isofroms in low quantities will go unidentified and some of the modifications may have occurred *ex-vivo* following sampling [97]. A further small study assessing oxidised DJ-1 derived from erythrocytes found that 43 PD patients had significantly raised levels compared to 8 MSA patients, 13 PSP patients and 16 controls [98]. DJ-1 has also been assessed in saliva. Once saliva composition and total protein content had been accounted, there were similar levels in 16 PD cases and 22 controls in one study and 74 PD cases with 12 controls in a further study, with inconsistent correlation using various clinical scores of severity [99,100].

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Lipid peroxidation changes secondary to oxidative stress: Reactive oxygen species have a clear affinity for lipids and the resulting lipid peroxidation can be considered a surrogate marker for oxidative stress. Malondialdehyde (MDA) is the most researched candidate and has been found to have significantly elevated levels in serum/plasma compared to controls consistently in study sizes of 20, 56 and 80 PD patients [101–103]. It also correlated inversely with disease progression in a much larger study of 211 PD patients and 135 controls [78]. Raised lipid hydroperoxides were also associated with late PD only [102]. However, this has not been replicated in CSF analysis of 21 PD patients and 20 controls [104].

Recently developments in metabolic profiling of many constituents involved in fatty acid oxidation have highlighted a number of decreased long chain acylcarnitines in early PD in studies involving 40 and 254 PD patients [105,106]. Previous studies have also shown other products of fatty acid oxidation such as F2-isoprostanes (F2-IsoPs), neuroprostanes (F4-NPs) and hydroxyeicosatetraenoic acid products (HETEs) being altered in PD but plasma levels of F2-IsoPs in PD subjects were not significantly different from controls in a study of 47 PD patients assessing plasma and a further similarly sized study assessing plasma and urine [107,108]. A third study subsequently showed plasma F2-IsoPs, F4-NPs and HETEs were significantly higher in 61 PD subjects compared to controls [79].

Urate: Urate is a product of purine metabolism and is also an antioxidant present in both brain and blood. Urate was initially investigated as a potential biomarker when epidemiology studies found a link between high urate levels and a reduced risk of PD. A recent meta-analysis analysing all relevant epidemiological studies confirmed that high serum urate (defined as a serum urate above 6.8 mg/dl) is associated with a reduced risk of

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PD by 33% in a dose-dependent manner [109]. This prompted further research as a biomarker and a further recent meta-analysis analysing all studies assessing serum urate and PD, amounting to 2379 PD patients and 2267 controls. Serum urate was significantly lower in PD patients than in controls with no geographic or differences between sex. Due to the variations in study designs it was difficult to correlate urate with PD severity but later stages appeared to have a more marked reduction still of urate levels compared to early or mild PD [110]. Urate levels may help to differentiated between PD and PSP or vascular parkinsonism, but not between PD and MSA [111].

Mitochondrial DNA: A Chinese study quantified mtDNA copy number in the serum of 414 PD cases and 231 controls using real-time quantitative PCR. Mean mtDNa copy numbers were significantly decreased by around 40% compared to controls. In total 71% of PD patients exhibited a significant decrease of mtDNA copy number compared to controls. The mtDNA copy number also had a significant difference between age of onset in PD cases with 75% of cases with onset over the age of 50 having a significantly lowered mtDNA copy number compared to 49% of cases with onset less than 50 years. Interestingly, there was also a significant reduction in mtDNA copy number in patients with compound heterozygous *POLG1* mutations [112].

A further study assessed mtDNA copy number in the serum of 363 white blood cell samples, 151 post mortem samples of substantia nigra pars compacta (SNpc) and 120 samples of frontal cortex in PD cases with matched controls for all samples. Quantitative PCR was use to assess mtDNA copy number. A significant decrease in mtDNA copy number in PD

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patient blood cells independent of age and gender, and this was mirrored in the post mortem samples of SNpc and frontal cortex [113].

Methylated mitochondrial DNA (mtDNA) was reported to be reduced in the CSF of 56 PD patients with no association between age, gender or clinical presentation and severity [114]. A further recent CSF study was able to differentiate iPD from PD caused by LRRK2 mutations in that the 20 LRRK2 PD patients had markedly elevated levels of cell-free circulating mtDNA levels compared to 26 asymptomatic LRRK2 mutation carriers, 32 iPD patients and 21 controls [115].

Summary and outlook: Currently, most of the work attempting to identify biomarkers reflecting mitochondrial dysfunction in PD identified viable candidates but any attempts to correlate these to disease severity and progression were inconsistent and replication in independent cohorts is typically lacking.

