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The unresolved role of mitochondrial DNA in Parkinson's disease: An overview of published studies, their limitations, and future prospects

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ABSTRACT

Parkinson's disease (PD), a progressive neurodegenerative disorder, has long been associated with mitochondrial dysfunction in both sporadic and familial forms of the disease. Mitochondria are crucial for maintaining cellular homeostasis, and their dysfunction is detrimental to dopaminergic neurons. These neurons are highly dependent on mitochondrial adenosine triphosphate (ATP) and degenerate in PD. Mitochondria contain their own genomes (mtDNA). The role of mtDNA has been investigated in PD on the premise that it encodes vital components of the ATP-generating oxidative phosphorylation (OXPHOS) complexes and accumulates somatic variation with age. However, the association between mtDNA variation and PD remains controversial. Herein, we provide an overview of previously published studies on the role of inherited as well as somatic (acquired) mtDNA changes in PD including point mutations, deletions and depletion. We outline limitations of previous investigations and the difficulties associated with studying mtDNA, which have left its role unresolved in the context of PD. Lastly, we highlight the potential for further research in this field and provide suggestions for future studies. Overall, the mitochondrial genome is indispensable for proper cellular function and its contribution to PD requires further, more extensive investigation.

1. The mitochondrial genome

Mitochondria are primarily responsible for generating adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS). Besides nuclei, they are the only other cellular organelles harbouring their own genome (mtDNA). mtDNA is a compact (16 569 bp), circular, double-stranded genome, comprised of 37 genes including 13 which encode essential subunits of the OXPHOS enzymes (Fig. 1) (Andrews et al., 1999). More recently mtDNA has been reported to additionally encode two short open reading frames (sORFs) within the ribosomal RNA genes; *MT-RNR1* and *MT-RNR2* (Lee et al., 2015). These sORFs can be translated into mitochondrial-derived peptides (MDPs) which have important functions including regulating nuclear gene expression in response to metabolic stress (Kim et al., 2018).

Mitochondrial genomes differ to their nuclear counterparts with regards to their replication, repair and inheritance mechanisms. Unlike nuclear DNA (nDNA), mtDNA is primarily maternally inherited, although rare cases of biparental inheritance have recently been reported (Luo et al., 2018). Moreover, it exists as multiple copies (known as polyploidy) within individual mitochondria. Hundreds to thousands of mtDNA copies can exist per mitochondria and thus per cell, depending on the energetic demand of the cell (Miller et al., 2003). Compared to nDNA, mtDNA is highly susceptible to accumulating errors due to the lack of protective histones and its close proximity to damaging by-products of OXPHOS e.g. reactive oxygen species (ROS) (Khrapko et al., 1997). In addition, reduced fidelity of the mtDNA polymerase, mtDNA polymerase gamma (POLG1), has been suggested to contribute to a higher mutational rate of mtDNA (Song et al., 2005). As a result, most

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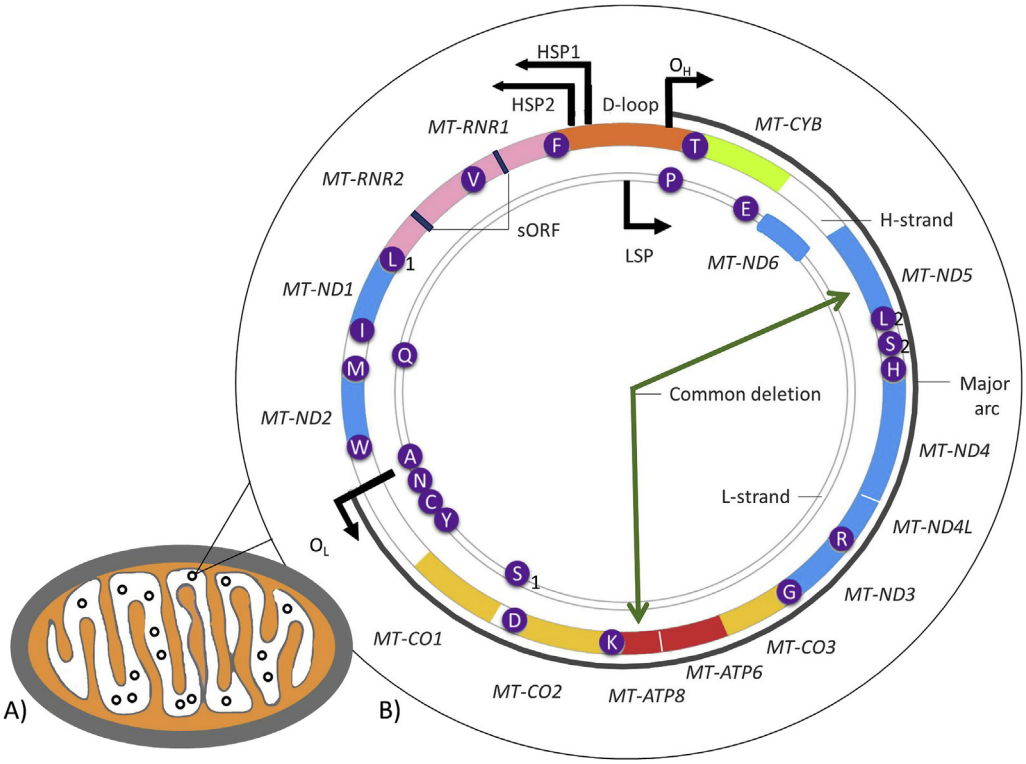

Fig. 1. The mitochondrion and structure of human mitochondrial DNA A) Basic structure of a mitochondrion containing multiple copies of mitochondrial DNA (mtDNA) represented as black circles. B) Structure of mtDNA: a 16 569 bp circular, double-stranded molecule consisting of a guanine-rich heavy strand (H-strand) and a light strand (L-strand), rich in cytosine. Approximately 93% of the genome is coding, having only one significant non-coding control region which includes the displacement loop (D-loop). This stretch of DNA contains the origin of H-strand replication (OH), the H-strand transcriptional promoters (HSP1 and HSP2) as well as the L-strand promoter (LSP). The DNA contains 37 primarily contiguous genes, nine on the L-strand and 28 on the H-strand. Twenty-four genes encode mature RNA products: one 12 S rRNA (small ribosomal subunit) and one 16 S rRNA (large ribosomal subunit), and 22 mitochondrial tRNAs (genes highlighted in purple). The remaining 13 mtDNA genes encode polypeptide components of the electron transport chain (ETC) involved in energy production via oxidative phosphorylation (OXPHOS). These include seven subunits (MT-ND1,2,3,4,4L, 5,6) of complex I (genes highlighted in blue), one subunit (MT-CYB) of complex III (gene highlighted in light green), three subunits (MT-CO1,2, and 3) of complex IV (genes highlighted in yellow), and two subunits (MT-ATP6 and 8) of complex V (genes highlighted in red). mtDNA also encodes two mitochondrial-derived peptides, MOTS-c and Humanin, as short open reading frames (sORF) in the *MT-RNR1* and *MT-RNR2* genes (highlighted in pink) respectively. Deletions in mtDNA frequently occur in the major arc (indicated by the dark grey line) between the heavy and light strand origins of replication (OH and OL respectively). Among these deletions is the 'common deletion': a 4977-base-pair deletion located between the *MT-ATP8* and *MT-ND5* genes as indicated by the dark green arrows.

individuals harbour low levels (< 1%) of inherited and/or acquired mtDNA variants (Ye et al., 2014a). Most copies of mtDNA in a cell are identical, which is referred to as *homoplasmy*. A mix of both mutated and wild-type mtDNA is referred to as *heteroplasmy*. Levels of heteroplasmy can change over time through mechanisms of relaxed replication in postmitotic tissue and random segregation of mtDNA during cell division in mitotic tissues (Chinnery and Samuels, 1999). Although, the process of mammalian mtDNA replication is not yet completely understood, mtDNA is known to replicate independently of the cell cycle - undergoing life-long replication in both proliferating and post-mitotic cells (Yasukawa and Kang, 2018). Tissue-specific differences in mtDNA maintenance, replication, and expression exist (Herbers et al., 2019) which, together with the unique features of mtDNA, particularly polyploidy and heteroplasmy, pose a significant challenge for studying their role in diseases including Parkinson's disease (PD).

