Circulating Cell Free Mitochondrial DNA Copy Number Variation in Neurodegenerative Disease

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Abstract

Circulating cell free nuclear DNA (ccf-nDNA) is a widely observed phenomenon that has proved useful in non-invasive prenatal screening and as a potential biomarker of cancer. Circulating cell free mitochondrial DNA (ccf-mtDNA) has subsequently been identified, which has prompted a new wave of research to assess if this also has predictive ability in disease predisposition or progression. Previous studies of ccf-mtDNA in Alzheimer's disease (AD) and pilot work in our lab investigating ccf-mtDNA and Parkinson's disease (PD), suggest that ccf-mtDNA may indeed serve as a biological marker of neurodegenerative disease (NDD). Given the rising prevalence of NDD in our aged population and the limited accuracy of current clinical tests of NDDs considerable effort has been placed into the identification and validation of novel fluid based biomarkers of NDD.

With this in mind, I aimed to assess the utility of ccf-mtDNA as a biomarker for NDD as well as increase our understanding of the mechanism of ccf-mtDNA release. To achieve this I have used three, large, CSF cohorts: a diverse cohort of NDDs, a cohort of progressive multiple sclerosis (PMS) and a large-scale longitudinal study of PD. Ccf-mtDNA levels were assessed using qPCR and ccf-mtDNA integrity was assessed using both qPCR and next-generation-sequencing. Ccf-mtDNA levels and integrity were compared between cases and controls, correlating results to clinical and neuropathological measurements of disease as well as both nuclear and mitochondrially encoded protein levels.

Similar to previous work, we found significant differences in the abundance but not integrity of ccf-mtDNA between patients and controls, particularly in PMS patients. Large-scale replication of ccf-mtDNA in PD revealed that, although ccf-mtDNA levels were indeed lower in cases compared to controls, this was significantly modulated by a number of confounding factors such as treatment, treatment duration and comorbidity.

Whilst we conclude that ccf-mtDNA levels are unlikely a viable biomarker for NDD, this work has highlighted important considerations relating to the heterogeneity of ccf-mtDNA abundance and its utility as a biological marker in disease. Additionally, this work has revealed a novel interaction between therapeutic interaction and the release of ccf-mtDNA, which may expand our understanding of disease mechanism and serve as an independent measure of therapeutic efficacy in future trials.

This thesis is dedicated to my wonderful Grandad, William Lowes, the kindest, most humble human to grace this earth.

You will always be our sunshine.

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Authors Declaration

This thesis is submitted for the degree of Doctor of Philosophy at Newcastle University. I, Hannah Lowes, declare that the work described here is my own, unless where clearly acknowledged and stated otherwise. I certify that I have not submitted any of the material in this thesis for a degree qualification at this or any other university.

Hannah Lowes

List of publications

Lowes, H., Pyle, A., Duddy, M., Hudson, G. (2018) Cell-free mitochondrial DNA in progressive multiple sclerosis. Mitochondrion., S1567-7249(18)30101.

Burté F, Houghton D, **Lowes H**, Pyle A, Nesbitt S, Yarnall A, Yu-Wai-Man P, Burn DJ3, Santibanez-Koref M, Hudson G. (2017) Metabolic profiling of Parkinson's disease and mild cognitive impairment. Movement Disorders Jun;32(6):927-932.

Pyle A, **Lowes H**, Brennan R, Kurzawa-Akanbi M, Yarnall A, Burn D, Hudson G. (2016) Reduced mitochondrial DNA is not a biomarker of depression in Parkinson's disease. Movement Disorders Dec;31(12):1923-1924

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Chapter 1. Introduction

1.1 Mitochondria

1.1.1 Organisation and Function

Mitochondria are essential intracellular organelles that function to supply eukaryotic cells with adenosine triphosphate (ATP) through aerobic respiration. They are thought to have originated more than two billion years ago as a product of symbiosis between primitive aerobic alpha-proteobacterium and primordial anaerobic eukaryotic progenitor cells (Koonin, 2010; Stewart and Chinnery, 2015) according to the endosymbiotic theory proposed in the 1970's by *Dr Lynn Margulis (Margulis, 1975)*. This bacterial ancestry is evidenced in the evolved mammalian mitochondrion structure, comprised of an outer and inner structural membrane (OMM and IMM, respectively), an intermembrane space (IMS) and its own circular genome, the mitochondrial DNA (mtDNA) (Perier and Vila, 2012).

The OMM is a phospholipid bilayer, containing channel forming proteins called porins, which allow the bidirectional transportation and diffusion of small molecules (<10kDa). The IMM is the site of the oxidative phosphorylation system (OXPHOS), or the respiratory chain, which is made up of four protein complexes (NADH dehydrogenase, succinate dehydrogenase, cytochrome b, and cytochrome c oxidase) and an ATP synthase (regarded as the fifth complex), encoded by the mtDNA. Besides its primary function of energy production, the mitochondrion also regulates numerous metabolic processes such as the citric acid cycle, steroid and haem synthesis and β-oxidation of fatty acids, as well as regulation of apoptosis and reactive oxygen species generation (Chinnery and Hudson, 2013).

1.1.2 Mitochondrial genome

The mitochondria are distinctive because they contain their own double-stranded DNA (mtDNA); a circular molecule approximately 16.6Kb in length in humans (Schapira, 2006) that encodes 37 genes. Of these genes, 28 are localised to the Heavy-strand (H) and nine are on the Light-strand (L) (Chinnery and Hudson, 2013). The H-strand codes for two ribosomal RNA (rRNA) molecules: 12S and 16S, 14 transfer RNA (tRNA) molecules and 12 polypeptides, whilst the L-strand encodes just eight tRNA molecules and one single polypeptide (Taanman, 1999). All 13 polypeptides are components of the respiratory chain complexes. Seven are subunits of NADH dehydrogenase or complex I (*ND1, 2, 3, 4, 4L, 5 and 6*), three are subunits

of complex IV/cytochrome c oxidase (Cyt c oxidase I, II and III), two are subunits of complex V (A6 and A8) and one is a subunit of complex III (Cytochrome b) (Schon *et al.*, 2012; Alexeyev *et al.*, 2013).

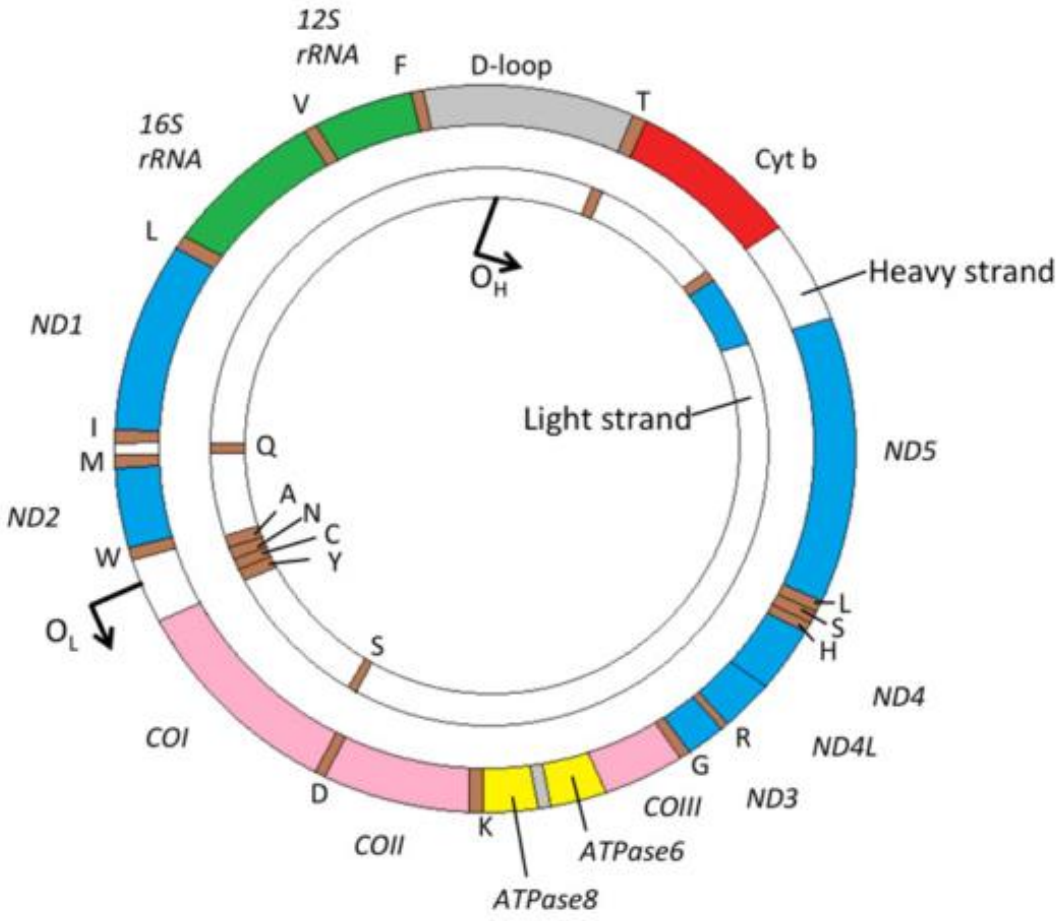

Figure 1.1. The mammalian mitochondrial genome, taken from Keogh and Chinnery (2015). Inner ring is the light strand, while the outer ring is heavy strand. D-loop, the major non-coding region is shown in grey. Genes encoding complex I subunits in blue, complex III in red, complex IV in pink, and ATP synthase in yellow. The two ribosomal RNA genes are shown in green, and letters denote the 22 tRNA genes. OH and OL denote the origins of heavy and light strand synthesis.

Compared to nuclear DNA (nDNA), the mtDNA is extremely efficient with >90% representing a coding region. The only significant non-coding region is the D-loop (shown as the grey section in Figure 1.1), consisting of two hypervariable regions: the control region (the site of interaction for nuclear-encoded transcription and replication initiation factors) and the origin of replication for the H-strand (O_H).

Furthermore, the mtDNA is exclusively maternally inherited and hence provides a 'molecular record' of human migration which can be traced back ~200,000 years to the 'Mitochondrial Eve'. Additionally, unlike nDNA the mtDNA lacks introns, histones and efficient DNA repair mechanisms, hence is far more susceptible to genetic mutation and often presents with far higher mutational burden (Taylor and Turnbull, 2005; Kumar *et al.*, 2009). A number of these mutations are hereditary, benign, single-nucleotide polymorphisms (SNPs) which can be phylogenetically analysed to categorise individuals into particular mtDNA haplogroups. Overall, 18 major haplogroups have been identified in the human mtDNA, nine of which (H, U, J, T, K, W, I, V, X) represent European ancestry. Other mutations are deleterious and can cause disease or dysfunction. For example, point mutations or deletions resulting in a change in the amino acid (AA) sequence or removal of part of a gene encoding a subunit of complex I can result in respiratory dysfunction.

As mtDNA is polyploid, the copies present within one cell can range between 100-100,000 depending on energy demand (Chinnery and Hudson, 2013). Tissues and cells with high energy demand such as brain, heart and neurons possess markedly higher mtDNA copies than those with lower energy demands (Kelly *et al.*, 2012). In addition to cellular and tissue-specific variation in mtDNA copy number, the presence of diseases and processes associated with aging can also affect mtDNA copy number. For example, conditions such as Parkinson's disease (Pyle *et al.*, 2015a; Dölle *et al.*, 2016; Grunewald *et al.*, 2016), cardiomyopathy (Ito *et al.*, 1992; Arbustini *et al.*, 1998; Huang *et al.*, 2016; Zhang *et al.*, 2017) and multiple sclerosis (Blokhin *et al.*, 2008) have been linked to mtDNA copy number depletion, whilst overall loss of mtDNA copy number control associates with aging (Clay Montier *et al.*, 2009). Additionally, due to this polyploidicity and mutational vulnerability, the mtDNA copies within a cell are not always identical. If only a proportion of the mitochondrial genomes develop a mutation, generating two sub-populations of mtDNA i.e. mutant and wild type, this is referred to as heteroplasmy and is expressed as a percentage of mutant to wild type copies. If all mtDNA genomes within the cell are identical this is called homoplasmy. This concept is particularly important when considering mtDNA mutations that cause disease, as heteroplasmic mutations often have to exceed a certain 'threshold level' of mutational burden before signs of biochemical dysfunction or clinical phenotypes are observed (Taylor and Turnbull, 2005).

1.1.3 Mitochondrial DNA copy number regulation

Currently, the overall mechanism controlling mtDNA replication and turnover (i.e. copy number) is unknown, however, it is well established that it does not coincide with the cell cycle and it occurs completely independently of nuclear DNA replication (Bogenhagen and Clayton, 1977). Despite this, a number of nuclear-encoded factors have been identified to be involved in mtDNA replication and maintenance, including, DNA polymerase γ (POLG), mitochondrial single-stranded DNA-binding protein (mtSSBP), Twinkle, and the mitochondrial transcription factor A (TFAM).

POLG is the only known DNA polymerase that localises within the mitochondria. It consists of three subunits; one catalytic subunit POLGA, which is essential for DNA repair and proofreading, and two POLGB accessory subunits, required for enzymatic stability (Kelly *et al.*, 2012). POLG is essential for mtDNA replication and maintenance, evidenced by pathological mutations in *PolgA,* which cause respiratory chain deficiencies and premature ageing (Stumpf and Copeland, 2011) and ablation studies, which show reduced mtDNA copy number after knock down of *PolgA (Hance et al., 2005)*. The other three factors are important at specific stages in mtDNA replication. TFAM, an essential component in the packaging of mtDNA into nucleoid structures, is necessary for mtDNA transcription and replication initiation (Clay Montier *et al.*, 2009) and total TFAM levels have been shown to be directly proportional to the amount of mtDNA (Ekstrand *et al.*, 2004). Whereas MtSSBP and Twinkle are important for helix destabilisation and POLG fidelity in mtDNA replication (Clay Montier *et al.*, 2009).

Whilst the exact mechanism of mtDNA copy number regulation is unclear, one theory has been postulated to explain how mtDNA copy number is maintained. The threshold theory hypothesises a model where mtDNA copy number is regulated by an upper and lower copy number limit (Figure 1.2). When mtDNA copy number falls below the lower threshold, this triggers an activation and upregulation of mtDNA replication factors to increase and restore mtDNA copy number levels, whereas if the upper threshold is exceeded, this triggers degradation factors which degrade the mtDNA and reduce mtDNA turnover to bring mtDNA copy number levels back down (Clay Montier *et al.*, 2009). However, currently, the ‘triggers’ of the replication and degradation machinery are unknown.

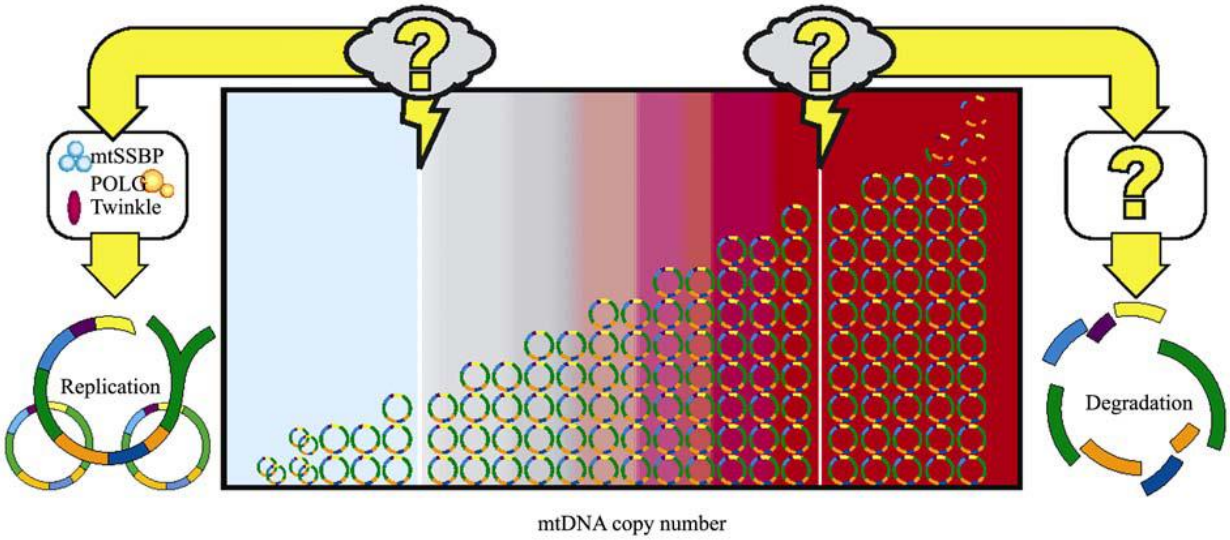

Figure 1.2. The threshold theory of mtDNA copy number regulation. Adapted from Clay Montier et al. (2009). Breaching of the lower threshold results in activation of replication machinery, whilst breaching of the upper threshold activates degradation machinery, thus mtDNA copy number is tightly regulated and maintained within these limits.