Imaging mitochondrial dysfunction in PD

Magnetic resonance spectroscopy (MRS) enables measurement of metabolites and hence investigation of cellular dysfunction non-invasively in humans *in vivo.* Whilst aspects of mitochondrial dysfunction may be captured using standard proton MRS, 31-phosphorus MRS (^{31}P-MRS) is considered the optimal modality to assess mitochondrial function, because most of the resolved molecules of interest are central to cellular bioenergetics, namely adenosine triphosphate (ATP), phosphocreatine (PCr), and cytosolic inorganic phosphate (Pi). In addition to phosphates involved in energy transduction pathways, ^{31}P-MRS allows

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measurements of phosphomonoesters and phosphodiesters that are the anabolic precursors and catabolic products of membrane phospholipids, intracellular pH, and cytosolic magnesium. Various methods are employed for absolute quantification, each with their own advantages and limitations. Absolute quantification can be time-consuming, no one method is absolutely superior to the others and there are technical challenges, for example, relating to variation in T1 and T2 relaxation times. To circumvent some of these issues, results are often presented as ratios of the signal intensities of two metabolites acquired from the same spectrum. However, interpretation of ratios can also be complicated because changes can be due to variations in the numerator, the denominator, or both.

Dynamic protocols can be used to investigate skeletal muscle bioenergetics and characterise metabolite changes following exercise. PCr kinetics are often reported because the rate at which PCr returns to resting state concentration after an exercise-induced decrease is considered a measure of mitochondrial oxidative capacity. Protocols applying the same principle to central nervous system areas, for example occipital lobe metabolite changes following visual stimulation, have been developed but are reported less frequently. In overt mitochondrial disease, such as mitochondrial cytopathies, ATP phosphorylation potential (a measure of the free energy of ATP) is diminished; this is associated with an elevation of Pi and calculated ADP as well as decreased PCr concentration [116]. Some authors have also hypothesised that dysfunctional mitochondria release Pi in the cytoplasm, where its concentration increases and can be measured by MRS [117].

^{31}P-MRS has been applied to investigate bioenergetic dysfunction in PD in both muscle and brain. Early studies sought evidence of bioenergetic dysfunction in the skeletal muscle of PD patients with mixed results. One of these studies applied a dynamic protocol to assess the

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bioenergetics of digital flexors following exercise [118]. No statistically significant differences between seven patients and 11 healthy controls were found, although the authors reported a non-significant delay of PCr recovery. The following year, a study assessed resting spectra acquired from the forearm muscles of 28 patients and 28 healthy controls [119]. An elevated Pi/PCr ratio was detected, and interpreted as evidence of mitochondrial dysfunction, as similar findings had been reported in patients with mitochondrial disorders. As the investigators did not conduct an exercise protocol, PCr recovery kinetics could not be assessed.

More recent studies have focused on the brain. In a comprehensive quantitative study, it was demonstrated that ATP and PCr concentrations were significantly reduced in the midbrain and putamen of 16 early and 13 late-stage PD patients compared to 19 healthy controls [120]. A relative decrease in ATP (reported as a percentage of the total phosphorus signal area) was found in another study [117] in the basal ganglia and brainstem of ten patients, compared to nine controls, and was interpreted as *in vivo* evidence of mitochondrial dysfunction. In contrast, a further study did not find any significant differences in ^{31}P-MRS parameters in the brains of 20 patients with early PD compared to 12 controls [121]. Interestingly, the authors of the latter study suggested that brain bioenergetics might be affected by oestrogen levels, as they found, in the same patient cohort, lower ATP concentrations in ten men with PD compared to ten women [122]. The discrepancy in the results might be ascribed to methodological and analysis differences.

Metabolite alterations have also been reported in PD beyond the nigrostriatal tract. Pi has been shown to be elevated in the temporoparietal [117], occipital [123], and frontal [124]

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lobes in PD. In the latter study, decreased phosphorylation potentials in ten patients compared to nine healthy controls were also detected. Further evidence for latent bioenergetic dysfunction in anatomical areas not considered primarily affected by the disease was provided by two studies from the same group [125,126]. The authors demonstrated that ATP and PCr concentrations in the occipital lobes remained constant during visual stimulation in both healthy controls and patients. However, in PD, the level of these metabolites decreased during the recovery phase, in contrast to an increase in healthy subjects. The authors concluded that mitochondrial dysfunction is unmasked during the post-activation period, hypothesising that this was a phase of particularly increased energy demand. The same group also showed that, in a patient with a compound heterozygous *PINK1* mutation, basal levels of ATP and PCr in occipital cortex were decreased, the concentration of these metabolites dropped further during visual activation, and then recovered almost to resting levels during the post-activation period [126]. In contrast, a different group detected elevated PCr in the putamen of two homozygous *PINK1* patients, and elevated ATP in one of them [127]. They also assessed nine heterozygous *PINK1* carriers, and found no changes in phosphorus metabolites compared to 23 healthy controls. It appears there may be heterogeneity in the metabolic phenotype of PD, and potentially genetic influences.