PD is a neurodegenerative movement disorder with a complex aetiology comprising both environmental and genetic factors. Progressive dopaminergic (DA) neuronal loss in the Substantia Nigra pars compacta (SNpc) is characteristic of PD, but pathological mechanisms are poorly understood. Despite decades of research and definite links to mitochondrial dysfunction in disease susceptibility and progression, the role of mtDNA in risk and pathogenesis of sporadic PD (sPD) remains equivocal. This review aims to appraise the literature to determine the reason(s) for this. We discuss results from published studies which have investigated mtDNA changes in PD and highlight inconsistencies between studies which have left the role of mtDNA in PD largely unresolved. We also draw attention to factors which complicate the study of mtDNA in PD and may have contributed to conflicting results. In doing so, we provide suggestions to guide and possibly improve future research in this field.

2. Parkinson's disease and mitochondrial dysfunction

The DA neuronal loss in PD results in an array of classical motor symptoms including bradykinesia, tremor and rigidity (Gelb et al., 1999). These, together with various non-motor symptoms (e.g. depression, anxiety and insomnia), arising from the loss of additional neuronal populations, significantly compromise patients' quality of life. A pathological hallmark of PD are Lewy bodies (LB); intracellular inclusions of the aggregate-prone, alpha-synuclein protein (Spillantini et al., 1997). Only approximately 10% of cases have familial PD (Elbaz et al., 1999) and around 30% of these have monogenic PD i.e. have mutations in nDNA-encoded genes (Kumar et al., 2011). Notably, most PD cases are sporadic and have an unknown aetiology of disease as well as an absence of a family history. However, both familial and sPD have been linked to mitochondrial dysfunction (Liu et al., 2009; Parker et al., 1989).

Although the generation of ATP is the most recognised function of mitochondria, the organelles also participate in critical processes which help ensure cell viability, including lipid biosynthesis, calcium homeostasis and apoptosis and even play diverse roles in immune response (Bulua et al., 2011; West et al., 2011). Mitochondrial dysfunction therefore impinges on a wide spectrum of cellular functions. This dysfunction is multifactorial in origin and can arise from impaired mitochondrial biogenesis, altered mitochondrial dynamics (e.g. fission and fusion), impaired mitochondrial quality control (mitophagy), compromised OXPHOS and calcium imbalances (Park et al., 2018).

Early evidence that mitochondrial dysfunction may play a central role in sPD pathogenesis resulted from the unintentional exposure of humans to the drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP), specifically its active derivative MPP+, causing parkinsonian symptoms (Langston et al., 1983; Davis et al., 1979). Primate models

subsequently reported SNpc DA cell loss and other parkinsonism symptoms including akinesia, tremor and rigidity in monkeys treated with a form of MPTP (Burns et al., 1983). Similarly, MPP + mouse models exhibited DA neuronal loss (Heikkila et al., 1984; Wallace et al., 1984) and showed that MPP + inhibits complex I activity, thereby interfering with OXPHOS (Heikkila et al., 1985; Mizuno et al., 1987). As such, an energy crisis was suggested as an important mechanism underlying neuronal degeneration leading to PD symptoms (Heikkila et al., 1985; Mizuno et al., 1987). Subsequently, in the late 1980's and early 1990's, defects of complex I were observed in various tissues of sPD patients, including skeletal muscle (Shoffner et al., 1991; Bindoff et al., 1991; Blin et al., 1994; Nakagawa-Hattori et al., 1992; Cardellach et al., 1993), the SNpc (Janetzky et al., 1994; Schapira et al., 1989) and platelets (Haas et al., 1995; Benecke et al., 1993; Yoshino et al., 1992; Krige et al., 1992). As a consequence of these initial studies, it was hypothesised that the observed complex I defect may be derived via the mitochondrial genome (Parker et al., 1989). Moreover, genes linked to early-onset forms of PD (e.g. *PINK1*, *PRKN* and *DJ-1*), encode proteins which participate in- or mediate some form of mitochondrial function, regulation or quality control (Park et al., 2018). For instance, as reviewed elsewhere (Trinh and Farrer, 2013), the proteins PINK1 and Parkin are involved in mitophagy, consequently, loss of function mutations in nDNA encoding these proteins affect mitochondrial biogenesis and induction of autophagy.

3. Early evidence for mtDNA involvement in PD from cybrids

In addition to nDNA mutations contributing to familial PD onset, mtDNA variation has been linked to sPD, as demonstrated through cybrid (cytoplasmic hybrid) cell line studies (Table 1) (Swerdlow, 2012). Briefly, ρ0 cells which are devoid of mtDNA but contain identical nDNA, are fused with mtDNA-containing platelets from either PD patients or controls. Following multiple cellular replication cycles, the cybrids generated share the same environmental and nDNA background, differing only with regards to their mitochondrial genomes (Ghosh et al., 1999; Swerdlow et al., 1996). Therefore, differences observed between cell lines with differing mtDNA content, from either a patient or control, are thought to be solely attributable to the mtDNA itself (Ghosh et al., 1999; Swerdlow et al., 1996; Esteves et al., 2008). As a result, p0 cybrid cells have provided a useful tool to investigate the role of mtDNA in cellular health and in disease.

Albeit an effective model to study mtDNA background *in vitro*, cybrid studies have limitations which contribute to discrepancies between studies. One of the most notable is the frequent use of tumour cell lines exhibiting aneuploidy or other forms of nuclear genomic instability (Wilkins et al., 2014). Despite potential limitations, sPD cybrid studies have consistently provided insight into the role which mtDNA background has on mitochondrial morphology and function between cell lines from patients and controls (Table 1).

Notably, emerging evidence implicates mitochondrial genomes in modulating nuclear gene expression by means of signalling pathways (Kenney et al., 2014; Vivian et al., 2017; Jandova et al., 2012). These include the integrated stress response, retrograde (mitochondria-nucleus) communication, proteostasis regulation and mitonuclear feedback signalling (Quiros et al., 2016). With mitochondrial biogenesis, maintenance and other mitochondrial processes heavily depending on nDNA gene expression (Dominy and Puigserver, 1500), mtDNA may influence these processes and could for instance account for variable biogenesis between sPD cybrids with differing mtDNA donors (Keeney et al., 2009).

Importantly, sPD cybrids have exhibited several features of mitochondrial dysfunction including decreased complex I activity, increased ROS, morphologically abnormal mitochondria and decreased maximum respiratory capacity, linking mtDNA to sPD (Table 1). These findings all highlight that mtDNA can substantially influence important mitochondrial processes and physiology. Consequently, cybrid studies spawned investigations aimed at identifying mtDNA variation which could account for mitochondrial abnormalities observed in sPD cybrids.

4. Studies investigating homoplasmic mtDNA variation

4.1. Early mtDNA sequencing studies

In the 1990's and early 2000's, a few studies sequenced the complex I genes of PD patients and controls to identify possible mtDNA variants associated with the disorder (Smigrodzki et al., 2004; Kösel et al., 1998; Richter et al., 2002; Simon et al., 2000; Kirchner et al., 2000). Standard Sanger sequencing techniques available during this time were primarily limited to identifying homoplasmic or high-frequency heteroplasmic variants (Howell et al., 2005). A few studies sequenced whole mtDNA (Ikebe et al., 1995; Ozawa et al., 1991), whilst others sequenced all of the mtDNA tRNA (Simon et al., 2000; Grasbon-Frodl et al., 1999; Brown et al., 1996) and rRNA genes (Brown et al., 1996). Furthermore, some employed restriction fragment length polymorphism (RFLP) methods to investigate whether certain mtDNA variants (e.g. m.3397A > G in *MT-ND1*) associate with PD (Bandmann et al., 1997; Mayr-Wohlfart et al., 1997).