1.2 Cell-free DNA

1.2.1 Origin and function

Although the mtDNA is usually contained within the mitochondria of a cell, it is also identified to circulate extracellularly, coined circulating cell-free mtDNA (ccf-mtDNA). Circulating cell-free nucleic acids (ccf-nucleic acids) were first described in human serum in the late 1940's by Mandel and Metais (Mandel and Metais, 1948), initially discovering the presence of cell-free nuclear DNA (ccf-DNA), later followed by ccf-mtDNA (Zhong *et al.*, 2000). To date, subsequent research has focussed on the difference in the quantity of ccf-DNA and ccf-mtDNA levels in various body fluids from healthy individuals compared to disease states, particularly focussing on its potential diagnostic, prognostic and biomarker potential. However, despite huge research interest and the vast and relatively non-invasive availability of ccf-nucleic acids (ccf-DNA and ccf-mtDNA), the underlying biology has been greatly overlooked and current understanding regarding the origin and function is limited.
At present, there are two main contending theories to explain the origin and subsequent expulsion of cell-free nucleic acids into circulation; *(i)* cellular breakdown, and *(ii)* active DNA release (Aucamp *et al.*, 2018).

i) *Cellular breakdown*

Cellular breakdown mechanisms, including cell apoptosis, necrosis and autophagy have been implicated in triggering the release of DNA into extracellular fractions (Fournie *et al.*, 1995; Berezin, 2015), with supporting evidence from studies investigating both ccf-DNA and ccf-mtDNA. For example, ccf-DNA and ccf-mtDNA copy number in the plasma are found to correlate with the necrotic processes induced as a result of traumatic injury (Lo *et al.*, 2000; Lam *et al.*, 2004), invasive surgery (McIlroy *et al.*, 2015) and sepsis (Kung *et al.*, 2012; Di Caro *et al.*, 2016; Timmermans *et al.*, 2016) and could be valuable prognostic markers in these patients (Wang *et al.*, 2014; Gögenur *et al.*, 2017). Furthermore, cytotoxic compounds, including ionizing radiation and halogenated hydrocarbons causing cell apoptosis through direct or indirect (free radical production, oxygen starvation) processes, are also associated with ccf-nucleic acid level (Budnik *et al.*, 2013; Borghini *et al.*, 2015). This suggests that ccf-nucleic acids may be being expelled from lysing necrotic and apoptotic cells, and thus in theory, ccf-nucleic acid levels in circulation may directly

reflect the degree of cellular damage occurring as a result of trauma or toxin exposure.

Differently to ccf-DNA, and although ccf-mtDNA is associated with trauma, elevation of plasma ccf-mtDNA copy number does not appear to correlate with necrosis (McIlroy *et al.*, 2015) or cell apoptosis (Aucamp *et al.*, 2018). Alternatively in traumatic injury or surgical trauma, ccf-mtDNA is considered to serve as a damage-associated molecular pattern (DAMP) (Timmermans *et al.*, 2016; Gögenur *et al.*, 2017), important in activating the immune response and promoting inflammation after injury (Collins *et al.*, 2004; Zhang *et al.*, 2010; Nakayama and Otsu, 2018). This trauma associated ccf-mtDNA is possibly released from neutrophil extracellular traps (NETs); extracellular structures composed predominantly of mtDNA, generated from dying or dead cells (Yousefi *et al.*, 2009). These structures have been shown to release mtDNA into circulation, particularly in response to reactive oxygen species (ROS) rather than cell death, however the exact mechanism is currently unknown (Yousefi *et al.*, 2009; McIlroy *et al.*, 2014).

ii) *Active release*

Alternatively, ccf-nucleic acids may be actively secreted from living cells in a process relating to cell turnover or cell signalling, likely encapsulated in vesicles (microvesicles/exosomes/apoptotic bodies). Early *in vitro* work showed that living bacterial, plant and animal cells can spontaneously release DNA into extracellular spaces and culture medium (representative of circulation) (Rogers *et al.*, 1972; Stroun and Anker, 1972; Anker *et al.*, 1975; Melentijevic *et al.*, 2017), whereas dead cells cannot, suggesting an underlying active mechanism in ccf-nucleic acid release. Additionally, ccf-nucleic acids have been localised to extracellular vesicles in cell culture studies (Guescini *et al.*, 2010; Balaj *et al.*, 2011; Waldenstrom *et al.*, 2012; Fernando *et al.*, 2017; Wang *et al.*, 2017), supporting the notion that ccf-nucleic acids may act as signalling factors.

It has also been postulated that ccf-nucleic acids may be actively targeted for release as part of a quality control mechanism. Recent *in vitro* work has shown that mutant mtDNA is expelled from healthy neurons into culture medium, most likely in a protective process to maintain mitochondrial function and subsequent cell viability (Melentijevic *et al.*, 2017).

1.2.2 Clearance of ccf-nucleic acids

The clearance and degradation of ccf-nucleic acids is also poorly understood. Ccf-DNA is thought to be rapidly cleared from circulation, with studies showing complete clearance of foetal ccf-DNA from the mother's blood in less than 2 hours (Lo *et al.*, 1999). In addition ccf-DNA is vulnerable to degradation in the extracellular fluid due to DNase activity in lysosomes after phagocytosis (Manuelidis, 2011). Conversely, ccf-mtDNA appears to be more resistant to this degradation (Tepper and Studzinski, 1993; Foran, 2006; Manuelidis, 2011), and thus persists in circulation for longer than ccf-DNA.

1.2.3 Methods of quantifying ccf-nucleic acids

Quantitative PCR was originally introduced in 1996 (Heid *et al.*, 1996), and has since become the established method for determination of mtDNA and ccf-nucleic acid copy number. The principle of qPCR utilises a fluorescent reporter molecule to measure the amplification of a specific DNA amplicon in real time as the reaction progresses (Arya *et al.*, 2005). At the outset of the reaction, fluorescence remains at undetectable background levels, but eventually accumulates to yield a detectable signal. The cycle number at which this fluorescence signal is observed is called the quantification cycle (Cq) which is representative of the initial amount of template present. Essentially, samples with a large amount of template at the beginning of the reaction will require less cycles to accumulate a detectable signal, and thus a lower Cq value, whereas samples with low template will have a markedly higher Cq value.

Cellular mtDNA copy number determination is based on the relative proportion of a nuclear housekeeping gene (to account for cell count) to a mitochondrial reference gene (Malik *et al.*, 2011), typically a conserved gene in the minor deletion arc i.e. *MTND1* (Krishnan *et al.*, 2007; Pyle *et al.*, 2015b; Shen *et al.*, 2015), or D-loop region (Phillips *et al.*, 2014). Whereas determination of ccf-mtDNA copy number does not require this calculation. Instead, the absolute ccf-mtDNA copy number is calculated by taking the average quantity (Cq) of the mitochondrially encoded gene relative to the standard curve (Schmittgen and Livak, 2008) (Figure 1.3, A). In most cases a nuclear housekeeping gene will still be measured to assess cellular contamination (Pyle *et al.*, 2015b).

In addition, qPCR can be used to measure the level of deleted mtDNA within a sample, whereby the ratio of two mtDNA genes; one conserved i.e. *MTND1* (minor deletion arc) and one commonly deleted region i.e. *MTND4* (major deletion arc), is calculated to yield a deletion percentage level (Krishnan *et al.*, 2007; Grady *et al.*, 2014).

More recently, droplet digital PCR (ddPCR) has come into use for the measurement of ccf-mtDNA copy number. DdPCR technology utilises water-oil emulsion to partition nucleic acid samples into nanoliter-sized droplets within which, PCR amplification is then carried out (Hindson *et al.*, 2011) (Figure 1.3, B). Similarly to qPCR, specific nDNA or mtDNA amplicons are targeted but without the need for standard template curve comparison as DNA quantity is generated as copies per droplet, thus allowing detection of extremely low levels of DNA (Henrich *et al.*, 2012; Miotke *et al.*, 2014; Belmonte *et al.*, 2016). This greatly reduces costs, errors, and background noise compared to qPCR and increases the statistical power of copy number analysis, as a single sample can generate tens of thousands of data points using ddPCR rather than a single result generated in qPCR (Whale *et al.*, 2012; Hayden *et al.*, 2013).

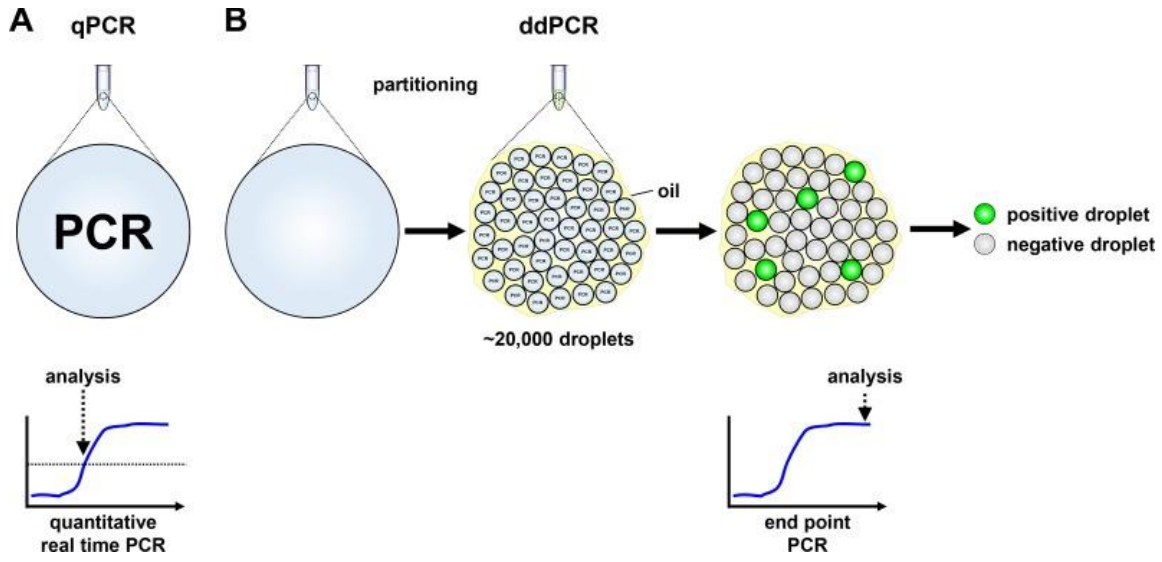

Figure 1.3. Schematic of quantitative polymerase chain reaction (qPCR) and droplet digital PCR (ddPCR). A) Signal detection in qPCR is calculated in a single reaction, measured in real-time from the exponential phase of the reaction and unknown copy number is extrapolated from a standard curve. B) Droplet digital PCR partitions a sample into thousands of individual qPCR reactions and measures the end point to determine absolute copy number from the proportion of reactions that contain the target molecule (positive) compared to those which do not (negative). Adapted from Lodrini et al. (2017).

1.2.4 Ccf-nucleic acids in disease

Following the initial discovery of ccf-nucleic acids in human body fluids (Mandel and Metais, 1948) numerous studies have demonstrated potential diagnostic and prognostic applications for this non-invasive phenomenon (Table 1.1).

i) Oncology and ccf-nucleic acids

Work by Leon *et al.* (1977) was the first to highlight the profound implication of ccf-nucleic acids in the field of oncology, demonstrating elevated levels of ccf-DNA in the serum of metastatic disease compared to non-metastatic patients and correlating this to poorer survival (Leon *et al.*, 1977). Subsequent work verified these initial findings and confirmed the tumoural origin of ccf-DNA (Stroun *et al.*, 1989; Sorenson *et al.*, 1994; Vasioukhin *et al.*, 1994). Since then, numerous studies have confirmed the existence of significant differences between ccf-DNA levels in the blood of healthy individuals compared to cancer patients (reviewed in Ziegler *et al.* (2002)), with more recent studies also implicating ccf-mtDNA (Mehra *et al.*, 2007; Ellinger *et al.*, 2008; Zachariah *et al.*, 2008; Ellinger *et al.*, 2009; Kohler *et al.*, 2009; Ellinger *et al.*, 2012; Yu *et al.*, 2012; Hou *et al.*, 2013; Mahmoud *et al.*, 2015; Lu *et al.*, 2016), Table 1.1). For example, ccf-DNA and ccf-mtDNA has been reported to be elevated in the blood of patients with epithelial ovarian cancer (Zachariah *et al.*, 2008), breast cancer (Mahmoud *et al.*, 2015), urological cancers (Mehra *et al.*, 2007; Ellinger *et al.*, 2008; Ellinger *et al.*, 2009; Ellinger *et al.*, 2012; Lu *et al.*, 2016) and lung cancer (Hou *et al.*, 2013) compared to healthy controls and individuals with benign tumours. Additionally, levels of ccf-nucleic acids were found to correlate to survival (Mehra *et al.*, 2007) and disease stage (Hou *et al.*, 2013; Mahmoud *et al.*, 2015).

Other forms of cancer have been associated with a reduction in ccf-mtDNA copy number i.e. hepatocellular cancer (Li *et al.*, 2016) and Ewing's sarcoma (Yu *et al.*, 2012). Despite these minor discrepancies, work on ccf-nucleic acids in oncology has indicated an accurate and specific prognostic and diagnostic ability of ccf-DNA and ccf-mtDNA. Subsequently, analysis of ccf-nucleic acids may soon be recommended into clinical use in cancer management to provide non-invasive analysis of tumour heterogeneity, cancer origin, disease burden, prognosis and response to treatment (reviewed in Stewart and Tsui (2018)).

ii) *Pregnancy and ccf-nucleic acids*

Alongside the emergence of diagnostic applications of ccf-nucleic acids in oncology, other research, recognising the pseudo-malignant nature of the placenta, demonstrated the presence of foetal ccf-DNA in the maternal blood (Lo *et al.*, 1997). The identification of this phenomenon presented a potential non-invasive usage of ccf-DNA in prenatal screening as an alternative to very invasive conventional methods i.e. amniocentesis and chorionic villious sampling (reviewed in Rafi and Chitty (2009) and Tsang and Dennis Lo (2007)). After decades of research, non-invasive prenatal testing (using foetal ccf-DNA) is now clinically recommended for the detection of common foetal trisomies, such as Downs syndrome (Committee Opinion No. 640., 2015; Norton *et al.*, 2015), in foetal sex determination (Honda *et al.*, 2002) and for the identification of pregnancies at high risk of rhesus disease by identifying foetal rhesus D status (Gautier *et al.*, 2005).

iii) *Other conditions and ccf-nucleic acids*

Ccf-nucleic acids have also been implicated as prognostic and diagnostic markers in a variety of other health conditions (Table 1.1, *Other Conditions*). As a prognostic marker, elevated ccf-DNA levels are indicative of tissue rejection following organ transplantation (Beck *et al.*, 2015; Bloom *et al.*, 2017; Schütz *et al.*, 2017); elevated ccf-mtDNA copy number indicates high risk of coronary heart disease in diabetes mellitus (Liu *et al.*, 2015; Liu *et al.*, 2016); and elevated ccf-mtDNA but reduced ccf-DNA indicate better survival after heart failure (Arnalich *et al.*, 2012; Dhondup *et al.*, 2016). Likewise, ccf-mtDNA levels in infectious diseases such as Hepatitis B and HIV relate to risk of secondary complications (Table 1.1). As a potential diagnostic marker, plasma ccf-DNA and ccf-mtDNA levels are observed to be elevated in stroke (Tsai *et al.*, 2011; Wang *et al.*, 2015), meningitis infection (Lu *et al.*, 2010) and heart attack (Wang *et al.*, 2015) compared to controls and can also indicate exposure to harmful carcinogens (Budnik *et al.*, 2013; Borghini *et al.*, 2015).

iv) *Neurodegenerative diseases and ccf-nucleic acids*

More recently, ccf-mtDNA copy number has been associated with neurological and neurodegenerative conditions (Table 1.1, *Neurodegenerative and Neurological conditions*), such as Parkinson's disease (PD) (Pyle *et al.*, 2015b; Podlesniy *et al.*, 2016b), Alzheimer's disease (AD) (Podlesniy *et al.*, 2013; Podlesniy *et al.*, 2016a; Cervera-Carles *et al.*, 2017) and Multiple Sclerosis (MS) (Varhaug *et al.*, 2017; Leurs

et al., 2018). Details of these studies are discussed in the respective disease sections titled ‘‘disease’ and ccf-mtDNA copy number’ (Sections 1.5.7, 1.6.6, 1.7.6). Due to the neurological nature of these diseases, ccf-mtDNA copy number is quantified from the cerebrospinal fluid (CSF) of the brain or spinal cord.