Overall, to date, ^{31}P-MRS studies have detected some evidence of bioenergetic dysfunction *in vivo* in PD, consistent with the experimental literature. However, there are at present only a limited number of studies, and great heterogeneity in terms of acquisition and analysis techniques. An important gap in the literature is that no longitudinal studies assessing variations of phosphorus spectra over time have yet been conducted. This is crucial to determine whether ^{31}P-MRS could be used as a biomarker of progressive energy metabolic

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failure, because the demonstration of target engagement by emerging therapies that influence mitochondrial biology in PD appears an exciting practical future application.

Clinical trials with mitochondrial rescue agents

The strong evidence of mitochondrial dysfunction in both sporadic PD and all forms of familial PD as well as in a multitude of different model systems has made the rescue of mitochondrial dysfunction an obvious target for neuroprotective intervention . In this final section, we will briefly discuss clinical trials which investigated compounds for their neuroprotective effect in PD patients, based on the assumption that these drugs would slow down neuronal cell death and thus disease progression by restoring energy production, reducing oxidative stress or both.

Selegiline and tocopherol/vitamin E: The first trial investigating drugs with putative rescue mechanisms on mitochondrial function and oxidative stress was the DATATOP study [128]. 25 years later, it is still a landmark study. Both selegiline as a monoamine oxidase B inhibitor at a dose of 10 mg per day and tocopherol (a component of vitamin E that traps free radicals) at a dose of 2000 IU per day were assessed together with two matched placebos in 800 patients, randomly assigned to one of the four treatment arms. The primary endpoint was the onset of disability prompting the clinical decision to administer levodopa. The results of the tocopherol arm were clearly negative after a mean of 14 +/- 6 months. The selegiline treatment and matched placebo arms were extended up to 24 months. The use of selegiline had a beneficial effect which occurred largely during the first 12 months. The results and interpretation of the DATATOP study have been hotly debated but it is currently felt that

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the apparent beneficial effect of selegiline medication is most likely to be due to its symptomatic effect rather than a neuroprotective effect.

MitoQ and ubiquinone: MitoQ consists of the lipophilic cation triphenylphosphonium (TPP) covalently attached to the ubiquinone moiety of the edogenous antioxidant CoQ10 [129]. The TPP cation enables MitoQ to cross membranes and to accumulate several-hundred fold within mitochondria due to the large membrane potential [130]. A 13-centre in New Zealand and Australia recruited 128 newly diagnosed untreated patients with PD in a double-blind study of two doses of MitoQ compared with placebo. There was no difference in the UPDRS score at 12 month follow-up. The much larger QE3 study investigated the potential neuroprotective effect of ubiquinone (CoQ10) in 600 drug-naive participants with PD who were randomly assigned to receive placebo, 1200 mg/d or 2400 mg/d of CoQ10. All participants also received 1200 IU/d of vitamin E. Patients were observed for 16 months or until symptomatic treatment with dopaminergic medication was required. The study was powered to detect a 3-point difference between the active treatment and placebo. CoQ10 was safe and well tolerated but did not show any clinical benefit [131].

Creatine: Creatine acts as an energy source after conversion to phosphor-creatine. In one of the largest studies ever undertaken in PD, 1741 patients were recruited into two treatment arms, placebo vs creatine monohydrate at a dose of 10 g/d. Patients were followed up for an impressive minimum of 5 years with a maximum follow-up of 8 years. The trial was terminated early for futility based on the results of a planned interim analysis of participants enrolled at least 5 years prior to the date of the analysis (n=995). Creatine treatment did not improve the outcome in patients with early or treated PD [132].

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Pioglitazone: Pioglitazone is an FDA-licensed drug for the treatment of type 2 diabetes. As a peroxisome proliferator-activated receptor γ (PPAR-γ) agonist, it acts to reduce insulin resistance. Its co-factor PGC-1α is a transcriptional coactivator and often referred to as a mitochondrial master regulator. There is strong evidence of impaired PGC-1α regulated transcriptional networks in PD [133]. 210 patients were enrolled in a phase 2, multicentre, double-blind, randomised, placebo-controlled, futility trial with three treatment arms, namely either 15 mg/day or 45 mg/day pioglitazone or placebo. The primary outcome was the change in total MDS-UPDRS score between baseline and 44 weeks. The results were negative and suggested that pioglitazone is unlikely to modify progression in early PD [134].