A handful of studies identified 'novel' variants in PD patients (Kösel et al., 1998; Richter et al., 2002; Grasbon-Frodl et al., 1999; Brown et al., 1996) and reported variants at an increased frequency in cases compared to controls (Kirchner et al., 2000; Mayr-Wohlfart et al., 1997). However, their findings may have been coincidental. This is

Table 1
Features of mitochondrial dysfunction observed in sporadic Parkinson's disease cybrids.

Phenotype	References
Altered mitochondrial ultrastructure: e.g. rounded, swollen mitochondria; mitochondrial matrix with few or disrupted cristae; presence of intramitochondrial inclusions	(Esteves et al., 2008, 2009; Swerdlow et al., 1998; Trimmer et al., 2000)
Decreased complex I activity	(Swerdlow et al., 1996; Esteves et al., 2008, 2009, 2010a; Gu et al., 1998)
Mitochondrial depolarisation	(Esteves et al., 2008)
Reduced mitochondrial ability to buffer cytosolic calcium/altered calcium homeostasis	(Esteves et al., 2010b; Sheehan et al., 1997)
Reduced ATP levels	(Esteves et al., 2008, 2009, 2010c)
Apoptosis: altered levels of apoptosis-related proteins	(Esteves et al., 2008; Onyango et al., 2005a, 2005b)
Reduced mtDNA content	(Keeney et al., 2009; Borland et al., 2009)
Microtubule depolymerisation	(Esteves et al., 2009, 2010c)
Increased alpha-synuclein oligomerization	(Esteves et al., 2009, 2010b, 2010c)
Existence of fibrillar and vesicular inclusions (cybrid Lewy bodies)	(Esteves et al., 2009; Trimmer et al., 2004)
Reduced respiratory reserve capacity	(Esteves et al., 2010a)
Increased toxin susceptibility	(Swerdlow et al., 1996; Esteves et al., 2008)
Significantly reduced mitochondrial axonal transport	(Borland et al., 2009)
Increased ROS production	(Swerdlow et al., 1996; Esteves et al., 2009)

ATP = Adenosine triphosphate; mtDNA = Mitochondrial DNA; ROS = Reactive oxygen species

because the sample sizes were often too small to draw statistically-powerful conclusions. Additionally, sequencing was still in its infancy, making it more likely to identify false positives due to the limited number of known variants which had been reported. In support of this statement, most of these 'novel' variants have now been reported in individuals without PD on the MITOMAP database (Lott et al., 2013); https://www.mitomap.org/MITOMAP), although not at high frequencies (Supplementary Table 1). Based on these early sequencing studies, no homoplasmic nor high-frequency heteroplasmic mtDNA variants were conclusively implicated in PD pathogenesis or risk.

4.2. Haplogroup association studies

The sequential accumulation of new variation in maternal lineages over thousands of years has generated stable mtDNA sequence variation which is homoplasmic. Subsets of such stable mtDNA variation define haplogroups which are primarily restricted to particular populations and geographic areas (Torroni et al., 2006). Details of variants which define individual mtDNA haplogroups are recorded on PhyloTree (Van Oven and Kayser, 2009); http://www.phylotree.org/).

Over the past two decades, the associations between mtDNA haplogroups and PD risk have been extensively studied using the 'haplogroup association approach', particularly in populations of European ancestry (Table 2 and Supplementary Table 2). This approach associates haplogroups with a disease phenotype and suggests that one or more common population variants may modify disease risk. As a result of these studies, multiple European haplogroups including J, K, U, and some super-haplogroups (e.g. UK and JT) have been associated with a reduced risk of PD (Gaweda-Walerych et al., 2008; Georgiou et al., 2017; Ghezzi et al., 2005; Hudson et al., 2013; Khusnutdinova et al., 2008; Mehta et al., 2009; Pyle et al., 2005; Van Der Walt et al., 2003). However, a number of additional studies were unable to replicate these findings and found no significant association between PD and haplogroups or common haplogroup variants (Mehta et al., 2009; Fachal et al., 2015; Huerta et al., 2005; Latsoudis et al., 2008; Ross et al., 2003; Autere et al., 2004) (Table 2 and Supplementary Table 2). For instance, Mehta et al. (Mehta et al., 2009) were unable to replicate previous associations between PD risk and haplogroups J and K in a large Australian cohort of European ancestry. Additionally, Van Der Walt et al. (Van Der Walt et al., 2003) and Huerta et al. (Huerta et al., 2005) identified the *MT-ND3* single nucleotide polymorphism (SNP) m.10398A > G to be protective against PD, whilst multiple other studies could not replicate this finding (Gaweda-Walerych et al., 2008; Ghezzi et al., 2005; Hudson et al., 2013; Latsoudis et al., 2008). Asian haplogroup association studies have also produced somewhat inconsistent results (Table 2 and Supplementary Table 2). For instance, although Wu et al. (Wu et al., 2018) and Liou et al. (Liou et al., 2016) both reported the Asian haplogroup B5 to be protective against PD, studies done by Chen et al. (Chen et al., 2007) and Chen et al. (Chen et al., 2015) reported no significant overall association between any of the common Asian haplogroups and PD risk.

Overall, the evident variability in results and lack of reproducibility across haplogroup studies has made it difficult to conclude whether common mtDNA population variants contribute to PD risk.

4.2.1. Limitations of past haplogroup association studies and possible sources of inter-study variability

To improve the design of future studies, it is important to identify all possible factors which are introducing variability into, and between studies in order to limit false positive or erroneous findings. In the following section we therefore highlight possible methodological concerns which may contribute to discrepancies between study findings and should be addressed in future work.

4.2.1.1. Population stratification. mtDNA haplogroup association studies are often confounded by high levels of population stratification, referring to systematic differences in allele frequencies between subpopulations in a population, due to different ancestry (Hellwege et al., 2017). This is because mtDNA itself is more prone to population substructure than nDNA, given that its effective population size is four times smaller than that of nDNA due to its uni-parental inheritance. Even when cases and controls are well matched in other regards (e.g. age, sex) substructure can be a confounding factor which may result in false positives. A study done by Otaegui et al. (Otaegui et al., 2004) with a cohort of Spanish PD patients, from different origins (Basque and non-Basque), and non-Basque controls, highlighted the importance of conducting case-control association studies in ethnically homogeneous populations (Table 2 and Supplementary Table 2). The authors found no association between PD and the m.10398A > G SNP ($P = 0.088$) when comparing all cases to controls but observed a significant overrepresentation of this SNP specifically in PD patients of Basque origin when compared to the non-Basque control population ($P = 0.0221$).

4.2.1.2. Replication cohorts and multiple testing. Several case-control association studies, including some of the 'PD haplogroup' studies listed in Table 2, lack replication cohorts (Salas and Elson, 2015). Such replication cohorts or even a second control group (e.g. as used by Ghezzi et al. (Ghezzi et al., 2005), Pyle et al. (Pyle et al., 2005) and Chen et al. (Chen et al., 2015)) can be useful for detecting population substructure which affects the validity of results if not taken into consideration (Salas et al., 2009).

Additionally, multiple PD haplogroup association studies failed to correct their P-values/significance levels for multiple testing (Table 2 and Supplementary Table 2). This may also have resulted in false positive findings (Pardo-Seco et al., 2013).