Condition Category	*Condition*	*Ccf-nucleic acid*	*Body Fluid*	*Method*	*Finding*	*Reference*
Pregnancy	Prenatal Testing	DNA (foetal)	Plasma	Various	Indicates foetal sex, trisomy, rhesus status and Mendelian diseases	(Tsang and Dennis Lo, 2007; Rafi and Chitty, 2009)
Cancer	Various	DNA (tumour)	Serum	Radiolabelled hybridisation	Ccf-DNA significantly higher in cancer compared to control	(Leon *et al.*, 1977)
	Breast	DNA (tumour) & mtDNA	Plasma	qPCR	Ccf-DNA significantly higher but ccf-mtDNA significantly lower in malignant and benign cancer compared to controls	(Kohler *et al.*, 2009)
					ccf-DNA and ccf-mtDNA significantly higher in cancer compared to control and associated with prognosis	(Mahmoud *et al.*, 2015)
	Ovarian	DNA (tumour) & mtDNA	Serum/ plasma	qPCR	Plasma but not serum ccf-DNA and ccf-mtDNA significantly higher in cancer compared to controls and benign tumour group	(Zachariah *et al.*, 2008)
	Urological	DNA (tumour) & mtDNA	Serum/ plasma	qPCR	Ccf-mtDNA significantly higher in malignancies compared to controls, ccf-DNA no different	(Mehra *et al.*, 2007; Ellinger *et al.*, 2008; Ellinger *et al.*, 2009; Ellinger *et al.*, 2012; Lu *et al.*, 2016)
	Ewing's Sarcoma	mtDNA	Serum	qPCR	Ccf-mtDNA significantly lower in cancer compared to controls	(Yu *et al.*, 2012)
	Lung	mtDNA	Serum	qPCR	Ccf-mtDNA significantly lower in cancer compared to controls	(Hou *et al.*, 2013)

Other Conditions	Organ transplant rejection	DNA	Plasma	ddPCR and qCPR	Ccf-DNA significantly higher in episodes of rejection	(Beck *et al.*, 2015; Bloom *et al.*, 2017; Schütz *et al.*, 2017)
	Hepatitis B	mtDNA	Serum	qPCR	Lower ccf-mtDNA indicated greater risk of hepatocellular carcinoma	(Li *et al.*, 2016)
	Meningitis	DNA & mtDNA	Plasma	qPCR	Ccf-DNA and ccf-mtDNA significantly higher in bacterial meningitis and correlated to measures of daily function	(Lu *et al.*, 2010)
	HIV	mtDNA	Plasma	qPCR	Ccf-mtDNA significantly higher in HIV and associated with inflammation	(Cossarizza *et al.*, 2011; Dai *et al.*, 2015; Arshad *et al.*, 2018)
		mtDNA	CSF	ddPCR	Ccf-mtDNA associated with viral load and neurocognitive impairment in HIV	(Perez-Santiago *et al.*, 2016; Mehta *et al.*, 2017)
	Trauma	DNA & mtDNA	Plasma	qPCR	Ccf-DNA and ccf-mtDNA significantly higher after trauma and correlated to prognosis	(Lo *et al.*, 2000; Lam *et al.*, 2003; Lam *et al.*, 2004; Gu *et al.*, 2013; Simmon *et al.*, 2013; Yamanouchi *et al.*, 2013; Timmermans *et al.*, 2016)
	Sepsis	DNA & mtDNA	Plasma	qPCR	Ccf-DNA and ccf-mtDNA significantly higher in sepsis patients and correlated to prognosis	(Kung *et al.*, 2012; Yamanouchi *et al.*, 2013; Di Caro *et al.*, 2016)
	Coronary Heart Disease	mtDNA	Plasma	qPCR	Higher ccf-mtDNA associated with greater risk of coronary heart disease in diabetes mellitus	(Liu *et al.*, 2015; Liu *et al.*, 2016)

	Myocardial Infarction	DNA & mtDNA	Plasma	qPCR	Ccf-DNA and ccf-mtDNA higher in acute myocardial infarction, but decrease after percutaneous coronary intervention	(Wang *et al.*, 2015)
	Heart Failure	DNA & mtDNA	Plasma	qPCR	Higher ccf-mtDNA and lower ccf-DNA indicates better survival after heart failure	(Arnalich *et al.*, 2012; Dhondup *et al.*, 2016)
	Stroke	DNA & mtDNA	Plasma	qPCR	Ccf-DNA and ccf-mtDNA higher after stoke compared to controls and correlate to neurological outcome	(Tsai *et al.*, 2011; Bustamante *et al.*, 2016)
			CSF	qPCR	Ccf-DNA and ccf-mtDNA higher after stoke compared to controls	(Wang *et al.*, 2013)
	Radiation	DNA & mtDNA	Serum	qPCR	DNA and mtDNA higher after occupational exposure to low level radiation	(Borghini *et al.*, 2015)
	Pesticides	mtDNA	Serum	qPCR	Ccf- mtDNA higher after exposure to halo-alkane pesticides	(Budnik *et al.*, 2013)
	Alzheimer's Disease	mtDNA	CSF	qPCR	Ccf-mtDNA significantly lower in preclinical AD and diagnosed AD compared to FTLD and controls	(Podlesniy *et al.*, 2013)
				ddPCR	Ccf-mtDNA lower compared to CJD and non-AD Dementia	(Podlesniy *et al.*, 2016a)
				ddPCR	Ccf-mtDNA higher in AD compared to controls	(Cervera-Carles *et al.*, 2017)

Neurodegenerative/ Neurological disease	Parkinson's Disease	mtDNA	CSF	qPCR	Ccf-mtDNA significantly lower in PD patients compared to controls	(Pyle *et al.*, 2015b)
				ddPCR	Ccf-mtDNA significantly higher in familial PD compared to idiopathic PD and asymptomatic mutation carriers	(Podlesniy *et al.*, 2016b)
	Relapsing Remitting MS	mtDNA	CSF	qPCR	Ccf-mtDNA significantly higher in RRMS compared to controls	(Varhaug *et al.*, 2017)
	Progressive MS	mtDNA	CSF	ddPCR	Ccf-mtDNA higher in PMS compared to non-inflammatory neurological disease controls	(Leurs *et al.*, 2018)

Table 1.1. Table lists a number of important studies that report a diagnostic or prognostic utility of circulating cell free nuclear DNA (ccf-DNA) or mitochondrial DNA (ccf-mtDNA) in numerous health conditions, analysed by quantitative PCR (qPCR) or droplet digital PCR (ddPCR) in human plasma, serum or cerebrospinal fluid (CSF).

1.3 Cerebrospinal Fluid

The cerebrospinal fluid (CSF) is the clear, colourless fluid that circulates around the ventricles of the brain (ventricular CSF) and within the cranial and spinal subarachnoid spaces (lumbar CSF) (Brinker *et al.*, 2014) (Figure 1.4). It is predominantly secreted from the choroid plexus and to a minor extent from ependyma, capillaries and brain interstitial fluid and is ultimately absorbed into the venous blood at the level of the arachnoid villi or into the lymphatic system along cranial nerves (Pollay, 2010). It is continually produced at a rate of 0.3-0.4ml min^{-1} and completely renews itself every six to eight hours through a complex filtering system (Brinker *et al.*, 2014). Its major function is hydromechanical protection of the brain and central nervous system. However, it also acts to maintain cranial pressure, regulate brain interstitial fluid homeostasis and remove unwanted metabolic waste products, antibodies and chemicals into the bloodstream.

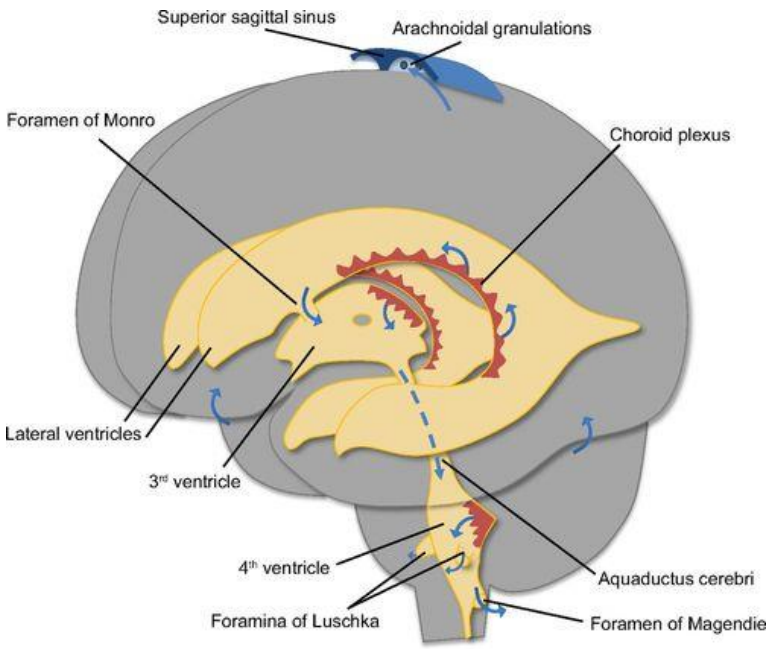

Figure 1.4. Schematic of the brain ventricular system, taken from Damkier et al. (2013). Brain parenchyma is grey, ventricles are yellow and choroid plexus is red. Blue arrows indicate the direction of flow of CSF.

The protein composition of the CSF varies depending on location (ventricular or lumbar), age, and health (Gerber *et al.*, 1998; Sommer *et al.*, 2002; Khoonsari *et al.*, 2016; Simon and Iliff, 2016), but is generally around 15 to 60 mg/dL. The majority of CSF proteins are derived from the blood, entering the CSF at the choroid plexus and circulating accordingly (Huhmer *et al.*, 2006), however, approximately 10% are believed to be directly synthesised and released by neurons in the brain. Accordingly, and due to the direct contact between the CSF and brain interstitial fluid/parenchyma (Khoonsari *et al.*, 2016), metabolic changes or pathological events arising from biochemical or cellular alterations in the brain can leave resultant biological markers in the CSF (Mollenhauer, 2014). For example, protein components of the neuropathological hallmarks (amyloid β plaques and neurofibrillary tangles) of Alzheimer's disease (AD) such as amyloid β, tau and phospho-tau can be detected within the CSF and can accurately distinguish AD patients from controls (Olsson *et al.*, 2016). Similarly, the widespread CNS inflammation that occurs in multiple sclerosis can be detected within the CSF by identification of elevated immunoglobulin or presence of multiple oligoclonal bands, and is now widely used in MS diagnosis (Polman *et al.*, 2011).

Therefore, CSF is believed to be the optimal fluid to discover novel biological markers of neurodegeneration and neuroinflammation, and research into biomarkers for neurodegenerative diseases (NDDs) has focused on this body fluid due to its proximity to the affected tissue site (Jeromin and Bowser, 2017).

1.4 Neurodegenerative Disease

Neurodegenerative diseases (NDD) comprise a diverse group of incurable nervous system disorders characterised by progressive and selective neuronal death and synaptic loss (Lin and Beal, 2006). The resulting phenotypes are specific to the affected population of neurons and brain regions they innervate, typically beginning as mild manifestations of disease, such as discoordination and mild memory loss, eventually progressing to severely disabling and debilitating conditions of cognitive and behavioural impairments and chronic motor dysfunction. The aetiology of NDD is particular complex. Risk factors are multifactorial, related to environment (Brown *et al.*, 2005), genetics (Bertram and Tanzi, 2005) and aging (Wyss-Coray, 2016) and biomarkers are limited in their reliability, specificity and utility, as they generally relate to post-symptomatic disease (reviewed in Rachakonda *et al.* (2004)). Due to this, diagnosis largely relies on the presentation of clinical phenotypes or post-mortem neuropathological exam and current treatments are targeted towards alleviation of symptoms rather than disease modification.

In an increasingly aged population, this lack of pre-symptomatic diagnosis and disease management presents NDD as a huge financial, societal and emotional burden. The global population aged 60 and old is expected to more than double by 2050, reaching almost 2.1 billion people (over one quarter of the global population) (Wyss-Coray, 2016) and as aging is the major risk factor for NDD (Niccoli and Partridge, 2012), this increasing age is paralleled with an increasing prevalence of NDDs. For example, the prevalence of Alzheimer's Disease; the most common NDD, is expected to triple by 2050. Thus, tremendous effort has been made to identify more accurate methods for early stage diagnosis, particularly focussing on the discovery of specific neuropathological, biochemical and genetic biomarkers relating to the onset or progression of disease or response to treatment (Rachakonda *et al.*, 2004).

1.5 Parkinson's disease

Parkinson's disease (PD) is a progressive, incurable neurological disorder. The first clinical description of PD dates back two centuries, reported in James Parkinson's 'An Essay on the Shaking Palsy', in 1817 (Goetz, 2011) and is now the second most common NDD, affecting 2-3% of the population over 65 years of age (Poewe *et al.*, 2017).

1.5.1 Clinical phenotype

PD is characterised by motor dysfunction, recognisable as four cardinal motor symptoms (T.R.A.P). These are resting tremor (T), muscular rigidity (R), bradykinesia (A) and postural gait instability (P) (Kalia and Lang, 2015). However, these motor features in patients are heterogeneous, leading to sub-categorisation of PD patients into the less disabling tremor-dominant and more disabling non-tremor-dominant disease groups (Marras and Lang, 2013). Although PD is traditionally regarded as a motor disorder, non-motor features such as olfactory dysfunction, cognitive impairment, sleep disorders, autonomic dysfunction, pain and psychiatric conditions (i.e. depression, hallucinations) are also observed (Kalia and Lang, 2015). Whilst nonmotor features such as cognitive decline and hallucinations are considered key milestones in the latter stages of PD, other features, such as rapid eye movement behaviour disorder, constipation and depression are regarded as prodromal markers of PD, with potential diagnostic utility due to their presentation long before classical motor symptoms (Jankovic, 2008).

1.5.2 Neuropathology

The major pathophysiology of PD is the selective loss of dopaminergic neurons from the midbrain substantia nigra pars compacta (SNpc) (Figure 1.6 A). The dopaminergic neurons of the SNpc project to the striatum and act on D1 and D2 receptors expressed there. Loss of these neurons leads to nigrostriatal dopamine depletion and results in the reduced activation of the direct (D1) and indirect (D2) nigrostriatal pathways to the thalamus.

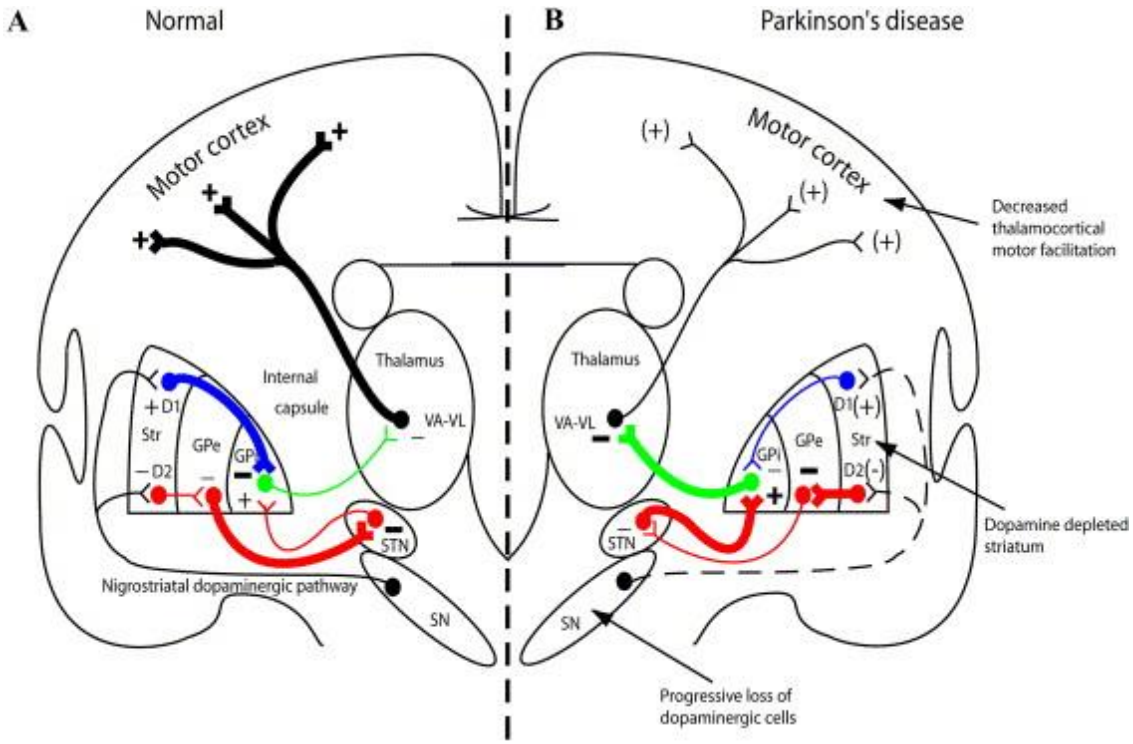

Figure 1.5. (A) Illustrates the normal nigrostriatal circuitry in the human brain. The striatum modulates the activity of the globus pallidus pars interna (GPi) via a direct (blue) and indirect (red) pathway. Excitatory input is denoted by (+), whilst (-) denotes inhibitory input. Bold lines indicate facilitated pathways, whereas thin lines demarcate depressed pathways. Normal dopaminergic input to the striatum results in net inhibition of the (green) inhibitory interneuron in the GPi and thus facilitation of thalamocortical motor activity. (B) Demonstrates the nigrostriatal pathway in Parkinson's disease. Reduced dopamine input to the striatum leads to augmentation of the (green) inhibitory interneuron in the GPi causing depression of the thalamocortical motor circuitry, hence Parkinsonian symptoms. Schematic derived from Bjarkam and Sørensen (2004).

In healthy individuals (Figure 1.5, A), excitation of D1 receptors by dopamine causes inhibition of the inhibitory interneuron in the Globus Pallidus pars interna (GPi, Figure 1.5, A, blue line). Whereas dopamine action at D2 receptors results in disinhibition of the inhibitory interneuron of the Globus Pallidus externa (GPe) allowing inhibition of the excitatory interneuron projecting from the subthalamic nucleus (STN) to the GPi (Figure 1.5, A, red line). The net result of dopaminergic stimulation of the direct and indirect pathway is a reduction in inhibitory output from the GPi interneurons and thus facilitation of thalamocortical motor activity (Figure 1.5, A, green line) (Bjarkam and Sørensen, 2004). In PD, due to the selective loss of dopamine neurons, there is a major striatal dopamine depletion, therefore the D1 and D2 pathways are impaired and eventually lost completely. Loss of these pathways results in an increase in

inhibitory output from the GPi, thereby reducing the thalamocortical innervation causing the classical motor symptoms associated with PD (Figure 1.5, B). Another important pathological hallmark of PD is the presence of Lewy body and Lewy neurite aggregations in the surviving neurons of the SNpc (Moon and Paek, 2015) and other regions of the central and peripheral nervous system. These intracytoplasmic proteinaceous inclusion bodies are composed of a granular core and surrounded by a filamentous halo primarily comprised of alpha synuclein (α-synuclein) (Olanow and Brundin, 2013) (Figure 1.6, B).