Exenatide: Exenatide a glucagon-like peptide-1 (GLP-1) agonist which is again licensed for the treatment of type 2 diabetes. Its precise mechanism in PD brains remains uncertain but preclinical data on GLP-1 agonists include reports of a protective effect in the MPTP-mouse model of PD and a stimulating effect on mitochondrial biogenesis *in vitro* [135,136]. A single-centre, randomised, double-blind, placebo-controlled trial randomly assigned 62 patients with moderate PD to receive subcutaneous injections of 2 mg or placebo once weekly for 48 weeks, followed by a 12-week washout period. At 60 weeks, off-medication scores on part 3 of the MDS-UPDRS had improved by 1.0 points in the exenatide arm but worsened by 2.1 points in the placebo group, an adjusted mean difference of -3.5 points in the practically defined off-stage [137].

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Summary and outlook: The lack of a beneficial effect for all drugs described above other than exenatide made some authors question the rationale for targeting mitochondria and oxidative stress at all in PD [130]. However, negative data from one study only question the therapeutic usefulness of a specific compound at its best and at least some studies such as the MitoQ study appear underpowered as well. Furthermore, data on blood-brain barrier penetrance in PD patients is not consistently available for the tested compounds and none of these studies had a biomarker arm which would have confirmed target engagement. As described at the beginning of this article, there is strong evidence of mitochondrial dysfunction in both sporadic and all forms of genetic PD. General doubts about the relevance of mitochondrial rescue as a promising strategy for neuroprotection in PD are therefore ill justified.

Conclusions and perspectives

There is enormous momentum in the field of mitochondrial research in PD but the field also seems rather fragmented. It will be crucial to have better integration and meaningful translation from the bench to the bedside and back to make real progress. Our next goal has to be to identify those PD patients who have particularly marked mitochondrial dysfunction and then develop mitochondrial biomarkers which correlate with disease progression and provide evidence of target engagement for putative mitochondrial rescue agents in future clinical trials.

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Figure 1. Mitochondrial abnormalities in Parkinson's Disease. Central to several subtypes of PD are abnormalities of the mitochondria. Cells and/or animal models with mutations or reduction in the protein levels of Parkin, PINK1 and DJ1 typically develop a complex I defect resulting in reduced ATP production; whereas LRRK2 mutations result in reduced complex IV activity, again resulting in ATP levels. Reductions in the mitochondrial membrane potential (MMP), aberrations in the mitophagy pathway and disturbed mitochondrial morphology have also been demonstrated as a consequence of impaired function for all of the above genes.

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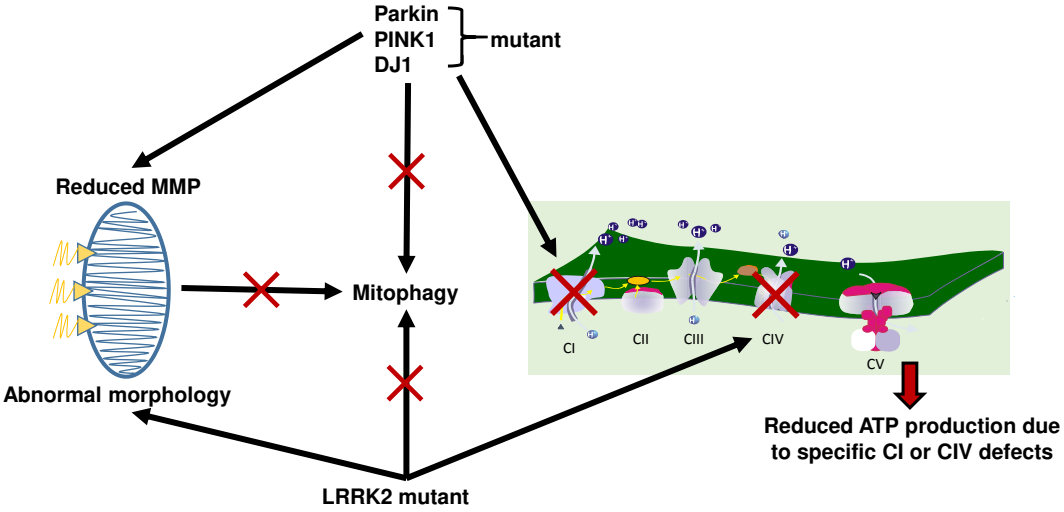

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