4.2.1.3. Power. Samuels et al. (Samuels et al., 2006) demonstrated that when using the haplogroup association hypothesis, very large sample sizes of cases and controls are necessary in order to reliably detect an association between haplogroups and complex human disease. To demonstrate this, the authors gave the example that studies with 500 cases and 500 controls have 90% power to detect a greater than 35% change in the frequency of the common haplogroup H. Therefore, several of the PD haplogroup studies are underpowered (Table 2 and Supplementary Table 2). Additionally, such underpowered haplogroup association studies are frequently analysed in ways which violate the assumptions of the statistical tests used, thereby inflating Type 1 error (Salas and Elson, 2015).

4.2.1.4. Pseudo-haplogroups. Pseudo-haplogroups are usually composed of low frequency haplogroups present in the study cohort, clustered together in ways which cannot be justified by phylogenetic knowledge (Fachal et al., 2015). Interestingly, this artificial clustering of haplogroups is commonly observed in neurological association studies including those done by Pyle et al. (Pyle et al., 2005), Ghezzi et al. (Ghezzi et al., 2005), Gaweda-Walerych et al. (Gaweda-Walerych et al., 2008) and Latsoudis et al. (Latsoudis et al., 2008) on PD risk. The creation of such pseudo-haplogroups results in erroneous inferences and false positive findings (Fachal et al., 2015). This is because pseudo-haplogroups are not biologically meaningful; they do not share a set of variants characterising an exclusive phylogenetic branch. To demonstrate this, Fachal et al. (Fachal et al., 2015) artificially merged haplogroups U and V of a migraine cohort from Cantabria. This resulted in the UV pseudo-haplogroup being significantly associated with migraines ($P = 0.0268$) even though it did not make phylogenetic sense. Thus, emphasising the risk of false associations when pseudo-haplogroups are used.

In summary, PD haplogroup studies have reported inconsistent findings. Evidently, there are many methodological concerns around haplogroup studies which may have influenced the findings of past studies. These concerns should carefully be addressed in future.

Table 2
Summary of mtDNA haplogroup association studies in Parkinson's disease

Ethnicity (ancestry)	Study participants	N	Multiple-test correction	Estimates of statistical power determined	Outcome/ Main findings	Reference
American (European)	Cases Controls	609 340	NS	NS	Haplogroups J (P = 0,02) and K (P = 0,02) associated with reduced PD risk. SNP m.10398A > G was reported to be protective against PD (P = 0,0001). SNP m.9055G > A in *MT-ATP6* reduced PD risk in women (P = 0,03). SNP m.13708G > A reduced PD risk in individuals > 70 years of age (P = 0,010).	Van Der Walt et al. (2003)
Irish (European)	Cases Controls	90 129	Yes (Bonferroni correction)	NS	No significant association between haplogroup J and PD found. SNP m.4216T > C, in linkage with mtDNA TJ cluster, was associated with increased PD risk (P = 0,014).	Ross et al.(2003)
Finnish (European)	Cases (PD) Cases (PDD) Controls	210 28 104	NS	NS	Supercluster JTWIX was associated with an increased risk of PD (P = 0,27) and PD with dementia (PDD) (P = 0,18).	Autere et al.(2004)
Spanish from Asturias, Northern Spain (European)	Cases Controls	271 230	Yes (Bonferroni correction)	NS	No Haplogroups were associated with PD risk. SNP m.4336T > C associated with increased PD risk only in females (P = 0,011). SNP m.10398A > G was protective against PD (P = 0,009).	Huerta et al.(2005)
English (European)	Cases Control group 1 Control group 2 (Birth cohort) Control group 3 (Post-mortem brain tissue from AD patients)	455 269 178 185	NS	NS	Haplogroups J and K not significantly associated with reduced PD risk. UKJT cluster reduced PD risk (P < 0,0001).	Pyle et al. (2005)
Italian (European)	Cases Control group 1 (CT1) Control group 2 (CT2)	620 1486 509	NS	NS	Haplogroup K (P = 0,048) but not J associated with reduced PD risk. SNP m.10398A > G did not significantly alter PD risk.	Ghezzi et al.(2005)
Taiwanese (Asian)	Cases Controls	416 372	Yes (Bonferroni correction)	NS	No haplogroups associated with PD risk.	Chen et al.(2007)
Polish from central, South and North-Western Poland (European)	Cases Controls Cases for gender stratified haplogroup J analysis Controls Additional cases for haplogroup K analysis Additional controls for haplogroup K analysis	241 277 304 316 91 137	NS	Yes	Haplogroup J (P = 0,0014) associated with reduced PD risk in males (after stratification by gender). Sub-lineages U4 +U5a1 + K+J1c + J2 also reduced PD risk (P = 0,027). SNP m.10398A > G did not significantly alter PD risk.	GawedaWalerych et al. (2008)
Greek from Crete (European)	Cases Controls	224 383	NS	NS	No haplogroups associated with PD risk. SNP m.10398A > G did not significantly alter PD risk.	Latsoudis et al.(2008)
Russian, Tatar (European)	Cases Controls	157 183	NS	NS	Haplogroup H associated with an increased PD risk (P = 0,0001). UK cluster associated with a decreased PD risk (P = 0,003).	Khusnutdinova et al. (2008)
Australian from New South Wales and Queensland (European)	Cases Controls	890 3491	NS	Yes	No significant associations between PD risk and haplogroups J and K found, nor the pooled UJKT haplogroup cluster.	Mehta et al. (2009)
English (European)	Cases (Discovery phase) Controls (Discovery phase) Cases (Replication phase) Controls (Replication phase) Cases (Meta-Analysis) Controls (Meta-Analysis)	1719 2889 851 2717 6140 13280	NS	Yes	**Association study**: No association between haplogroups and PD nor SNP m.10398A > G and PD. Super-haplogroup JT was associated with a protective effect against PD (P = 0,0354). Mitochondrial variants m.2158T > C (discovery: P = 0,024; replication: P = 0,0245) and m.11251A > G (discovery: P = 0,0292; replication: P = 0,0012) were associated with a reduced risk of PD in both the discovery and replication cohorts. **Meta-analysis**: Haplogroups J (P = 0,0122), K (P = 0,00363), T (P = 0,0245) and super-haplogroup JT (P = 0,000584) associated with reduced PD risk. Increased PD risk was associated with cluster HV (P = 0,00364).	Hudson et al. (2013)
Spanish from Pamplona, North-East Spain (European) Spanish from Santiago de	Cases Controls Cases Controls	478 394 305 293	Yes (NS)	NS	No haplogroups were associated with PD risk	Fachal et al. (2015)

(*continued on next page*)

Table 2 (*continued*)