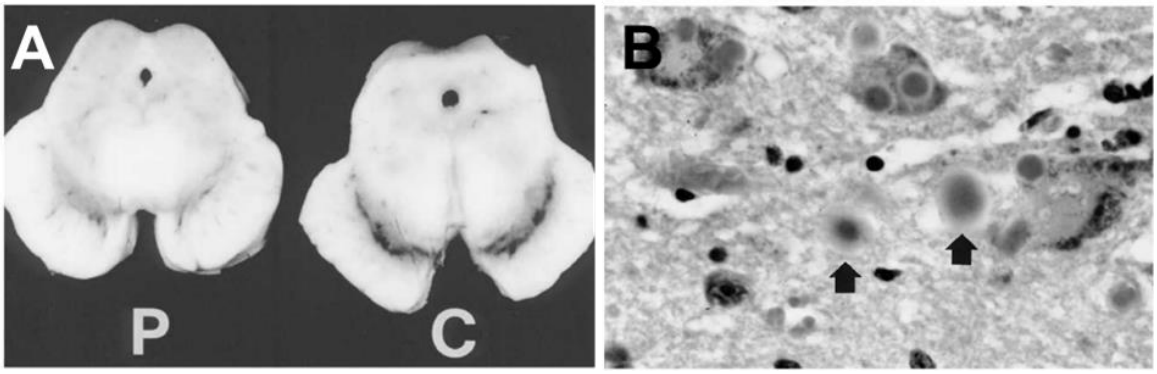

Figure 1.6. (A) Horizontal section through midbrain of PD patient (P) and control (C). Pigmented dopaminergic neurons of the substantia nigra are shown by the dark band. (B) Haematoxylin and eosin stained electron micrograph of the Substantia Nigra in Parkinson's disease. Lewy bodies are highlighted in neurons and neuropil by arrows. Taken from Mackenzie (2001).

Alpha synuclein protein expression is abundant throughout the central nervous system, with specific localised expression within the presynaptic nerve terminals (Maroteaux *et al.*, 1988). At a peripheral level, α-synuclein is found to be localised within the cardiac plexus, enteric nervous system, skin and pharyngeal muscles (Mu *et al*., 2013), as well as circulating in the blood contained within red blood cells and freely within the cerebrospinal fluid and plasma (Barbour *et al*., 2008).

Alpha synuclein is thought to be involved in a multitude of cellular functions including synaptic vesicle release, dopamine synthesis (through its interaction with tyrosine hydroxylase) (Venda *et al.*, 2010) and calcium homeostasis. In its monomeric form, α-synuclein is typically unfolded, however, both wild-type and mutant α-synuclein can oligomerise to form protofibrils which accumulate into lewy body plaques. These α-synuclein protofibrils are kinetically stabilised by dopamine (Mor *et al.*, 2017), possibly explaining the vulnerability of dopamine neurons in synuclein related

disorders. Loss of functional α-synuclein and aggregation of mutant α-synuclein is known to be cytotoxic and is found to disrupt catecholamine homeostasis, causing excess cytosolic dopamine (Perez and Hastings, 2004); and disrupts synaptic vesicle release, inhibiting neurotransmission (Cooper *et al.*, 2006; Larsen *et al.*, 2006). The presence of cytosolic dopamine has been found to promote the accumulation of toxic α-synuclein protofibrils (Rochet *et al.*, 2004), thereby revealing the possibility of a vicious pathogenic cycle specifically targeted to dopaminergic neurons. In this cycle, oligomerisation of α-synuclein causes leakage of dopamine from synaptic vesicles into the cytosol, which in turn promotes the additional accumulation of α-synuclein protofibrils (Mosharov *et al.*, 2006).

As one of the major neuropathologies of PD, α-synuclein or Lewy body accumulation and distribution in the brain is now used as a measure of PD severity and progression, using Braak staging, described by Braak *et al.* (2003). This theory suggests that synucleinopathy begins in the lower brainstem, involving Lewy body and Lewy neurite lesions in the dorsal motor nucleus, intermediate reticular zone and anterior olfactory nucleus (Stage 1. Figure 1.7, A), and extending upwards to the locus coeruleus (Stage 2. Figure 1.7, A). Lewy pathology then spreads rostrally to the midbrain, limbic cortex and amygdala (Stage 3/4. Figure 1.7, B) and eventually up to the neocortex, where the prefrontal cortex and sensory association cortices are initially affected (Stage 5), with the primary motor and sensory areas being infiltrated in the final stages (Stage 6, Figure 1.7, C) (Braak *et al.*, 2003; Jellinger, 2014). Clinically, the initial Braak stages of PD are considered to be pre-symptomatic and are associated with the non-motor symptoms (autonomic and olfactory) that precede motor dysfunction. Stages 3-4 are correlated with the onset of typical somatomotor complications of PD i.e. T.R.A.P phenotype, whilst stage 5-6 are associated with cognitive disturbance.

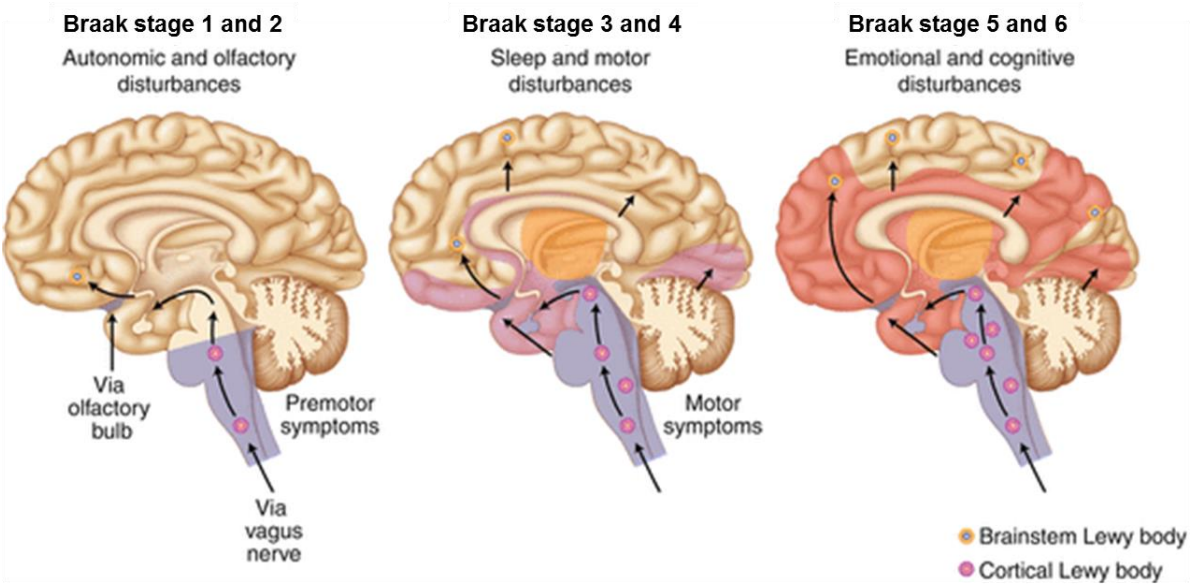

Figure 1.7. Representation of the Braak stages of PD, showing initial lesions sites in the brain stem (medulla oblongata and pons) and olfactory bulb, spreading up to the midbrain, limbic and neocortex structures (amygdala and SNpc), eventually infiltrating the cortical regions. Taken from Jellinger (2014).

1.5.3 Aetiology

Currently, the exact cause of PD is unknown. A minority of PD cases (~10%) are of a clear familial pedigree (Klein and Westenberger, 2012) in which a single gene mutation is responsible for disease. The most commonly reported and extensively studied gene mutations linked to monogenic PD are in *SNCA, LRRK2, Parkin, PINK1* and *DJ-1* genes (Klein and Westenberger, 2012).

The *SNCA* gene was the first reported autosomal-dominant cause of PD, typically associated with the early-onset (<50 years of age, EOPD) and more rapidly progressing form of disease (Klein and Westenberger, 2012). This gene encodes the α-synuclein protein, and missense mutations appear to cause a toxic gain of function, through impairment of the amino-terminal domain, resulting in formation of stable β sheets, rather than α-helices, thereby promoting toxic oligomer and fibril formation (Bertoncini *et al.*, 2005).

LRRK2 mutations are the most frequent known cause of autosomal-dominant PD and sporadic PD. These mutations are generally associated with late-onset and slow-progressing PD. *LRRK2* encodes the cytoplasmic protein leucine-rich repeat kinase 2

(LRRK2), and over 50 different mutations have been reported, with 16 being pathogenic (Nuytemans *et al.*, 2010). However, the exact pathogenic mechanism is unknown.

Parkin, *PINK-1*, *DJ-1* genes are all linked with autosomal recessive PD and clinically show identical PD phenotypes. *Parkin* is the second largest gene in the human genome and encodes the Parkin protein, an E3 ubiquitin ligase. It is the most common cause of EOPD and accounts for up to 77% of all familial PD cases with an onset <30 years of age, with almost 900 different mutations being described (Klein and Westenberger, 2012). *PINK1* mutations are the second most common cause of EOPD, after *Parkin* mutations and most commonly effect the kinase domain of the PINK1 protein kinase, thereby resulting in a loss-of-function of the kinase activity. *DJ-1* linked PD is particularly rare, with mutations only affecting around 1-2% of EOPD cases (Pankratz *et al.*, 2006). This gene encodes a protein that acts as a cellular sensor for oxidative stress; therefore mutations are likely related to loss of neuroprotective function and antioxidant activity.

The major proportion of PD occurs sporadically (~90%), and thus, the cause is far less clear. It is likely to be multifactorial in aetiology, involving interplay of polygenic influences i.e. GWAS identifying numerous risk loci for PD (Nalls *et al.*, 2014), environmental factors i.e. pesticide exposure found to increase risk, whilst smoking is found to decrease risk of PD (Gorell *et al.*, 2004; Dick *et al.*, 2007) and most prominently aging i.e. incidence of PD is 1% in over 60’s, which increases to 5% in over 85’s (Reeve *et al*., 2014).

1.5.4 Diagnosis, biomarkers and treatment

There is no definitive diagnostic test for PD. Instead diagnosis is based on the Movement Disorders Society (MDS) criteria (Postuma *et al*., 2015), focussing on clinical presentation of suspected PD patients. This criteria requires the presence of bradykinesia, in combindation with either resting tremor, rigidity or both, positive response to levodopa therapy and absence of features to suggest an alternative cause of PD (Postuma *et* al., 2015; Gelb et al., 1999). However, the reliability and validity of clinical criteria is not clearly established, with misdiagnosis occurring in 10-20% of patients (Jankovic, 2008) and typically neuropathological analysis at autopsy is needed to confirm diagnosis . Additionally, given that ~50% of dopaminergic nigral neurons are required to be lost before a clinical phenotype is expressed (Fearnley

and Lees, 1991), diagnosis needs to be focussed towards the presymptomatic phase of PD, to maximise therapeutic utility and enable disease modifying therapies to be successful.

For presymptomatic diagnosis to be made, accurate PD-specific biomarkers are needed, and although a diverse range of potential biomarkers exist for PD, none have been recommended into clinical practice. Potential biomarkers for PD can be categorised into four main subgroups; clinical, biochemical, genetic and imaging (Delenclos *et al.*, 2016).

Although clinical markers include the cardinal motor symptoms associated with the diagnosis of PD, they more importantly refer to the group of preclinical signs and conditions that precede the onset of classical PD (Sharma *et al.*, 2013). For example olfactory dysfunction is identified in >90% of all PD patients (Haehner *et al.*, 2007; Ross *et al.*, 2008), sleep disturbances and REM sleep behaviour disorder have been associated with parkinsonism (Boeve, 2013; dos Santos *et al.*, 2015) and constipation is reported to be prevalent in 25-65% of PD patients, potentially underlying pathophysiological changes occurring within PD (Stirpe *et al.*, 2016). A number of these nonmotor features have recently been included in a revised MDS criteria for prodromal PD, including REM behaviour disorder, olfactory dysfunction, constipation, daytime somnolence, erectile dysfunction, urinary dysfunction and depression (Berg *et al*., 2015). However, although these conditions have high concordance with PD and are non-invasive low cost tests, they also have limited specificity and sensitivity and are not specific to PD pathology (Delenclos *et al.*, 2016).

Imaging techniques, such as magnetic resonance imaging (MRI), transcranial sonography (TCS) and positron emission tomography (PET) can provide important information about the brain structure and function in PD, and are often used as an adjunct to clinical assessment to enable differentiation between PD and other motor disorders (Miller and O'Callaghan, 2015). However, in presymptomatic screening where neuroanatomical changes may be less prominent the use of imaging techniques is cost prohibitive (Politis *et al.*, 2017).

Genetic testing is exclusively useful for diagnosis of familial PD, whilst in sporadic PD the utility of genetic markers are not so clear and genome wide association (GWAS) and linkage studies have had little success in establishing reproducible candidate genes (Michell *et al.*, 2004).

Currently, the most promising biomarker avenue is biochemical testing focussing on tissue biopsy or body fluid based markers (i.e. salvia, blood, CSF), due to their less invasive nature and quicker processing time (Schapira, 2013). Due to links between PD and oxidative stress, several groups have investigated and identified potential oxidative stress related biomarkers for PD. Elevated levels of malondialdehyde and superoxide radicals (reactive oxygen species) in the CSF and blood have been associated with PD, whilst elevated urate (an anti-oxidant) in the serum is associated with lower risk of PD and slower PD progression (Shen *et al.*, 2013). However, these trends are not sufficiently robust to indicate use as diagnostic biomarkers in clinical practice (Michell *et al.*, 2004). Similarly, PD-associated proteins such as α-synuclein and DJ-1 have been explored as potential biomarkers for PD particularly in the CSF. These proteins typically have lower expression in PD CSF and can accurately distinguish PD from healthy controls (Hong *et al.*, 2010) (Tokuda *et al.*, 2006; Mollenhauer *et al.*, 2008; Mollenhauer *et al.*, 2011; Tateno *et al.*, 2012). However, there are conflicts within the literature with some reports indicating elevated levels of DJ-1 in the CSF (Waragai *et al.*, 2006) and others questioning the specificity of CSF α-synuclein in distinguishing PD (Ohrfelt *et al.*, 2009; Spies *et al.*, 2009; Aerts *et al.*, 2012). Therefore, larger longitudinal cohorts are required to clarify potential correlations and validate the biomarker utility of these proteins (Delenclos *et al.*, 2016).

Thus, at present, due to the lack of accurate biomarkers for presymptomatic diagnosis, treatment of PD is greatly limited and only functions to alleviate symptoms and improve quality of life, rather than slow or reverse the neurodegenerative process. The majority of treatments for PD focus on dopamine replacement therapy by pharmacological means, with the gold standard being Levodopa administration (Poewe *et al.*, 2010), alongside ‘activating’ therapies such as physical exercise, physiotherapy and dance interventions. However, disease-modifying treatments are in development. Administration of neurotrophic factors, such as glial cell-derived neurotrophic factor, have been shown to enhance midbrain neuronal survival *in vitro* and rescue degenerating neurons *in vivo* (Jankovic and Aguilar, 2008). However, clinical trials have been conflicting, with some reporting a significant clinical improvement (Gill *et al.*, 2003), some reporting no beneficial effects (Lang *et al.*, 2006) and others reporting dangerous side effects (Nutt *et al.*, 2003). Additionally, cell-replacement therapy, involving transplantation of stem cell derived dopaminergic neurons into the midbrain, is in development, however, this is still in preclinical *in vivo*

testing at present and results regarding efficacy and toxicity are conflicting (Barrow, 2015). These disease-modifying therapies would likely, be more efficacious if they could be instigated in the early prodromal phase of disease, thus biomarker research is crucial to allow this.