Ethnicity (ancestry)	Study participants	N	Multiple-test correction	Estimates of statistical power determined	Outcome/ Main findings	Reference
Compostela, North-West Spain (European)						
Han Chinese from Southern China (Asian)	Cases (Total) Cases EOPD (< 50 years of age) Cases LOPD (> 50 years of age) Controls (Total) Control team 1 Control team 2	279 63 216 510 118 392	NS	NS	Overall, no association between haplogroups and PD. Haplogroup B (P = 0,004) associated with a lower risk for EOPD in individuals younger than 50 years (after age stratification,). Haplogroup D associated with a higher risk of PD (P = 0,033) and Haplogroup B was associated with a lower risk of PD (P = 0,018) in individuals younger than 50.	Chen et al.(2015)
Han Chinese from Northern China (Asian)	Cases Controls	322 332	Yes (Bonferroni correction)	NS	SNP m.10398A > G (P = 0,001) significantly associated with increased PD risk in females (P = 0,0036).	Chu et al.(2015)
Taiwanese (Asian)	Cases Controls	725 744	Yes (Bonferroni correction)	NS	Haplogroup B5 associated with a reduced PD risk (P = 0,002).	Liou et al. (2016)
Greek from Cypriot (European)	Cases Controls	230 457	Yes (Bonferroni correction)	NS	Haplogroup U associated with reduced PD risk (P =0,03), supercluster LMN (P = 0,01) and cluster N(xR) (P = 0,006) were significantly protective against PD in females.	Georgiou et al.(2017)
Han Chinese from East China (Asian)	Cases Controls	500 505	NS	NS	**D-loop-sequencing**: SNPs m.151T > C (P = 0,023), m.189G > A (P = 0,03), m.16086C > T (P = 0,007 and m.16271C > T (P = 0,0497) were associated with increased PD risk. SNPs m.318C > T and m.16134T > C (P = 0,022) were associated with decreased PD risk. Haplogroup A5 (P = 0,039) was associated with increased PD risk. Haplogroup B5 (P = 0,068) was associated with a reduced disease risk. **Meta-analysis**: Haplogroup B5, but not B4, was protective against PD (P = 0,0003). Haplogroup G not associated with PD (P = 0,09).	Wu et al. (2018)

AD = Alzheimer's disease; EOPD = Early onset Parkinson's disease; LOPD = Late onset Parkinson's disease; mtDNA = Mitochondrial DNA; N = sample size; NA = Not applicable; NS = Not specified; PDD = Parkinson's disease with dementia; PD = Parkinson's disease

5. Somatic mtDNA changes in PD

Somatic mtDNA variation, including point mutations and deletions, accumulate over time in post-mitotic tissue, including the ageing human brain (Lin et al., 2002; Bender et al., 2006; Arnheim and Cortopassi, 1992). This gives rise to a mixed population of mutant and wild-type mtDNA molecules i.e. heteroplasmy. The acquired variants may additionally clonally expand in individual cells through relaxed replication and random intracellular drift (Elson et al., 2001). During relaxed replication, mtDNA molecules are randomly selected for replication. Thus, the proportion of mtDNA heteroplasmy in a cell can significantly increase over time if mutant mtDNA molecules are replicated more frequently by chance than the wild-type mitochondrial genomes (Elson et al., 2001). When the proportion of mutant mtDNA molecules with harmful mtDNA changes exceeds a critical threshold level, a cellular defect in OXPHOS will arise (Chinnery and Hudson, 2013). Consequently, a number of studies have investigated high levels of heteroplasmic mtDNA point mutations and mtDNA deletions in the post-mortem brain tissue of PD patients. Both deletions and point mutations in these studies were considered somatic rather than germline.

5.1. Somatic mtDNA point mutations and deletions

With the advent of sequencing technologies sensitive enough to detect low-level heteroplasmies (< 10%), a handful of studies sequenced mtDNA in PD cases and controls extracted from post-mortem brains (Table 3 and Supplementary Table 3). They hypothesised that multiple, individually rare mtDNA point mutations in either the entire mitochondrial genome, or subset of mtDNA genes, could collectively constitute a high variant burden in the brains of PD patients. This burden would ultimately lead to neuronal loss. However, only two studies reported significantly higher burdens of point mutations in the brains of PD cases compared to controls (Coxhead et al., 2016; Lin et al., 2012). Others reported no significant difference between case and control groups (Table 3 and Supplementary Table 3) (Smigrodzki et al., 2004; Dölle et al., 2016; Simon et al., 2004; Wei et al., 2017).

In addition, acquired mtDNA deletions have also been suggested to play an important part in the selective neuronal loss in the ageing and PD brain (Bender et al., 2006; Kraytsberg et al., 2006; Reeve et al., 2008; Nido et al., 2018). Mechanisms, such as breakpoints in tandem repeats of mtDNA or replication errors (Nissanka et al., 2019) give rise to these deletions (Reeve et al., 2008; Nido et al., 2018). Notably, DA metabolism has been shown to drive the generation of mtDNA deletions in *in vitro* and *in vivo* models (Neuhaus et al., 2013). DA is readily oxidised, leading to ROS and neurotoxic quinone production (Halliwell and Gutteridge, 1984). ROS, such as H_2O_2, have been shown to cause single and double stranded breaks in mtDNA *in vitro* (Shokolenko et al., 2009). As such, SNpc DA neurons appear to be particularly susceptible to accumulation of mtDNA deletions and most of these deletions are located between the heavy and light strand origins of replication (the major arc; Fig. 1) (Bender et al., 2008).

The 'common deletion' is a 4977-base-pair deletion located between the *MT-ATP8* and *MT-ND5* genes, spanning four genes coding for complex I (*MT-ND3*, *MT-ND4*, *MT-ND4L* and *MT-ND5*) (Fig. 1). It is thought to bring about a complex I defect, thus an energy crisis (Ikebe et al., 1990). The frequency of mtDNA carrying this deletion in PD cases compared to controls has been investigated using Southern blotting, competitive- and kinetic-PCR techniques as well as *in situ* hybridisation techniques (Ikebe et al., 1990; Kösel et al., 1997; Mann et al., 1992; Zhang et al., 2002; Ozawa et al., 1990). Despite two studies reporting a greater burden of this deletion in patients compared to controls (Ikebe et al., 1990; Ozawa et al., 1990), others could not replicate these findings (Kösel et al., 1997; Mann et al., 1992; Zhang et al., 2002). Instead, they reported no difference between cases and controls, suggesting that the common deletion may be a result of the natural ageing process rather than disease pathogenesis. Gu et al. (Gu et al., 2002) later reported that the number of mtDNA deletions (not limited to the common deletion) was increased significantly in the SN homogenate of PD patients compared to age-matched controls, patients with multiple system atrophy, Dementia with Lewy Bodies, and those with Alzheimer's disease.

Additional studies compared the total burden of mtDNA carrying deletions in the major arc, between brains of PD cases and controls using quantitative PCR (qPCR) (Bender et al., 2006; Dölle et al., 2016; Grünewald et al., 2016; Bury et al., 2017). Such qPCR methods typically measure the ratio of a gene not commonly deleted (e.g. *MT-ND1*) in mtDNA to one which is frequently deleted (e.g. *MT-ND4*) using either relative or absolute quantification methods. The latter requiring a standard curve. Two studies reported significantly higher burdens of mtDNA deletions in individual DA SNpc ($P = 0.004$) (Dölle et al., 2016) and pedunculopontine cholinergic neurons ($P < 1 \times 10^{-4}$) (Bury et al., 2017) of PD cases compared to controls. The others reported no significant differences in deletion levels between SN neurons of cases and aged control groups (Bender et al., 2006; Grünewald et al., 2016).

Based on the evidence outlined above, it is reasonable to argue for or against a role of somatic point mutations and/or deletions in PD pathogenesis. However, variability between study designs (but also small sample sizes) makes it difficult to compare findings across studies and makes it challenging to draw a reliable conclusion regarding the contribution of somatic mtDNA deletions and point mutations to PD.

5.2. Changes in mtDNA copy number

In addition to possible point mutations and deletions in mtDNA molecules, mtDNA depletion should also be considered as a possible predisposing factor for PD. The importance of adequate mtDNA in cells is highlighted by mtDNA depletion syndromes – a group of autosomal recessive disorders characterised by severe mtDNA depletion which results in impaired energy production (Suomalainen and Isohanni, 2010). Since mtDNA encodes essential OXPHOS complex components, a sufficient amount of mtDNA is required for the production of these OXPHOS subunits and to manage cellular energy demands (El-Hattab and Scaglia, 2013). Therefore, there has also been growing interest in understanding mtDNA levels (mtDNA copy number) in PD.

Frequently, studies investigating mtDNA copy number have used qPCR methods to quantify the ratio of *MT-ND1* to a nuclear housekeeping gene. Recent studies have reported significant differences in mtDNA copy number values between PD patients and controls in the SN, with lower mtDNA copy numbers observed in the patients (Dölle et al., 2016; Grünewald et al., 2016; Pyle et al., 2016). Notably, in one study mtDNA copy number in DA neurons was observed to increase with age in controls (Dölle et al., 2016). Therefore, despite the age-related accumulation of mtDNA deletions, the pool of wild-type mtDNA was maintained. In contrast to the controls, no compensatory increase in mtDNA copy number was observed in patients, resulting in relative depletion of wild-type mtDNA in the SNpc. This was suggested to ultimately result in respiratory deficiency and neuronal loss. Bury et al. (Bury et al., 2017), who observed increased levels of both mtDNA deletions and mtDNA copy number in pedunculopontine cholinergic neurons of PD cases compared to controls, suggested that different neurochemical cell types and brain regions in PD patients may differ in response to mtDNA deletion accumulation. However, reasons for the lack of possible compensatory mechanisms in the SNpc of the PD brain remain unclear. Hence, further research is warranted on how mtDNA levels are regulated in different cell-types.

mtDNA depletion has also been recognised as a potential biomarker for PD detection because significant reductions in mtDNA copy number have not only been observed in the SN of PD patients (Dölle et al., 2016; Grünewald et al., 2016; Pyle et al., 2016) but also in peripheral blood (Pyle et al., 2016; Gui et al., 2015) and cerebrospinal fluid (Pyle et al., 2015). Overall, reports on mtDNA depletion in PD appear promising, although further research in this field is required, particularly with

Table 3
Summary of mtDNA sequencing studies on somatic variation in Parkinson's disease

Study participants	N	Brain region	Variants considered	Pathogenicity scoring	Method	mtDNA region sequenced	Outcome/ Main findings	Reference
Cases Controls (< 10 years old) Controls (12-24 years old) Controls (Elderly) Cases Controls (< 10 years old) Controls (12-24 years old) Controls (Elderly)	8 2 6 7 8 3 4 10	FCtx (Tissue homogenate) SN (Tissue homogenate)	All point mutations; G:C to T:A and T:A to G:C transversions	NS	PCR-cloning-sequencing strategy	*MT-ND4*	No significant difference in heteroplasmic point mutation burdens between cases and controls.	Simon et al. (2004)
Cases Controls	6 6	FCtx (Tissue homogenate)	Nonsynonymous point mutations	Yes	PCR-cloning-sequencing strategy	*MT-ND1*; *MT-ND2*; *MT-ND3*; *MT-ND4L*; *MT-ND4*; *MT-ND5*; *MT-ND6*	No significant difference in heteroplasmic point mutation burdens between cases and controls.	Smigrodzki et al. (2004)
Cases (Early PD + ILBD) Cases (Late PD) Controls	9 8 23	SN (Isolated dopaminergic neurons; Glial cells)	All point mutations; GT/CA transversions	NS	PCR-cloning-sequencing strategy	*MT-ND5*; D-loop	Significantly elevated levels of heteroplasmic point mutations in neurons of early PD + ILBD cases compared to controls (P < 0,0001) and late stage PD cases (P = 0,0006). No significant difference in mtDNA point mutation levels in SN glia from early PD + ILBD cases compared to controls (P = 0,73).	Lin et al. (2012)
Cases Controls Cases Controls	114 34 125 30	SN (Tissue homogenate) FCtx (Tissue homogenate)	All point mutations; nonsynonymous point mutations	Yes (MutPred scoring)	NGS (Illumina sequencing)	Whole mtDNA	Significantly higher heteroplasmic variant burden in the SN (P = 0,012) and FCtx (P = 0,005) of PD patients compared to controls.	Coxhead et al. (2016)
Cases Controls	10 10	SNpc (Single dopaminergic neurons-184 in total)	SNVs; transitions and transversions; deletions	NS	Ultra-deep NGS (Illumina sequencing)	Two rRNA (*MT-RNR1*, *MT-RNR2*); 10 tRNA (*MT-TV, MT-TL1, MT- TI, MT-TQ, MT-TM, MT-TW, MT-TA, MT-TN, MT-TC, MT-TY*); two Complex I genes (*MT-ND1*, *MT-ND2*)	Significantly higher levels of mtDNA deletions (P = 0,004), but not point mutations, in patients compared to controls. Significant mtDNA depletion in PD cases compared to controls (P = 0,006).	Dölle et al. (2016)
Cases (DLB-PD) Controls Controls (Young)	89 351 110	Cerebellum; Cerebral Cortex; Other brain regions (Tissue homogenate)	All point mutations; nonsynonymous point mutations	Yes (MutPred and Polyphen-2)	NGS (Illumina sequencing)	Whole mtDNA (Extracted from whole exome sequencing data)	No significant difference in heteroplasmic point mutation burdens between cases and controls.	Wei et al. (2017)

DLB-PD = Dementia with Lewy Bodies or Parkinson's Disease; FCtx = Frontal cortex; ILBD = Incidental Lewy body disease; mtDNA = Mitochondrial DNA; N = Sample size; NA = Not applicable; NGS = Next Generation Sequencing; NS = Not specified; SN = Substantia Nigra; SNVs = Single nucleotide variants; PD = Parkinson's disease

regards to the mechanisms underlying mtDNA depletion and its utility as a biomarker.

5.3. Challenges of studying somatic mtDNA changes in PD

Studying acquired mtDNA changes in PD is particularly challenging, rendering the role of mtDNA in PD unresolved. Many factors can substantially influence study findings and can contribute to inconsistent reports such as those detailed above. In the following section we highlight important methodological and biological factors to consider when studying somatic mtDNA changes in PD case-control studies.

5.3.1. Techniques and technologies

Technologically, many well-established molecular techniques used to study nDNA variation may not be suitable for the study of mtDNA. A review by Moraes et al. (Moraes et al., 2003) drew attention to techniques including Southern blotting, PCR amplification, and RFLP, and reviewed their respective advantages and disadvantages when analysing heteroplasmic mtDNA point mutations and deletions. The authors highlighted that these techniques can yield misinterpreted results for mtDNA given the genome's unique properties of heteroplasmy and polyploidy. Thus, the use of Southern blotting and different PCR techniques in earlier mtDNA deletion studies could account for inconsistent findings. For instance, Ikebe et al. (Ikebe et al., 1990) and Ozawa et al. (Ozawa et al., 1990), employed PCR and kinetic-PCR techniques respectively, and reported significantly higher mtDNA common deletion levels in cases than in controls. In contrast Mann et al. (Mann et al., 1992) and Kösel et al. (Kösel et al., 1997) who employed Southern blotting and competitive PCR techniques respectively found no significant increase of this deletion in cases compared to controls. Newer techniques used to quantify mtDNA deletions also have disadvantages. For instance, the relative quantification of levels of *MT-ND1* to *MT-ND4* fails to quantify rarer mtDNA deletions which extend into the minor arc and may not reflect the true extent of deleted mtDNA.

Moreover, the ability to confidently detect and quantify low levels of point mutation heteroplasmies in mtDNA varies between sequencing technologies (Pareek et al., 2011; Greaves et al., 2009). Some technologies such as Sanger sequencing are not sensitive enough to detect individually-rare mtDNA variants below 15% heteroplasmy, thereby potentially underestimating heteroplasmic variant levels (Zhang et al., 2012). Others, such as post-PCR cloning strategies, are at risk of overestimating heteroplasmic variant loads because of DNA polymerase enzyme transcriptional errors which are indistinguishable from true variants (Greaves et al., 2009). As a result, sequencing data generated using such technologies may not be truly representative of the heteroplasmic mtDNA levels in a sample and have likely contributed to some inconsistent findings between published studies (Table 3).