1.5.5 PD and mitochondrial dysfunction

Over the last 30 years, multiple lines of evidence have sustained a link between PD and mitochondrial dysfunction. This link was first described in the early 1980's when a group of drug abusers presented with a PD-like syndrome following intoxication with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a synthetic analogue of heroin (Langston *et al.*, 1983). Once injected into the body MPTP is taken up into astrocytes and is oxidised into its active form, MPP+, by MAO-B. MPP+ selectively accesses dopaminergic neurons through the plasmalemmal dopamine transporter where it accumulates bound to neuromelanin until reaching toxic levels, inhibiting mitochondrial complex I activity (Vila and Przedborski, 2003). As a result of this, mitochondrial respiration is impaired leading to decreased ATP production, increased generation of ROS and RNS and excitotoxicity and culminating in neuronal death (Vila and Przedborski, 2003; Winklhofer and Haass, 2010). Moreover, *in vivo* studies of other environmental toxins such as rotenone, paraquat and maneb (manganese ethylenebis-dithiocarbamate) provide further support that mitochondrial dysfunction is central to PD pathogenesis. Rotenone potently inhibits complex I activity (Betarbet *et al.*, 2000); paraquat stimulates free radical generation (Brooks *et al.*, 1999); and maneb potently inhibits complex III activity (Thiruchelvam *et al.*, 2000). Induction of any of these three toxins to rodents or primates generates animal models with the characteristic motor phenotype of PD and common SNpc neuropathology.
Furthermore, human studies have confirmed a biochemical link implicating mitochondrial dysfunction in PD as numerous studies have shown a complex I deficiency in post-mortem brain samples from PD sufferers (Schapira *et al.*, 1989; Keeney *et al.*, 2006; Parker Jr *et al.*, 2008).
In addition, familial and sporadic PD appear to converge at the level of the mitochondria and numerous PD-related nuclear gene mutations i.e. *SNCA, Parkin, PINK1, LRRK2, DJ-1, ATP13A2*, have been identified to be associated with vast mitochondrial dysfunction; summarised in Table 1.2. Most notably, PINK1 and Parkin are crucial proteins in the regulation of mitophagy, the selective removal of damage

mitochondria (Narendra *et al.*, 2008). Briefly, PINK1 accumulates and is stabilised to dysfunctional mitochondria (due to their low membrane potential) where it recruits Parkin from the cytosol. Parkin then ubiquitylates the mitochondrial proteins and initiates the clearance of the mitochondria by lysosomes (Youle and Narendra, 2011). Therefore, loss of function of either of these genes results in an inability to remove dysfunctional mitochondria and thus leads to neuronal demise. Numerous in vivo models of *PARK2* and *PARK6* knock-out have confirmed that removal of these genes impair mitophagy and cause cardinal symptoms of PD (reviewed in Pickrell and Youle (2015)) thereby directly implicating mitochondrial dysfunction in the pathogenesis of PD.

Gene	***PD locus***	***Chr locus***	***Inheritance***	***Protein encoded***	***Link to mitochondria***
SNCA	PARK1/4	4q21-q23	autosomal dominant	α-synuclein	Involved in mitochondrial dynamics and homeostasis. Causes direct mitochondrial toxicity (Mullin and Schapira, 2013)
Parkin	PARK2	6q25-q27	autosomal recessive	Parkin - component of E3 ubiquitin ligase	Regulates mitophagy, mitochondrial biogenesis, maintenance and repair (Kazlauskaite and Muqit, 2015)
PINK1	PARK6	1p36-p35	autosomal recessive	PINK1 - mitochondrial protein kinase	Regulates mitophagy and regulates mitochondrial transport proteins (Kazlauskaite and Muqit, 2015)
LRRK2	PARK8	12p11-q13	autosomal dominant	LRRK2 - multidomain protein	Involved in mitochondrial function, homeostasis and OXPHOS (Rideout and Stefanis, 2014)
DJ-1	PARK7	1p36	autosomal recessive	DJ-1 protein	Regulates mitophagy (Thomas *et al.*, 2011)
ATP13A2	PARK9	1p36	autosomal recessive	Lysosomal P-type ATPase	Involved in mitochondrial maintenance and bioenergetics (Gusdon *et al.*, 2012)

Table 1.2. Parkinson's disease-related nuclear genes, including their PARK locus, genetic locus, mode of inheritance, encoded protein and their link to the mitochondria. Chr; chromosome, SNCA; alpha synuclein, PINK1; PTEN-induced kinase 1, LRRK2; Leucine rich repeat kinase 2, DJ-1; protein deglycase DJ-1, ATP13A2; ATPase.

1.5.6 PD and mtDNA

A number of studies have reported specific mitochondrial SNP's and haplogroups associated with the risk of PD (van der Walt *et al.*, 2003; Autere *et al.*, 2004; Ghezzi *et al.*, 2005; Huerta *et al.*, 2005; Pyle *et al.*, 2005; Hudson *et al.*, 2014). Initial investigations carried out by van der Walt *et al.* (2003) showed an underrepresentation of haplogroups J and K in the PD population compared to controls, perhaps indicating a protective effect of these haplogroups. Further, this group also reported a reduced frequency of the mitochondrial SNP 10398G in the PD population. This SNP is found in both haplogroups J and K and causes a non-conservative amino acid change from threonine to alanine within the NADH3 gene, a subunit of complex I, possibly conferring the protective advantage by its reduction of complex I activity, excitability and ROS production (Hudson *et al.*, 2014).
Subsequent findings have been conflicting, and whilst the majority of studies have given support to the reported association between mtDNA and PD (Autere *et al.*, 2004; Huerta *et al.*, 2005; Pyle *et al.*, 2005), others have not been so convincing (Ghezzi *et al.*, 2005). For example, Pyle *et al.* (2005) extended the work by van der Walt and colleagues and compared the frequency of the pooled phylogenetic cluster UKJT in a large cohort of PD patients and controls. In their own study and through re-analysis of van der Walt et al (2003), they confirmed a reduced frequency of supercluster UKJT in PD cases compared to controls. They concluded that this supercluster haplogroup was associated with a 22% risk reduction for PD, thus supporting the notion that mtDNA may contribute to the genetic cause of PD. Another report published that same year described the same protective association of haplogroup K and PD as previous, but conversely stated that there was no difference in the frequency of haplogroup J and mitochondrial SNP 10398G in PD cases and controls in an Italian population (Ghezzi *et al.*, 2005). Thus, highlighting the difficult nature of genetic association studies and demonstrating the possible risk of false-positive results due to geographical differences and small cohort size.
More recently, Hudson *et al.* (2013) performed a 2-stage association study of mtDNA variants in >3000 PD cases and >5000 controls, identifying nine PD-associated mtDNA variants. Phylogenetically linked SNP's m.2158T>C and m.11251A>C were the only two variants identified in both the discovery and replication phase and were both associated with a reduced risk of PD. However, further exposing the difficulties surrounding genetic association studies, the study was unable to identify consistent

association with variants m.4336T>C and m.10398A>G and PD, despite reports of association in earlier studies (van der Walt *et al.*, 2003; Autere *et al.*, 2004; Huerta *et al.*, 2005).

Differently, somatic mtDNA mutational burden may be altered in PD. Previous literature had reported that mutant mtDNA heteroplasmy must exceed 60% to cause loss of the dopaminergic neurons from the SNpc (Rossignol *et al.*, 2003; Bender *et al.*, 2006), therefore, mtDNA mutational load is expected to be higher in PD sufferers if mitochondrial dysfunction is implicated in disease pathogenesis. Recent studies have confirmed this hypothesis and have shown a significant increase in the number of mtDNA deletions in the SNpc (Bender *et al.*, 2006) and the mean heteroplasmic mtDNA variant burden in the SNpc, frontal cortex (Coxhead *et al.*, 2016) and single neurons (Lin *et al.*, 2012) of PD patients compared to controls.

Furthermore, mtDNA copy number within particular brain regions has also been associated with PD. Pyle *et al.* (2015a) quantified the mtDNA copy number of peripheral blood cells (PBC's), SNpc tissue and frontal cortex tissue from PD cases and controls via qPCR. This demonstrated a significantly lower mtDNA copy number in the samples taken from the PD patients compared to controls (Pyle *et al.*, 2015a), suggesting a possible imbalance in the turnover of mitochondria and mtDNA in the PD brains.

1.5.7 PD and ccf-mtDNA

Ccf-mtDNA copy number has also been quantified in the lumbar CSF (lCSF) of PD patients and compared to controls in a pilot study carried out by Pyle *et al.* (2015b). The ccf-mtDNA copy number of 53 idiopathic PD patients and 10 age-matched controls was quantified using multiplex qPCR at two different time points, baseline (t=0) and 18 months later (t=18). They discovered that ccf-mtDNA (*MTND1* and *MTND4*) copy number was significantly lower in the lCSF of PD patients compared to controls (Figure 1.8, A) and this was further exaggerated at the 18 month follow up. Additionally, ROC curve analysis suggested that ccf-mtDNA copy number was accurate and reliable in predicting the disease state of individuals (i.e. healthy control or PD) based on the area under the curve of >0.80 (Figure 1.8, B), leading to the conclusion that low ccf-mtDNA may be a useful marker of PD.

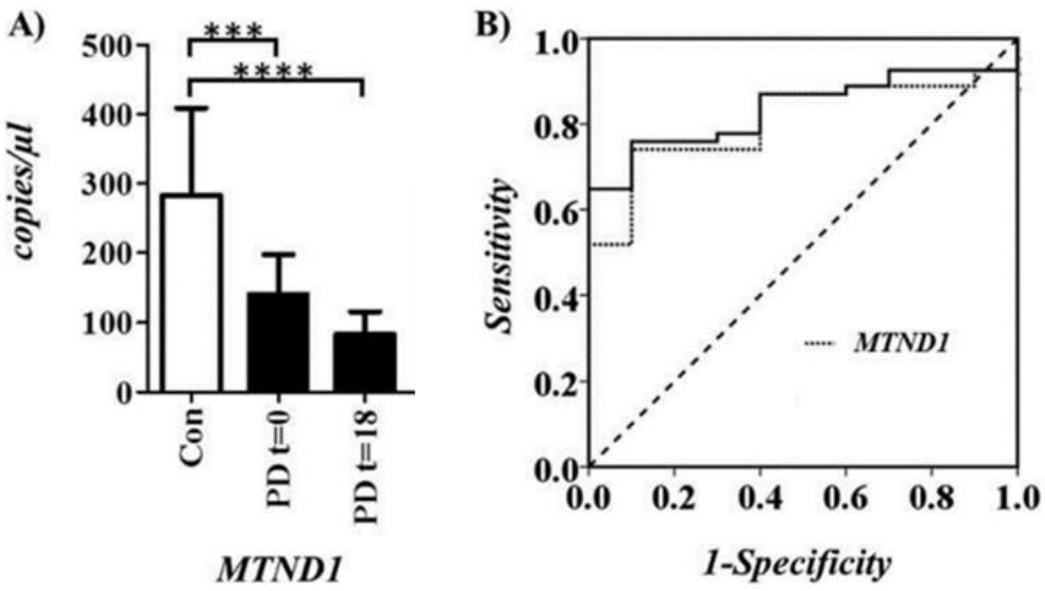

Figure 1.8. Results taken from Pyle et al., 2015; (A) Ccf-mtDNA copy number in the lumbar CSF of 10 controls compared to 53 PD patients at baseline (t=0) and 18 month follow-up (t=18). (B) ROC analysis showing area under the curve of 0.81 and 0.84 for MTND1 and MTND4, respectively, where dashed line indicates the reference line.

Similarly, another study compared lCSF ccf-mtDNA levels in idiopathic PD, familial LRRK2^{G2019S} mutation PD patients, and two control groups (asymptomatic LRRK2^{G2019S} carriers and first-degree relatives of LRRK2 patients without the mutation). LRRK2^{G2019S} PD patients were found to have significantly higher ccf-mtDNA levels than other groups (Podlesniy *et al.*, 2016b), suggesting that lCSF ccf-mtDNA copy number can distinguish familial LRRK2-related PD from idiopathic PD and controls. However, conversely to Pyle *et al*., (2015) idiopathic PD patients were not found to have significantly lower copy number compared to controls and non-manifesting carriers.

Therefore, due to conflicting conclusions and greatly limited sample sizes (only 10 controls and 53 idiopathic PD patients in Pyle *et al.,* (2016) and 21 controls, 26 carriers, 20 LRRK2^{G2019S} PD and 31 idiopathic PD patients in Podlesniy *et al.,* (2016)) the potential biomarker utility of lCSF ccf-mtDNA in PD is unclear. As with all novel biomarker studies, further replication is needed in an independent cohort to validate these findings (Hunter *et al.*, 2010).

1.6 Alzheimer's disease

AD was first described by Alois Alzheimer in 1907, reporting psychological and behavioural observations of a patient, Auguste Deter. A century later, and it is now the most common cause of dementia (accounting for 60-80%) and the most common NDD with an estimated prevalence of 7-10% in the population over 60 years of age (Prince *et al.*, 2013).

1.6.1 Clinical phenotype

AD is a chronic and progressive NDD characterised by impairment of cognitive functions and changes in behavioural or personality traits. The major symptoms of AD can be categorised into three distinct groups: (1) cognitive dysfunction i.e. memory loss, difficulties with language and executive function, (2) psychiatric/behavioural changes i.e. depression, hallucinations and delusions and (3) difficulty performing activities of daily living i.e. dressing, shopping and eating unaided (Burns and Iliffe, 2009). Typically symptoms appear mild in early stages of AD, presenting with minor memory loss and cognitive changes, gradually progressing to severe dementia and debilitating psychiatric and cognitive deficits, thereby increasing a patient's dependence on external support for everyday activities.

1.6.2 Neuropathology

The core pathology seen in AD is characterised by the presence of positive lesions such as amyloid plaques (Figure 1.9, A) and neurofibrillary tangles (Figure 1.9, B), and negative features of neuronal and synaptic loss.

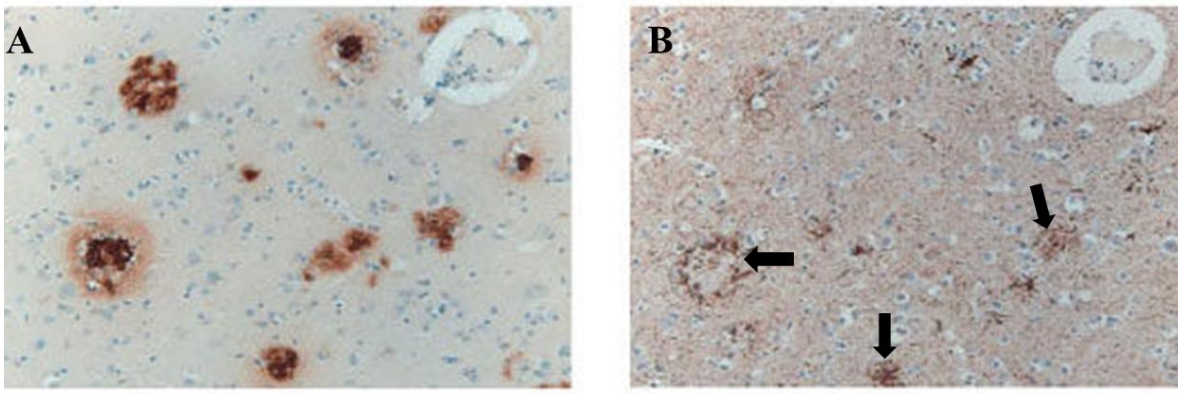

Figure 1.9. Cortical tissue sections from Alzheimer's disease patient showing the major disease-associated pathology; (A) amyloid plaques shown by brown stain and (B) neurofibrillary tangles shown by black arrows. Adapted from Nicoll et al. (2003).

Amyloid plaques and neurofibrillary tangles are formed by the accumulation of abnormally folded amyloid β (Aβ) and hyper-phosphorylated tau protein, respectively. The deposition of Aβ protein was put forward as a causative agent of AD in the 1990's in the amyloid cascade hypothesis (Hardy and Higgins, 1992).

This hypothesis states that amyloid precursor protein (APP) can be processed via two pathways involving a combination of secretases; either α- and γ- or β- and γ-secretases (Figure 1.10). Cleavage by α-secretase generates a long secreted form of APP (sAPPα) and C-83. This is further cleaved to nontoxic, non-amyloidogenic products P3 and AICD50 by γ-secretase. Conversely, cleavage with β-secretase generates sAPPβ and C-99. Secondary cleavage of C-99 by γ-secretase generates AICD50 and Aβ protein. These Aβ protein fragments oligomerise and fibrillise to form cardinal amyloid plaques (Chow *et al.*, 2010; Ballard *et al.*, 2011; Kumar *et al.*, 2015) which in turn triggers the changes in tau to form neurofibrillary tangles. The link between the two is not fully understood, but it's thought that toxic Aβ accumulation promotes tau hyperphosphorylation (Kumar *et al.*, 2015) which reduces the biological activity of tau leading to abnormal sequestration and microtubular destabilisation. This hyperphosphorylation also promotes oligomerisation of tau monomers which aggregate into a β sheet before forming neurofibrillary tangles (Ballard *et al.*, 2011). The toxic build-up of these proteinaceous inclusions leads to synaptic and neuronal loss, eventually causing gross cortical and hippocampal shrinkage.

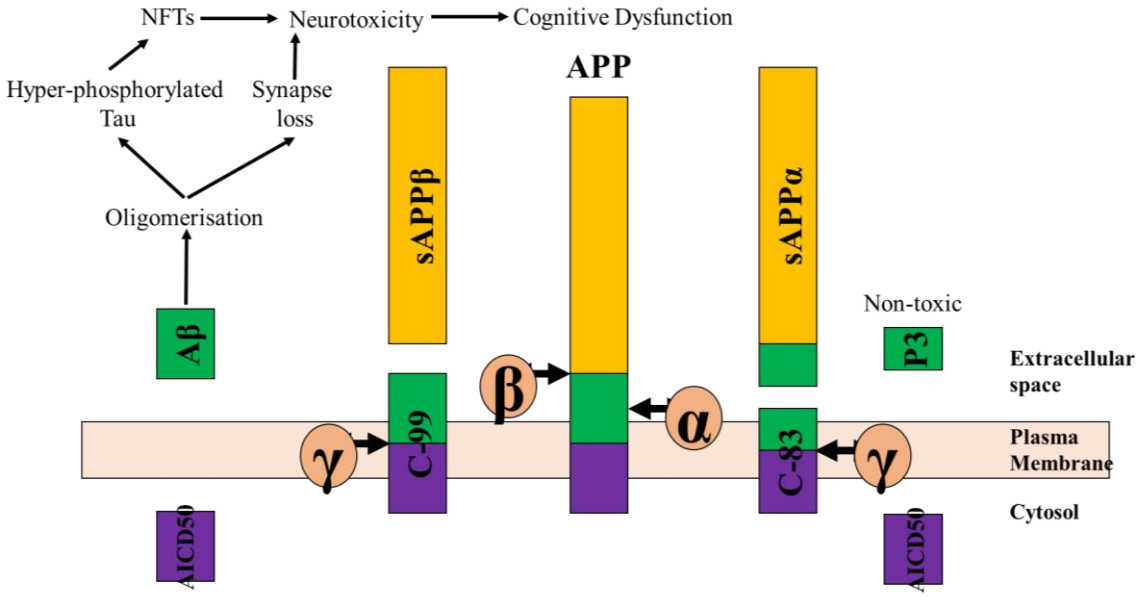

Figure 1.10. Amyloid cascade hypothesis showing the cleavage of amyloid precursor protein into (i) nontoxic products by alpha (α) and gamma (γ) secretases and (ii) toxic amyloid beta protein by beta (β) and gamma (γ) secretases.