When employing newer, next generation sequencing approaches (NGS), sequencing depth affects the level of heteroplasmy detected (Ye et al., 2014b). This level varies between NGS approaches. Ultra-deep sequencing of the mtDNA, with a depth of several tens of thousands is required to confidently detect very low levels of heteroplasmies (~1–10%). Additionally, technical artefacts and sequencing errors need to be controlled for in NGS to achieve accuracy of quantification and detection specificity (Ye et al., 2014b). To control for these errors and artefacts, NGS studies should integrate effective quality-control criteria. Web-based servers such as mtDNA-Server (https://mtdna-server.uibk.ac.at) are available specifically for the analysis of mtDNA NGS data. The workflow includes several quality control metrics, identification of artefacts and contamination, heteroplasmy detection, as well as variant annotation (Weissensteiner et al., 2016). The use of such servers could aid in standardising heteroplasmy detection and quantification across studies.

Moreover, as mentioned, mtDNA in post-mitotic tissue is likely to accumulate deletions with age (Kraytsberg et al., 2006). Consequently, mtDNA amplification in aged, post-mortem brains, prior to sequencing, could also influence results of sequencing studies. For instance, amplifying mtDNA in two fragments which overlap the deletion-prone major arc may result in mtDNA sequence data which was unrepresentative of the entire mtDNA data population. This is because mtDNA carrying deletions in the primer binding sites may not be amplified given that primers may not bind and not amplify the mtDNA effectively.

Finally, predicting the functional impact of mtDNA variants in sequencing studies is important. This is because the levels of variation accumulating with age in controls could be similar to those accumulating in PD patients but may collectively be less pathogenic. It should be stressed that studies should use *in silico* tools specifically developed for mtDNA variation since those developed for nDNA may not be suitable to predict the pathogenicity of mtDNA variants (Bris et al., 2018). Examples of mtDNA tools include APOGEE (Castellana et al., 2017), MToolBox (Calabrese et al., 2014) and MitoTIP (Sonney et al., 2017). Tools like eKLIPse (Goudenège et al., 1407) are also available for identifying and quantifying mtDNA deletions (and other rearrangements) and should be considered for future studies.

5.3.2. Tissue and brain region

There are tissue specific differences in mtDNA heteroplasmy accumulation (Samuels et al., 2013) and 'hot-spot' regions of heteroplasmy accumulation in mtDNA (Li et al., 2010; Stoneking, 2000). As a result, the brain tissue and mtDNA region selected for examination in post-mortem studies will influence results. As detailed in Table 3, published studies did not all sequence the same mtDNA region, nor examined DNA from the same brain region, which could explain conflicting results. Although multiple regions of the brain are affected in PD, the loss of DA neurons in the pathologically affected SNpc is the most extensive (Brichta and Greengard, 2014). The SNpc, in comparison to other brain regions, may therefore be expected to show the greatest difference in mtDNA changes between cases and controls, should such changes contribute to neuronal loss in PD, as hypothesised.

Depending on cellular energy demand, the number of mitochondria per cell and the copies of mtDNA per mitochondria can differ substantially between cell types in whole tissue (Veltri et al., 1990). As such, whole blood samples frequently used for mtDNA copy number assays may be biased by cellular composition. For instance, samples with more granulocytes than lymphocytes, may show lower mtDNA copy numbers than ones with a higher proportion of lymphocytes (Pyle et al., 2010). This is because granulocytes have been observed to have fewer mitochondria, and thus less mtDNA, than lymphocytes (Pyle et al., 2010).

Moreover, mtDNA deletion and point mutation studies using tissue homogenate rather than single cells are also likely biased by the cellular composition of samples. mtDNA changes identified in tissue homogenate may not reflect the true changes in mtDNA between cases and controls. For instance, although both glial cells and neurons acquire mtDNA variants, somatic variants accumulate preferentially in neurons (Cantuti-Castelvetri et al., 2005). In support of this, Lin et al. (Lin et al., 2012) reported that overall levels of mtDNA point mutations were similar in SN glia of early PD and incidental Lewy body disease (ILBD) cases (Braak stage 3) and controls. However, they were significantly elevated in the single SN neurons of early PD + ILBD cases compared to controls (Table 3). As such, a homogenised tissue sample consisting of predominantly glial cells may have less mtDNA variation than one which consists predominantly of neuronal cells. Given that post-mortem brains of PD patients typically represent advanced stages of the disease and that up to 98% of DA SNpc neurons can be lost during advanced PD (Damier et al., 1999), mtDNA molecules examined in PD brain homogenate most likely originate primarily from surrounding glial cells.

Moreover, although studying individual cells is advantageous to avoid the bias of tissue composition, it can still be problematic. The surviving neurons in post-mortem brain tissue of patients with advanced PD may have accumulated fewer somatic mtDNA changes

allowing the neurons to persist. On the other hand, already degenerated neurons are likely to have accumulated very high levels of detrimental, somatic errors. Consequently, the surviving neurons of late-stage PD may not differ significantly to those of aged controls with regards to mtDNA changes. Therefore, the pathological stage of disease of the patients from whom the tissue samples originated needs to be accounted for (Simon et al., 2017). Examining neurons from early-stage (~Braak stage 3) post-mortem tissue may be the best strategy.

6. Future studies

To date the role of mtDNA in PD has not yet been resolved largely due to the difficulty of studying mtDNA variation but also 'poor' study design. However, there is potential for improvement in this field. Future studies need to consider the limitations of previous studies and the challenges associated with studying mtDNA to improve future study designs. For instance, small sample sizes are a significant limitation and can be overcome with new collaborations (Button et al., 2013). Studies should also consider using newer, more standardised approaches, such as the variant load approach, which potentially circumvent pitfalls of past investigations. Alternatively, future studies could branch into studying nDNA variation in conjunction with mtDNA changes. These and additional options are discussed below.

6.1. Homoplasmic mtDNA variation: considering functional studies and the collective role of rare population variants

Several authors have highlighted the methodological deficiencies of haplogroup association studies, some of which have also been outlined in this paper (Fachal et al., 2015; Herrnstadt and Howell, 2004; Raule et al., 2007). Yet, the methods used in these studies have not changed significantly over the past 20 years.

The current 'haplogroup association' approach uses only a few mtDNA SNPs. This makes it a cheap and relatively simple method to perform, that doesn't require more high throughput technologies such as whole exome or complete mitochondrial genome sequencing and avoids complex bioinformatics analyses. We suggest that results from future haplogroup studies should ideally be validated using functional investigations (e.g. cybrid or histochemical studies) (Giannoccaro et al., 2017) although this might be challenging given that association genetics is based on very subtle effects. Nonetheless, additional work assessing mitochondrial homeostasis regulation and function (e.g. measuring ROS production, cellular respiration etc.) can provide functional evidence to support the genetic associations made. For example, Liou et al. (Liou et al., 2016) were able to validate their findings using cybrids. In their study, cybrid results revealed that the B5 haplogroup cybrid, harbouring the m.8584G > A/m.10398A > G variants showed more resistance to rotenone than the B4 cybrid lacking these variants. Briefly, rotenone is a pesticide and a complex I inhibitor, implicated in PD pathogenesis (Betarbet et al., 2000). Furthermore, low ROS production and low apoptosis rates were also observed in the B5 cybrid (Liou et al., 2016), supporting the hypothesis that the B5 haplogroup variants have a protective effect against PD. Another recent study reported that haplogroup K1 increased mtDNA copy number and demonstrated a resistance to rotenone (Strobbe et al., 2018). These findings support haplogroup studies which associated haplogroup K with a PD protective effect (Ghezzi et al., 2005; Hudson et al., 2013), and highlight the value of such studies.