1.6.3 Aetiology

The majority of AD cases are late-onset and sporadic, however, approximately 2-3% of cases have familial origin and autosomal dominant inheritance, causing an early-onset form of disease (Petrozzi *et al.*, 2007). The most common mutations associated with familial AD are found in three genes associated with amyloid β-peptide (Aβ) production; amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) (Ballard *et al.*, 2011). As described earlier, cleavage of APP can be non-amyloidogenic or amyloidogenic. Familial mutations in *APP* are found to preferentially promote the processing of APP through the amyloidogenic pathway of β- and γ-secretase cleavage (Goate *et al.*, 1991) leading to Aβ plaques and AD pathology. *PSEN1* and *PSEN2* encode the presenilins, which make up the catalytic domain of γ-secretase. Familial mutations in *PSEN1* or *PSEN2* are thought to cause Aβ plaque formation and AD pathology through alteration and promotion of the production of the more hydrophobic amyloidogenic form of Aβ; Aβ42 (Kelleher and Shen, 2017).

Similarly to PD, the sporadic form of AD likely arises due to a complex interaction between environmental and genetic factors, which individually are not sufficient to develop the disease. At present the major risk factors for sporadic AD relate to both ageing and the allele ε4 of the apolipoprotein E (ApoE4) (Piaceri *et al.*, 2012).

1.6.4 Diagnosis, biomarkers and treatment

Similarly to PD, AD can only be definitively diagnosed post-mortem. However, a set of criteria has been established and subsequently revised since the early 1980's to standardise and improve the accuracy of the clinical diagnosis of AD; relying on patient history, phenotypic presentation, physical examination and neuropsychological tests (McKhann *et al.*, 1984; Jack *et al.*, 2011). Physical examination may include blood tests to measure thyroid function and vitamin B12 levels, MRI or PET scans to measure synaptic dysfunction, gross morphological changes to the brain and to rule out other conditions such as tumours or stroke (Sperling *et al.*, 2011). Neuropsychological tests may include basic screening techniques such as the mini-mental state exam (MMSE) or Montreal cognitive assessment (MoCA), or the more diagnostic Addenbrooke's cognitive examination (Larner, 2007), to identify behavioural, psychological, and higher executive function changes associated with AD.

Differently to PD and other NDDs, AD also has well-established and validated CSF-based biomarkers which significantly increase the diagnostic accuracy of sporadic AD (reviewed in Blennow *et al.* (2010)). These biomarkers are associated with the neuropathological hallmarks of AD, including amyloid β_{42} - the main constituent of the amyloid plaques; and total tau and phospho-tau - constituents of the neurofibrillary tangles. In the CSF amyloid β_{42} is found at low concentrations, due to cortical deposition, whereas tau and phospho-tau are found at high concentrations, due to neuronal loss and tangle formation (Olsson *et al.*, 2016). Quantitative analysis of all three CSF proteins together has the greatest diagnostic validity, with a sensitivity of >95% and specificity of >85% (Humpel, 2011).

Despite accurate clinical diagnosis, current treatment strategies for AD still rely on symptomatic relief, typically using cholinesterase inhibitors to increase acetylcholine levels and promote synaptic plasticity, or using glutamate antagonists to reduce excitotoxicity and thereby enhance neuronal survival (Mendiola-Precoma *et al.*, 2016). However, disease modifying treatments are in development and are designed to target and prevent key aspects of AD pathology such as amyloid deposition (with tramiprosate), tau deposition, inflammation and oxidative damage (Galimberti and Scarpini, 2011; Yiannopoulou and Papageorgiou, 2013).

1.6.5 AD and mitochondrial dysfunction

Defects in mitochondrial function are prominent throughout AD and have been proposed to be the primary event causing Aβ deposition, synaptic degeneration and neurofibrillary tangle formation by Swerdlow *et al.* (2010), in the mitochondrial cascade hypothesis of AD. A number of biochemical changes such as oxidative damage, energy deficiency and mitochondrial abnormalities appear as fundamental characteristics of the early stages of AD, prior to significant Aβ plaque pathology (Beal, 2005; Petrozzi *et al.*, 2007). Furthermore, key enzymes of the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) are found to have impaired activity in AD brains, possibly explaining the reduction in brain metabolism seen in the cerebral cortices of AD patients by positron emission tomography (PET) (Blass *et al.*, 2000). For example, activity of the TCA cycle enzymes, pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase complex, are found to be decreased in AD brains (Bubber *et al.*, 2005). Similarly, complex IV (COX) activity of the ETC has been found to be deficient in post-mortem hippocampal tissue (Maurer *et al.*, 2000;

Bosetti *et al.*, 2002) and frontal, temporal and parietal cortices of the brain of AD patients (Kish *et al.*, 1992; Mutisya *et al.*, 1994; Maurer *et al.*, 2000). It is speculated that this COX deficiency is in part regulated by Aβ accumulation localised to the mitochondria (Reddy *et al.*, 2004; Reddy and Beal, 2008; Pagani and Eckert, 2011) and blocking of complex IV translocation into the mitochondria by TOM and TIM by the amyloid precursor protein (APP) (Morán *et al.*, 2012). Several studies have also reported alterations in mitochondrial dynamics. Typically, AD patient's and AD animal models show a switch towards increased mitochondrial fission (division), showed by elevated mitochondrial fission 1 (Fis1) protein levels and reduced dynamin related protein1 (Drp1), mito-fusin-1 and 2 (Mfn1 and Mfn2) protein levels, resulting in mitochondrial dysfunction (Wang *et al.*, 2009; Calkins *et al.*, 2011).

Changes to the mitochondrial genome have also been reported in AD, however, studies attempting to identify and understand the contribution of these mtDNA mutations to AD have been conflicting. For example, Coskun *et al.* (2004) studied the sequence of the mtDNA control region (CR) in the frontal cortex of AD patients and controls and discovered the presence of the T414G mutation in 65% of AD brains but none of the controls. Furthermore, AD brains were found to harbour 63% more heteroplasmic mutations in the mtDNA CR than controls, particularly in the key regulatory elements of L-stand transcription and H-strand replication (Coskun *et al.*, 2004). However, a previous larger scale study failed to detect the T414G mutation in AD patients, DLB patients or controls (Chinnery *et al.*, 2001). Moreover, differences in the representation of mtDNA haplogroup-associated polymorphisms in AD compared to controls has further implicated mtDNA changes in AD risk and development (Chagnon *et al.*, 1999; van der Walt *et al.*, 2004). However, evidence of an etiological role of these haplogroup-linked mutations is conflicting (Elson *et al.*, 2006; Hudson *et al.*, 2012).

Similarly to PD, mtDNA copy number has also been found to be depleted in AD patients compared to controls. Brain regions including the cerebellum (Wei *et al.*, 2017), frontal cortex (Rodriguez-Santiago *et al.*, 2001; Coskun *et al.*, 2004) and hippocampal pyramidal neurons (Rice *et al.*, 2014) from post-mortem AD patients have been found to contain significantly lower mtDNA copies compared to controls.

1.6.6 AD and ccf-mtDNA

Research into the levels of ccf-mtDNA copy number in AD patients has been conflicting. An initial study by Podlesniy *et al.* (2013) indicated that low lCSF ccf-mtDNA copy number was significantly associated with symptomatic AD compared to controls, as measured by real-time PCR. Low CSF ccf-mtDNA was also found to have predictive validity of an increased risk of AD, as it was also found to be associated with asymptomatic subjects who were anticipated to develop AD based on their amyloid-β and tau levels or due to their status as carriers of the pathogenic presenilin mutation (Podlesniy *et al.*, 2013). Furthermore, this reduction was specific to AD patients as frontotemporal lobar dementia patients and Creutzfeld-Jakob syndrome patients did not demonstrate this (Podlesniy *et al.*, 2016a). However, to further evaluate the accuracy of this potential diagnostic biomarker of AD, Cervera-Carles *et al.* (2017) replicated work by Podlesniy *et al.,* (2013) in a more comprehensive cohort, using droplet digital PCR. Opposing that reported by Podlesniy *et al.*, (2013), this group discovered a significant increase in CSF ccf-mtDNA copy number in AD patients compared to controls but no difference in ccf-mtDNA copy number in preclinical AD subjects or mild cognitive impairment (MCI) patients. Furthermore, this group concluded that ccf-mtDNA copy number dispersion was too large to accurately distinguish AD patients from controls, suggesting ccf-mtDNA had limited usage as a biomarker of AD.

However, although this work has been independently replicated and conclusions suggest limited utility of ccf-mtDNA copy number as a biomarker of AD, these studies are still limited by low population validity. It would be useful to validate these findings in a large and longitudinal cohort to assess if ccf-mtDNA copy number could accurately predict the conversion to AD in high risk individuals.

1.7 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic autoimmune disease which attacks the myelinated axons in the central nervous system, destroying the myelin and thereby slowing neural signalling. More than 2.5 million people suffer with a form of the condition, typically presenting with initial symptoms in early to mid-adulthood (aged 20-45 years) (Goldenberg, 2012). MS is classically considered an inflammatory condition due to the clinical and pathological symptoms of the disease, however, recent research has suggested that later stages of MS possess clear neurodegenerative elements.

1.7.1 Clinical Phenotype

The course and clinical presentation of MS is varied and unpredictable. In the majority of patients, the disease is initially characterised by reversible episodes of neurological deficits including optic neuritis, cerebellar dysfunction (causing limb and gait ataxia), spinal myelitis (causing asthenia), brainstem defects and bladder and bowel dysfunction (Tsang and Macdonell, 2011). This is then followed by progressive neurological degeneration over time.

MS was originally divided into four clinical categories based on the course of disease; relapsing-remitting, primary progressive, secondary progressive and progressive-relapsing (Lublin and Reingold, 1996). However, since then, MS has been reclassified into two primary clinical groups; relapsing-remitting disease (RRMS) and progressive disease (PMS) (Lublin, 2014).

RRMS can be further divided into two categories; active RRMS and inactive RRMS. Both forms are characterised by episodes of reversible neurological deficit (described previously), however, for patients to be considered active, a symptomatic episode must be followed by new clinical or radiological evidence i.e. lesions on an MRI scan, within a specified time frame. PMS was originally classified as two separate entities; primary progressive (PPMS) and secondary progressive (SPMS). PPMS defined patients with no relapses or remissions, but a progressive course from disease onset whereas SPMS referred to patients who transitioned from RRMS to a progressive course towards the later stages of disease (Goldenberg, 2012). However, this terminology is now considered outdated due to the almost indistinguishable clinical nature of PPMS and SPMS. Instead PMS is now classified as four sub-groups based their level of disability; 1) active with progression (attack and gradual worsening of

disease), 2) active without progression (attack with stable disease) 3) not active with progression (gradually deterioration of normal functions) and 4) not active without progression (stable disease) (Lublin, 2014).

1.7.2 Pathology

The major pathological hallmark of MS is focal demyelination of axons within the central nervous system, referred to as plaques or lesions, accompanied by inflammation and gliosis. Lesions can be found in both white matter and gray matter regions and have a predilection for brainstem and cerebellar regions, spinal cord and optic nerves (Popescu and Lucchinetti, 2012).

The reason as to why these lesions occur is currently unknown, but it is thought that the initial autoimmune attack is as a result of myelin specific T lymphocyte activation by antigen-presenting microglia (Espinosa-Parrilla *et al.*, 2016; Hohlfeld *et al.*, 2016) (Figure 1.11, 1). Once inside the CNS, infiltrating T cells secrete pro-inflammatory cytokines like TNF-α, ROS, interleukins and prostaglandins which stimulate the release of chemokines and recruit microglia, macrophages and lymphocytes (Figure 1.11, 2). These immune cells release more inflammatory factors which target the myelin sheath and oligodendrocytes to cause demyelination and neuroaxonal injury through direct cell contact-dependent mechanisms (Dendrou *et al.*, 2015) (Figure 1.11, 3).

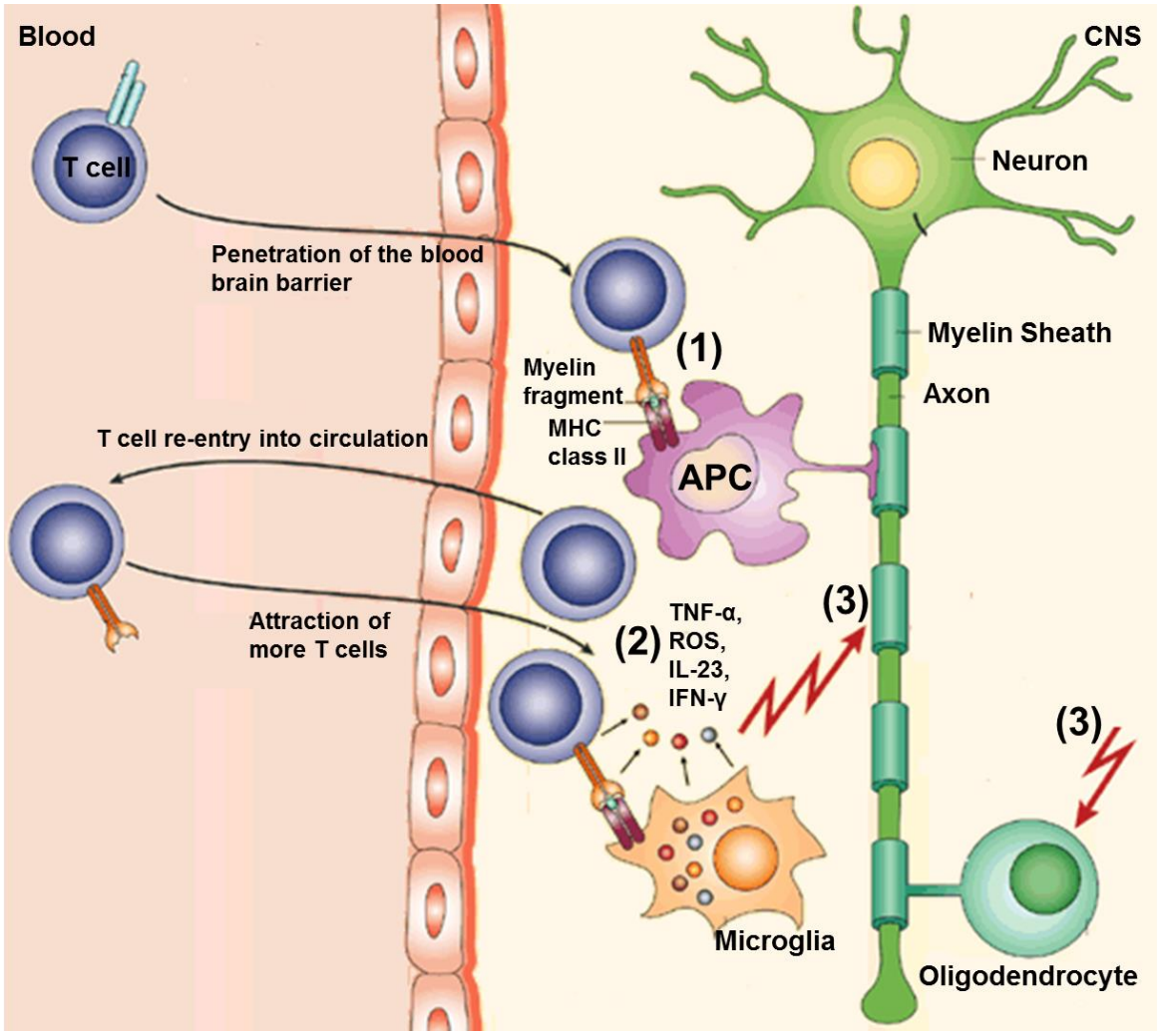

Figure 1.11. Schematic of the initial inflammatory phase of Multiple Sclerosis. (1) T cells cross the blood brain barrier to enter the central nervous system (CNS) in response to activation by an antigen presenting cell (APC). (2) Infiltrating T cells release proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), reactive oxygen species (ROS), Interleukin-23 (IL-23) and interferon-γ (IF-γ) which active and recruit more T cells, microglia and macrophages. (3) Cytokines target the myelin sheath to cause demyelination and oligodendrocytes to prevent remyelination leading to the initial demyelinating syndrome. Adapted from Steinman and Zamvil (2003).

In RRMS reparative and regenerative mechanisms are activated in response to the inflammatory insult, in which antioxidant and neurotrophic factors are upregulated and inflammation is reduced (Espinosa-Parrilla *et al.*, 2016). Surviving oligodendrocytes are activated to remyelinate the damaged axons, leading to the periods of remission, characteristic of RRMS. However, the myelin sheath can only be partially restored. If the inflammatory insult persists, remyelination becomes more difficult and increasing gliosis and inflammatory factors create a barrier between the oligodendrocytes and the axonal targets, called a glial scar. This converts lesions into chronic plaques, where demyelination is complete, indicative of progressive MS (Dendrou *et al.*, 2015). By this stage the immune response is no longer prominent due to chronic antigen-exposure, therefore inflammation declines and neurodegenerative processes take over.

1.7.3 Aetiology

The exact cause of MS is currently unknown, but lifestyle, environment and genetic predisposition are all thought to be key contributors. Factors such as smoking, low vitamin D levels, adult obesity, Epstein-Barr virus infection and night-shift work are all linked to increased risk of MS, whereas high coffee consumption, alcohol consumption and cytomegalovirus infection are all linked to a reduced risk of MS (Olsson *et al.*, 2017). MS is not considered a heritable disorder, however, genetic predisposition is reported to be a factor in risk, for example, population studies have estimated a 7-fold increase of risk in siblings (Westerlind *et al.*, 2014). Furthermore, over 100 associated genes or genetic loci have been confirmed for MS, with the strongest links being within the human leucocyte antigen (HLA) complex. HLA class II genes encode products that present antigens to CD4+ T lymphocytes, and the HLA-DRB1*15:01 variant has the most striking association with MS susceptibility, particularly in Caucasian populations (Greer, 2015). GWAS studies have identified various other SNPs in non-HLA genes, predominantly located in close proximity to or within genes that regulate adaptive or innate immunity (Olsson *et al.*, 2017).

1.7.4 Diagnosis, biomarkers and treatment

There is no single diagnostic test for MS. Instead clinical diagnosis can be made based on evidence of one or more of the following criteria based on biomarkers associated with MS: two lesions in different areas of the CNS identified by MRI, two different episodes in the disease course and chronic inflammation of the CNS, identified by spinal tap (Polman *et al.*, 2011). Another useful but not diagnostic biomarker of MS is the detection and analysis of oligoclonal bands in the CSF and more recently, the expression of a number of different proteins in the CSF (Housley *et al.*, 2015). For example, CSF neurofilament light chain (NFL) (Novakova *et al.*, 2017), glial fibrillary acid protein (GFAP) (Axelsson *et al.*, 2011), S100 beta (Barateiro *et al.*, 2016) and myelin basic protein (MBP) levels are all elevated in RRMS and PMS compared to controls and may have utility as prognostic biomarkers of MS. Recently, NFL, chitinase 3-like protein 1 (CHI3L1) and chitinase 3-like protein 2 (CHI3L2) have been identified as useful in predicting the conversion to MS after a clinically isolated syndrome i.e. optic neuritis (Teunissen *et al.*, 2009a; Canto *et al.*, 2015; Hinsinger *et al.*, 2015; Martinez *et al.*, 2015; Mollgaard *et al.*, 2016) and the ratio of CHI3L1 to CHI3L2 is reported to distinguish RRMS and PMS patients from one another (Hinsinger *et al.*, 2015). Nevertheless, none of these potential biomarkers have entered into clinical utility, therefore, further work to identify and validate prognostic biomarkers of MS is crucial to allow better disease management and treatment.

Similarly to neurodegenerative disease, there are currently no curative therapies for MS. Symptomatic treatment with corticosteroids are used in RRMS to treat acute exacerbations and shorten the duration of the attacks (Goldenberg, 2012) and a number of disease-modifying therapies, targeting the inflammatory response (i.e. interferon beta-1b (The IFNB Multiple Sclerosis Study Group, 1993) are also available to reduce the chance of relapse, decrease disease activity and progression and reduce new lesion formation (Vargas and Tyor, 2017). Conversely, treatments for PMS are greatly lacking, with current RRMS disease-modifying drugs mostly failing in progressive disease and limited research into novel drug development for PMS due to lack of understanding of the underlying mechanisms and biomarkers for PMS (Feinstein *et al.*, 2015).

1.7.5 MS and mitochondrial dysfunction

Increasing evidence suggests a link between mitochondrial dysfunction and MS pathology. For example, demyelinated spinal cord lesions of MS patients show dramatically reduced numbers of mitochondria (Dutta *et al.*, 2006) and chronic active brain lesions demonstrate functional deficits in mitochondrial respiratory chain complexes (Lu *et al.*, 2000; Dutta *et al.*, 2006; Mahad *et al.*, 2008). Moreover, oxidative stress is reported to play a role in the pathogenesis of MS due to the high levels of ROS and RNS released by microglia and macrophages in the respiratory burst (Gilgun-Sherki *et al.*, 2004; Witte *et al.*, 2014) and numerous reports have implicated elevated oxidative stress as mediators of demyelination and axonal damage (Smith *et al.*, 1999; Lu *et al.*, 2000; Smith and Lassmann, 2002; Gray *et al.*, 2008). Similar findings are reported in *in vivo* experiments, using the experimental autoimmune encephalomyelitis (EAE) model of MS, identifying mitochondrial injury as a crucial factor in axonal degeneration and showing that detoxification of ROS could rescue the mitochondrial injury and axonal degeneration (Nikić *et al.*, 2011). Further support comes from studies investigating the mitochondrial genetics of MS. Similarly to PD, reduced neuronal mtDNA copy number is associated with late-onset MS (Blokhin *et al.*, 2008) and multiple deletions of mtDNA can be detected in the gray matter of MS patients (Campbell *et al.*, 2011). In addition, various mtDNA variants (i.e. G866A uncoupling protein 2 (Vogler *et al.*, 2005)) and haplogroup associations have been shown to influence the susceptibility of individuals to develop MS (reviewed in (Andalib *et al.*, 2013)), with haplogroup J increasing the risk (Tranah *et al.*, 2015) and haplogroup K decreasing the risk (Ban *et al.*, 2008; Hudson *et al.*, 2014). In addition, MS typically presents in conjunction with a subset of Leber's hereditary optic neuropathy patients (LHON), a primary mitochondrial disorder characterised by mtDNA mutations in complex I subunits of the mitochondrial respiratory chain (Mojon *et al.*, 1999). Thus, current literature indicates a strong mitochondrial association to MS pathogenesis.

1.7.6 MS and ccf-mtDNA

Ccf-mtDNA copy number levels have recently been investigated in both inflammatory RRMS and neurodegenerative PMS. Varhaug *et al.* (2017) quantified the ccf-mtDNA copy number in lumbar puncture CSF taken from patients with RRMS and healthy controls and concluded that RRMS patients had significantly higher ccf-mtDNA copy

number compared to controls, perhaps owing to the active inflammatory processes occurring. Conversely, Leurs *et al.* (2018) carried out a larger scale study to look at differences in ccf-mtDNA in RRMS, PMS and controls, using digital PCR. Despite a large degree of variation in their dataset, they reported a significant increase in ccf-mtDNA copy number in PMS patients compared to controls, but no significant differences in the RRMS patients. Therefore, further work is need to clarify this to untangle the true relationship between MS and ccf-mtDNA copy number.

1.8 Other Neurodegenerative conditions and mitochondrial dysfunction

It appears that despite huge heterogeneity between NDDs, almost all of these conditions converge at the level of the mitochondria and share aspects of mitochondrial dysfunction linked to underlying neuronal loss.

Dementia with Lewy bodies (DLB) has prominent association with mitochondrial dysfunction based on the hypothesis that α-synuclein interacts with the mitochondria directly. Mitochondria have been shown to be drawn into Lewy body aggregations present in DLB brains where they are damaged and lose their integrity, indicated by higher mtDNA deletion levels in Lewy body positive neurons compared to Lewy body negative neurons (Muller *et al.*, 2013; Power *et al.*, 2017). Furthermore, due to the shared genetic background of DLB and PD, a number of the familial nuclear gene mutations linked to mitochondrial dysfunction and PD (discussed in Section 1.5.5, Table 2) are also implicated in DLB i.e. *SNCA*, *PARKIN*, *PINK1*.

Similarly, various familial mutations implicated in causing amyotrophic lateral sclerosis (ALS), the most common type of motor neuron disease (MND), have links with mitochondrial abnormalities. Transgenic mouse lines expressing mutant *TDP-43*, *FUS* and *SOD1* present with mitochondrial swelling, deformed cristae, defects in OXPHOS machinery and reduced mtDNA copy number (Johri and Beal, 2012; Magrané *et al.*, 2012). Additionally, mutant and wild type SOD1 protein localise to the IMS, but only mutant SOD-1 mice developed a progressive ALS-like disease, characterised by weight loss, muscle weakness and brain atrophy, alongside spinal motor neuron degeneration and mitochondrial dysfunction (Igoudjil *et al.*, 2011).

Even extremely rare neurodegenerative diseases have shown links with mitochondrial dysfunction. For example progressive supranuclear palsy (PSP) patients typically show OXPHOS deficiency and cybrid lines expressing mitochondrial genes from PSP patients show reduced complex I activity, impaired ATP production and oxygen consumption as well as oxidative damage suggesting that mtDNA aberrations occur in PSP and may exacerbate neuronal loss and dysfunction (Swerdlow *et al.*, 2000; Albers *et al.*, 2001).

1.9 Research Hypotheses and Aims

1.9.1 Research Hypothesis

The development of novel biomarkers for neurodegeneration will be crucial in enabling early disease stage intervention and in the development of new therapies. I hypothesise that ccf-mtDNA copy number levels will be associated with the development and progression of NDDs, such as Parkinson's disease, Alzheimer's disease (AD) and progressive Multiple Sclerosis (PMS). I hypothesise that ccf-mtDNA copy number will be significantly different between NDD patients and matched controls and will correlate to clinical measures of disease. In addition, based on previous literature, I hypothesise that ccf-mtDNA has neuronal or cellular origins, whereby it is released into the CSF by active or passive mechanisms relating to cellular or mitochondrial quality control, cell death or cell signalling.

1.9.2 Research Aims

To address my hypotheses, I propose the following aims;

1. To expand previous analysis to other NDDs (such as AD, PD, dementia with lewy bodies, motor neuron disease, progressive supranuclear palsy and dementia), I aim to detect and quantify ccf-mtDNA in the post-mortem ventricular CSF, contrasting the levels of ccf-mtDNA between NDD patients and matched controls.
2. To replicate and validate findings from the Incidence of Cognitive Impairment in Cohorts with Longitudinal Evaluation – Parkinson's Disease (ICICLE-PD) (pilot) study, I aim to utilise a larger longitudinal cohort to assess the potential biomarker ability of lumbar CSF ccf-mtDNA copy number level to distinguish healthy controls from PD patients.
3. To investigate the relationship between CSF ccf-mtDNA copy number and NDD clinical and pathological phenotypes, I aim to correlate ccf-mtDNA copy number to assessments of disease severity and progression (such as Movement Disorders Society – Unified Parkinson's Disease Rating Scale (MDS-UPDRS) and MoCA).
4. To identify links between CSF ccf-mtDNA copy number and protein indicators of NDDs and gain understanding about the origin or mechanisms of release of ccf-mtDNA, I aim to correlate CSF protein levels with ccf-mtDNA copy number.
5. To investigate the integrity of the ccf-mtDNA and assess if it is important in the release of ccf-mtDNA, I aim to analyse the deletion level and mutational burden of the ccf-mtDNA and compare this between NDD patients and matched controls.

Chapter 2. Materials and Methods

2.1 Sample cohorts

2.1.1 ICICLE-PD

The Incidence of Cognitive Impairment in Cohorts with Longitudinal Evaluation – Parkinson's Disease (ICICLE-PD) study is a twin centre longitudinal observational study with an overarching aim of improving the understanding of the mechanisms that underly the evolution of PD dementia from disease onset (Yarnall *et al*., 2014). The cohort includes newly diagnosed PD patients (diagnosed between 2009 and 2011) and controls, followed up at 18 month intervals to track the progression of their disease.

Lumbar CSF samples

The ICICLE lumbar CSF cohort was used in preliminary experiments (Pyle et al., 2015b) in the discovery phase prior to this project. The cohort consisted of 53 Parkinson's disease (PD) patients (male n=34, female n=19) lumbar CSF samples, collected from consenting participants in the lateral or recumbent position by lumbar puncture. No control CSF was collected as part of this study and thus 10 control lCSF samples (male n=6, female n=4) were included from a different study.

Serum samples

The ICICLE cohort also contained a vast repository of serum samples from both PD patients (n=230, male n=142, female n=88) and controls (n=93, male n=49, female n=44) comprising a total of 323 individuals (Williams-Gray *et al.*, 2016). Peripheral blood samples were collected from participants and serum was extracted for use.

Phenotype data

Baseline PD patients were clinically diagnosed by a local neurologist, according to the United Kingdom Parkinson's Disease Society Brain Bank Criteria. Subjects were clinically and neuropsychologically assessed at baseline and at 18- and 36-month follow ups. Assessments included disease duration, family history, medication history, Movement Disorders Society-revised Unified Parkinson's Disease Rating Scale (MDS-UPDRS) with Hoehn and Yahr stage, Mini Mental State Examine (MMSE), Montreal Cognitive Assessment (MoCA) and Geriatric Depression Scale-15 (GDS-15).

Variable	Serum		CSF	
	Control (n=93)	PD (n=230)	Control (n=10)	PD (n=53)
Gender (M/F)	49/44	142/88	6/4	34/19
Age (years)	68.0±8.0	66.4±9.5	61.2±7.6	66.5±10.5
Disease duration (months)	-	6.0±5.0	-	5.4±4.9

Table 2.1. Demographic characteristics of serum and cerebrospinal fluid (CSF) ICICLE-PD subjects at study enrolment. Values are mean±SD, except gender which is number of male/female. N numbers in brackets. PD; Parkinson's disease.

2.1.2 PPMI-PD cohort

The Parkinson's Progression Markers Initiative (PPMI) study is a multicentre observational clinical study which aims to identify biomarkers of PD progression through imaging, biological sampling and clinical and behavioural assessment. Clinical sites are based within the USA, Europe, Isreal and Australia with all data and samples made available to collaborators within a central database and biorepository (Parkinson's Progression Markers Initiative, 2018).

Lumbar CSF samples

The PPMI-PD cohort comprised a total of 905 lumbar puncture CSF samples from patients at study baseline (BL) and at a 3 year follow up (V08). These samples were obtained through the Parkinson Progression Markers Initiative (PPMI) in collaboration with the Michael J Fox foundation (MJFF). The baseline group consisted of 541 samples, divided into 169 controls (mean age 60.3±11.4, male n=108, female n=61) and 372 PD patients (mean age 61.3±9.7, male n=241, female n=131). Due to patient drop-out over time the follow-up group was reduced by one third to 114 controls (mean age 63.7±10.9, male n=72, female n=42) and 250 PD patients (mean age 63.5±9.3, male n=166, female n=84).

Phenotype data

All PD cases had a clinical diagnosis of PD for two years or less and were not taking PD medication for their symptoms at baseline. All control participants were 30 years or older, did not have PD or prodromal signs of PD and did not have a first degree

blood relative with PD. PD cases and healthy controls underwent clinical and neuropsychological testing and imaging and biospecimen assessment at enrolment and throughout the longitudinal study (Marek *et al.*, 2011). Assessments included disease duration, family history, medication history, MDS-UPDRS with Hoehn and Yahr stage, MoCA, GDS, self-reported autonomic symptoms in PD (SCOPA-AUT), sleep characterisation, olfactory testing (UPSIT), dopamine transporter imaging (DaTSCAN), magnetic resonance imaging (MRI), genotyping with NeuroX or Immunochip arrays (Nalls *et al.*, 2016) and numerous protein measurements (alpha-synuclein, amyloid, tau etc) in the blood and CSF.

Variable	***Baseline***		***36 months (V08)***	
	Control (n=169)	***PD (n=372)***	***Control (n=114)***	***PD (n=250)***
Gender (M/F)	108/61	241/131	72/42	166/84
Age (years)	60.3±11.4	61.3±9.7	63.7±10.9	63.5±9.3
Disease duration (months)	-	6.6±6.5	-	42.8±6.8

Table 2.2. Demographic characteristics of PPMI-PD subjects. Values are mean±SD, except gender which is number of male/female. N numbers in brackets. PD: Parkinson's Disease.

2.1.3 NDD-vCSF cohort

Samples in the NDD-vCSF cohort were obtained from the Newcastle Brain Tissue Resource (NBTR), a repository of fixed and frozen human brain tissue and fluid which have been donated for research into dementia and neurodegenerative disease (Newcastle University, 2018).

Ventricular CSF samples

A total of 136 post-mortem ventricular CSF samples made up the NDD-vCSF cohort, with 92 neurodegenerative disease (NDD) cases (PD n=8, AD n=45, DLB n=15, Dementia n=11, MND n=8, PSP n=5) and 27 age matched controls (mean age 75±14, male n=18, female n=26) obtained from the Newcastle Brain Tissue Resource (NBTR) (Newcastle University, Newcastle Upon Tyne). Another 17 age matched controls were obtained from the MS Society Tissue Bank (Imperial College, London).

Phenotype data

All NDD cases were clinically diagnosed in life and both cases and controls were neuropathologically confirmed post-mortem i.e. controls were negative for hallmarks of disease related neurodegeneration or inflammation. The NBTR provided demographic, phenotypic and neuropathological data when available, including age at death, disease duration, post-mortem interval, clinical diagnosis, neuropathological diagnosis, Tau Braak staging and dementia severity (MMSE).