Although haplogroup association studies can be improved with such additional functional work, or by using replication cohorts, correcting for multiple testing, and avoiding pseudo-haplogroup construction, the current discrepancies between studies also highlight the need for newer and more standardised models.

Future studies should study homoplasmic, rare population variants which are mildly deleterious and could collectively contribute to disease risk in an individual, instead of only investigating haplogroup-defining variants in PD risk. This hypothesis, known as the variant load hypothesis, distances itself from the study of haplogroups (Pienaar et al., 2017). The most up-to-date version of the variant load hypothesis excludes variants predicted to be likely benign from the analysis, as many of these variants are common polymorphisms. Such common variants are more likely to be subject to population stratification, thus if left in the analysis they might be the cause of false positive associations. The variant load approach additionally condenses the likely impact of an individual's mtDNA variation into a numerical value on a continuous scale rather than in the form of a letter as done in haplogroup studies. Consequently, more powerful parametric statistics can be applied and fewer comparisons are needed, thus granting this approach greater statistical power with smaller sample sizes than the traditional haplogroup association method (Venter et al., 2017). For instance, a recent study with a total sample size of 82 employed the variant load approach to investigate the role of mtDNA in oxidative stress and inflammation (Venter et al., 2019). This study had 80% power to detect a correlation (with moderate effect size) between markers of oxidative stress and inflammation and variant loads. Given the decreasing cost of NGS and the growing availability of pipelines which simplify mtDNA data analyses, whole mtDNA sequencing data can be used to test the variant load hypothesis.

6.2. Somatic mtDNA variation and mtDNA depletion

Studying the role of somatic mtDNA variation in PD is challenging especially since the availability of PD post-mortem brains is limited and the pathological stage of disease may vary. Moreover, not all molecular techniques used to study nDNA variation are suitable for mtDNA given the unique features of mitochondrial genomes (polyploidy and heteroplasmy). Future studies should therefore carefully consider which molecular techniques would be most suitable for the purpose of their investigation to ensure results are reliable. Moreover, somatic mtDNA studies should correct for tissue cellular composition bias where possible. A combination of the strategies employed in the herein reviewed studies could be best to study somatic mtDNA variation in PD, particularly mtDNA point mutations. For instance, ultra-deep sequencing technologies could be used to sequence mtDNA molecules of individual, early-stage PD and control SNpc neurons.

mtDNA depletion in PD is an emerging biomarker of disease pathology and may be more promising to understand PD than somatic mtDNA variation given that mtDNA depletion studies have reported few discrepancies (Dölle et al., 2016; Grünewald et al., 2016; Pyle et al., 2015, 2016; Gui et al., 2015). We suggest future studies should try to replicate these reports of mtDNA depletion in PD but also correct for cellular composition when possible, to make results more reliable and reproducible (Guyatt et al., 2019).

Future studies should also place greater focus on studying nDNA variation, in combination with mtDNA changes, which may underlie potentially high mtDNA variant burdens in PD brains, and/or mtDNA depletion. For instance, Gui et al. (Gui et al., 2015) reported that PD patients with a particular *POLG1* genotype in combination with additional *POLG1* variants, had significantly lower numbers of mtDNA than those without such variants. This suggested that *POLG1* variation may contribute to mtDNA depletion in PD cases. *POLG1* encodes a subunit of the DNA polymerase which replicates mtDNA. mtDNA mutator mice with mutations in nDNA encoding mtDNA replication components, including *Polg1*, have additionally demonstrated an accelerated accumulation of mtDNA point mutations and deletions, highlighting a potential role for nDNA underlying mtDNA changes (Edgar et al., 2009; Trifunovic et al., 2004, 2005; Vermulst et al., 2008). As a result of these mtDNA changes, the mutator mice demonstrate a progressive respiratory chain dysfunction as seen in PD patients and premature aging phenotypes (e.g. hair loss, osteoporosis, and progressive hearing loss) (Trifunovic et al., 2004). These studies again implicate somatic mtDNA changes, resulting from nDNA mutations, in PD which has been

hypothesised to be a form of accelerated aging (Barbeau, 1984; Collier et al., 2017). Mitochondrial transcription factor A (TFAM) is an essential DNA-binding protein required for transcription and maintenance of mtDNA. Knockout mice with a deleted *Tfam* gene demonstrate a respiratory chain deficiency and reduced mtDNA expression in DA neurons (Ekstrand et al., 2007). These mice also exhibit parkinsonism typical phenotypes including progressive motor function impairment and DA neuronal loss (Ekstrand et al., 2007) implicating defective mtDNA maintenance in PD, brought about by nDNA changes. Notably, components of the nDNA-encoded, mtDNA replication machinery including mitochondrial genome maintenance exonuclease 1 (MGME1), POLG1, and mtDNA replicative helicase (TWINKLE) have recently been implicated in degradation of damaged mtDNA (Peeva et al., 2018). Thus, nDNA variation in genes encoding these factors is of particular interest for further study given their dual role mtDNA synthesis and degradation (Nissanka et al., 2019).

6.3. Mitochondrial-derived peptides (MDPs)

Recently, MDPs, encoded as sORFs, in mtDNA have been identified as important regulatory peptides of metabolic activity and stress response (Lee et al., 2013, 2015; Kim et al., 2017). The discovery of MDPs underscores the potential existence of more sORFs hidden in mtDNA. Recent findings have implicated the mitochondrial open reading frame of the 12S rRNA-c (MOTS-c), a 16-amino-acid MDP encoded within the *MT-RNR1* gene, in the regulation of nuclear gene expression in response to metabolic stress (Kim et al., 2018). These findings suggest that nuclear and mitochondrial genomes cross regulate each other but they also highlight the potential of MDP involvement in disease (Lee, 2019). Further work needs to be conducted to better understand the role of MDPs and the mechanisms regulating their expression in PD.

7. Concluding remarks

PD is an immensely complex disorder of multifactorial origin, but one with clear links to mitochondria and their genomes. Early evidence directly implicates mtDNA of sPD patients in altering mitochondrial structure and function. However, despite a multitude of studies investigating mtDNA in PD, the contribution of the mitochondrial genome to this disorder remains elusive.

To date, studies investigating homoplasmic and heteroplasmic mtDNA changes in PD have produced conflicting results. In almost all of these studies, low statistical power resulting from small sample sizes is a significant limitation, which likely contributed to some discrepant findings. Most mtDNA haplogroup association studies additionally suffered from methodological deficiencies (e.g. population stratification, lack of-replication cohorts and correction for multiple testing) which could primarily contribute to such discrepancies. On the other hand, innate difficulties of studying somatic mtDNA changes (e.g. heteroplasmy and tissue specific differences in mtDNA) along with different methodological techniques applied have produced conflicting results in somatic mtDNA studies which either provide evidence for or against a role of somatic mtDNA changes in PD.

Consequently, a role for the involvement of mtDNA in PD cannot at this stage be ruled out and needs to be further investigated in a more critical and systematic way. Future studies should take into account limitations of past studies reviewed here to improve current study designs. Alternatively, they should consider the use of more standardised approaches such as the variant load approach. The limitations, challenges, and future prospects of studying mtDNA in PD are summarised in Supplementary Fig. 1. Although not caveat-free, the study of mtDNA depletion appears most promising and we propose that further research in this area is required, particularly regarding the mechanisms and possible nDNA variation underlying mtDNA depletion in PD patients.

Albeit small, relative to the nuclear genome, the mitochondrial genome is an integral component for the production and functioning of mitochondria, and overall eukaryotic cell homeostasis. The role of mtDNA therefore should not be underestimated, and future adequately powered and well-designed studies exploring current (mtDNA variation and levels) and new avenues (including MDPs) are needed to fully assess the magnitude of its contribution to PD pathogenesis.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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