Variable	***Control (n=44)***	***PD (n=8)***	***AD (n=45)***	***DLB (n=15)***	***Dementia (n=11)***	***MND (n=8)***	***PSP (n=5)***
Gender (M/F)	18/26	5/3	23/22	10/5	9/2	2/6	4/1
Age at death (years)	75±14	80±8	83±10	80±8	72±13	73±12	77±16
Disease duration (years)	-	10±5	7±4	8±4	6±4	2±1	4±2
Post-mortem interval (hours)	38±24	57±28	38±27	42±25	42±25	25±14	61±19

Table 2.3. Demographic characteristics of NDD-vCSF subjects at study enrolment. Values are mean±SD, except gender which is number of male/female. N numbers are in brackets. PD: Parkinson's Disease, AD: Alzheimer's Disease, DLB: Dementia with Lewy Bodies, MND: Motor Neuron disease, PSP: Progressive Supranuclear Palsy.

2.1.4 MS-vCSF cohort

Samples in the MS-vCSF cohort were obtained from the Multiple Sclerosis and Parkinson's Tissue Bank (MSSTB), a national collection of central nervous system tissue samples donated by individuals with MS, PD or related neuroinflammatory and neurodegenerative conditions (Imperial College London, 2018).

Ventricular CSF samples

The MS-vCSF consisted of 81 ventricular CSF samples. The controls used in this cohort (n=44) are the same as those used in the NDD-vCSF cohort along with 37

Progressive Multiple Sclerosis (PMS) samples (mean age 70±15, male n=19, female n=18) obtained from the MS Society Tissue Bank (MSSTB, Imperial College, London).

Phenotype data

All PMS cases were clinically diagnosed in life and re-confirmed neuropathologically post-mortem. The MS Society Tissue bank provided demographic and neuropathological reports for each patient including age at death, disease duration, post-mortem interval, MS diagnosis (RRMS, PMS), brain weight and CSF pH.

Variable	***Control (n=44)***	***PMS (n=37)***
Gender (M/F)	18/26	19/18
Age at death (years)	75±14	70±15
Disease duration (years)	7±10	26±14
Post-mortem interval (hours)	38±24	24±18

Table 2.4. Demographic characteristics of MS-vCSF subjects at study enrolment. Values are mean±SD, except gender which is number of male/female. N numbers are in brackets. PMS: Progressive Multiple Sclerosis.

2.2 CSF Sample Collection and Preparation

2.2.1 Lumbar CSF cohorts

ICICLE lumbar puncture CSF was carried out on participants in the recumbent or sitting position within 4 months of initial cognitive assessments. Samples were visually inspected for blood, then centrifuged at 2,000g for 10 minutes, within 15 minutes of collection. Supernatant was aliquoted into polypropylene cryovials which do not influence biomarker outcome (Teunissen *et al.*, 2009b) and frozen at -80°C.

PPMI lumbar CSF was collected by lumbar puncture between L3-L4, L4-L5 or L5-S1 spinal vertebrae from individuals in lateral recumbent or sitting position. Cell counts, total protein and glucose levels were assessed within 4 hours of collection and samples were visually inspected for blood contamination. Samples were transferred to polypropylene cryovials, centrifuged at 2,000g for 10 minutes at RT, then aliquoted and stored at -80°C at the central repository. Samples were shipped overnight on dry ice. Once received samples were thawed and mixed and 50µl aliquots were taken and stored in 96-well MicroAmp™ reaction plates (Thermo Fisher Scientific, Paisley, UK) at 4°C ready for quantitative PCR. Remaining CSF was stored at -80°C.

2.2.2 Ventricular CSF Cohorts

All ventricular CSF was obtained post-mortem, directly syringed from hemisected ventricles of the brain and immediately frozen at -80°C in polypropylene cryovials. Samples were prepared similarly to lumbar CSF. Briefly, samples were inspected for blood contamination, thawed and centrifuged at 2,000g, for 10 minutes. Supernatant was further centrifuged at 15,000g for 10 minutes, then 20µl aliquots were taken and stored in the same way as lumbar CSF.

2.3 qPCR Standard Template Generation

Nuclear gene *Beta-2-Microglobulin* (*B2M*) (1092bp; Genbank accession ID: NM_004048) was used as the reference housekeeping gene, whilst mitochondrial genes *MTND1* and *MTND4* (1040bp and 1072bp; Genbank accession IDs: *MTND1* and *MTND4* NC_012920) were used as the reference mtDNA for the standard curve. Opposing positions of *MTND1* and *MTND4* in the mtDNA are displayed in Figure 2.1.

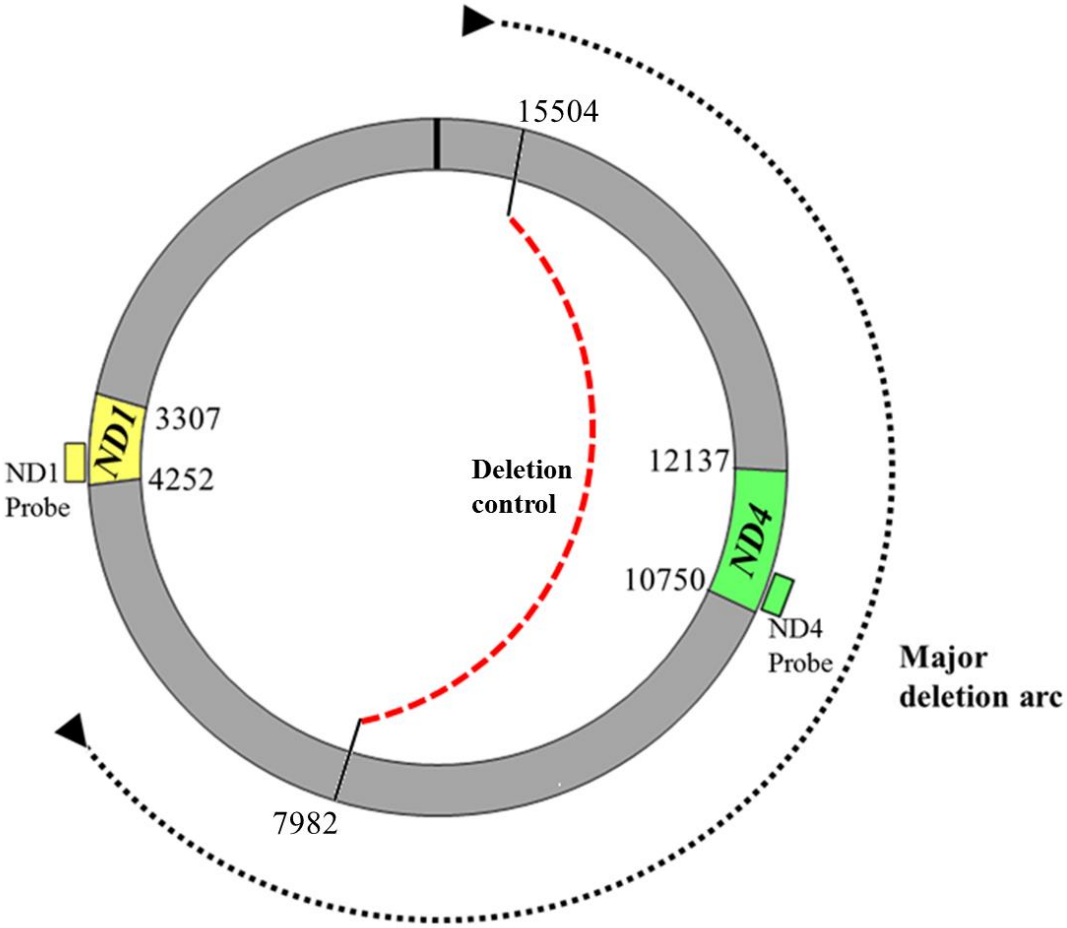

Figure 2.1. Schematic of the mitochondrial genome highlighting the base pair position of the MTND1 (yellow) and MTND4 (green) genes. Numeric values indicate base pair position. Approximate binding sites for the two mitochondrial Taqman probes are also shown in blocks. The outer black dotted line illustrates the major deletion arc of the mitochondrial genome. The inner red dotted line displays the region of large-scale deletion within the mtDNA deletion control.

Gene-specific forward and reverse primers (Integrated DNA Technologies, Leuven, Belgium) were used to amplify the control DNA, which was subsequently used to generate standard curves for comparative copy number analysis (Table 2.5).

	GENE	PRODUCT (BP)	FORWARD SEQUENCE	REVERSE SEQUENCE
PCR TEMPLATE:	*B2M*	1092	CGCAATCTCCAGTGACAGAA	GCAGAATAGGCTGCTGTTCC
	MTND1	1040	CAGCCGCTATTAAAGGTTCG	AGAGTGCGTCATAGTTGTTC
	MTND4	1072	ATCGCTCACACCTCATCTCC	TAGGTCTGTTTGTCGTAGGC
QPCR PRODUCTS:	*B2M*	231	CACTGAAAAAGATGAGTATGCC	AACATTCCCTGACAATCCC
	MTND1	111	ACGCCATAAAACTCTTCACCAAAG	GGGTTCATAGTAGAAGAGCGATGG
	MTND4	107	ACCTTGGCTATCATCACCCGAT	AGTGCGATGAGTAGGGGAAGG

Table 2.5. Forward and reverse 5'-3' primer sequence used in PCR and qPCR. Product size generated from PCR and qPCR are shown.

	GENE	FLUOROPHORE	QUENCHER	SEQUENCE
TAQMAN PROBES:	*B2M*	FAM	BHQ_1	CCGTGTGAACCATGTGACTTTGTC
	MTND1	HEX	BHQ_1	ACCCGCCACATCTACCATCACCCTC
	MTND4	Cy5	BHQ_2	CAACCAGCCAGAACGCCTGAACGCA

Table 2.6. 5'-3 sequence for Taqman probes. Gene specific fluorophore and quencher are displayed.

2.3.1 Polymerase Chain Reaction

All reactions were set up on ice. The total 24µl mastermix consisted of nuclease-free H2O, 1x MyTaq™ reaction buffer (contains MyTaq™ buffer, dNTPs, MgCl2, enhancers and stabilisers), 400nM forward and reverse primer and 1U MyTaq HS DNA Polymerase (Bioline, London, UK). 50ng DNA was loaded into 10 wells of a 96 well plate with the PCR mastermix, alongside a negative control containing only the PCR mastermix. The reaction was run on an Applied Biosystems® Veriti® 96 well thermal cycler (Thermo Fisher Scientific) with the following program: 1) activation step for one min at 95°C 2) 30 cycles of; denaturation for 15s at 95°C, annealing for 15s at 61°C and extension for 10s at 72°C 3) final extension for 10 mins at 72°C.

2.3.2 Agarose Gel Electrophoresis

The post-PCR products were pooled (n=10) and combined with Orange G loading dye (50% glycerol, Orange G powder (Sigma Aldrich Company Ltd, Dorset, UK) and 50% water). Positive PCR products were loaded into one single large well on a 1% agarose gel (1% w/v agarose (Bioline): 1g agarose:100ml 1x tris-acetate-EDTA (TAE) buffer (Omega Bio-tek, GA, USA), 4mg ethidium bromide (Invitrogen, Paisley, UK)). DNA ladder and negative products were loaded into separate wells, after combination with loading buffer. Agarose gels were electrophoresed at 70V for 45 mins in 1x TAE buffer (Lee *et al*., 2012).

2.3.3 Gel DNA extraction and quantification

Gels were imaged using the UVP GelDoc-It™ imaging system (UVP, Cambridge, UK) and the DNA bands were cut from the gel under ultraviolet light. DNA was extracted from the gel using QIAquick Gel extraction kit; microcentrifugation protocol, according to manufacturer's instructions (QIAGEN, Manchester, UK). DNA concentration (ng/µl) and 260/280nm wavelength ratio were calculated using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific). DNA was considered pure if the 260/280nm ratio was between 1.8 and 2.0.

2.4 Plasmid cloned DNA template

Due to the vast number of samples in the PPMI-PD cohort (Section 2.1.2), standard template generation was changed to the plasmid cloned method, described in detail in Rygiel *et al.* (2015). Essentially, this provides a large reserve of readily available template DNA to be reused in subsequent qPCR experiments and normalises the template material to be used for standard curve generation, thereby reducing inter-plate variability.

2.5 Copy number calculation

Equation 1 was used to calculate standard DNA copy number. All standard DNA was then diluted to approximately $1x10^{10}$ ng/µl and then multiplexed together to a dilution of $1x10^{9}$ng/µl in nuclease-free water.

$$Copy\ Number = [C \div (L\ x\ 2\ x\ 330)]\ x\ A$$

Equation 1: Formula used to calculate DNA copy number in copies/µl. C is DNA concentration in nanolitres (10^{-9}), L is amplicon length in base pairs and A is Avogadro's constant (6.023×10^{23}).

2.6 Quantitative PCR for mtDNA copy number quantification

The qPCR reaction plate was set up in a UV-treated PCR hood to minimise DNA contamination. A ten-fold serial dilution of $1x10^{8}$ to $1x10^{2}$ ng/µl was prepared using the multiplexed $1x10^{9}$ DNA standards. CSF samples were ran neat, whilst 2 plate calibrators were diluted 1:100 from stock and used as the deletion and non-deletion mtDNA controls. The deletion mtDNA control (provided by Professor CT Moraes, Miller School of Medicine, University of Miami, USA) was sourced from cybrid cell lines containing a single large-scale mitochondrial deletion in the major deletion arc (position m.7982 to 15504, highlighted in Figure 2.1 (red dotted line)) with approximately 70% heteroplasmy. Non-deletion control mtDNA was extracted from whole blood of healthy controls.

Nineteen µl of qPCR mastermix (1x iTaq™ Universal Probes Supermix (BioRad, Hertfordshire, UK), 70nM F&R *MTND1* and *MTND4* primers, 300nM F&R *B2M* primers, 200nM *MTND1*, *MTND4*, *B2M* probes and dH20) were loaded into a Hard-Shell® 96-well PCR plate (BioRad, Hertfordshire, UK) with 5ng DNA or 1µl CSF. Details of the qPCR primer sequences and Taqman probes can be found in Table 2.5 and Table 2.6. All DNA standards, controls and samples were ran in triplicate or quadruplicate on a CFX96 Touch™ Real-Time PCR detection system (BioRad, Hertfordshire, UK). The program was; initial denaturation (95°C for three mins), followed by 40 cycles of: denaturation (95°C for 10s) and annealing and extension (62°C for one min).

2.7 Copy number calculation

Bio-Rad CFX manager 3.1 software was used to analyse the qPCR data. Outlier replicates more than 0.5 Ct different from each other were excluded. The standard dilution series was analysed to generate the most efficient standard curve for each fluorophore using the control DNA plate normalisers. *B2M* (FAM) threshold was set and Ct values were recorded for deletion and non-deletion controls. Thresholds for *MTND1* (HEX) and *MTND4* (Cy5) were set relative to Ct values of *B2M* non-deletion control DNA. There was a consistent difference of approximately 6.9 Ct and 1.26 Ct values, for *MTND1* and *MTND4* respectively, between runs. Standard curves were only acceptable when negative control contamination was above 35 Ct values and when efficiencies were between 90-110%.

Once standard curves were running efficiently and parallel to each other, CSF samples were analysed. Again replicates greater than 0.5 Ct difference were removed and sample copy number was generated from an average of all included replicates. Samples with more than 10 nDNA copies (*B2M* SQ >10) were excluded from the study. *MTND1* and *MTND4* copy number was calculated as an average of the raw HEX and Cy5 SQ value, respectively.

2.8 Protein quantification

Total protein concentration of CSF samples was measured via the Qubit™ Protein Assay Kit (ThermoFisher Scientific) on the Qubit 3.0 Fluorometer. Pre-diluted BSA standards were used to calculate protein concentrations of the 200x diluted CSF samples.

2.8.1 Western Blot

Western blot techniques were optimised for ventricular CSF within Chapter 3 section 3.3.1. Finalised experimental protocols are presented below.

SDS PAGE Gel Electrophoresis

Samples were loaded in 20µg protein quantities, mixed with 4µl NuPAGE™ LDS Sample Buffer (4X; Life Technologies), 2.5µl NuPAGE™ reducing agent (ThermoFisher Scientific) and dH20 to make a total sample volume of 25µl. Samples were boiled at 70°C for 10 minutes and then loaded into a NuPAGE™ Novex™ 4-12% bis-tris polyacrylamide gel (ThermoFisher Scientific). Molecular weight of the sample bands was estimated by comparison to See-Blue™ Plus2 Pre-stained Protein standard (ThermoFisher Scientific). Samples were run in 1X NuPAGE™ MES SDS Running buffer (ThermoFisher Scientific), with 500µl NuPAGE™ Antioxidant additionally added to the internal chamber, at 160v for an hour.

Protein transfer

Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using an iBlot™ 2 dry blotting system and Invitrogen™ Midi Transfer stack (Thermo Fisher Scientific) and then blocked in 5% milk Tris buffered saline with Tween (TTBS) solution (20ml Tris-HCl pH 7.5, 29.2g NaCl, 1ml Tween20, made up to 1 litre with dH20) for 1 hour on a Stuart® see-saw rocker (Cole-Parmer).

Antibodies

Primary antibodies were diluted in 5% milk TTBS solution and incubated overnight at 4°C on a Stuart® roller-mixer (Cole-Parmer). The antibody was removed and membrane washed 3 X 10mins in TTBS. Appropriate HRP conjugated secondary antibody was diluted in 5% milk TTBS solution, incubated with the membrane at RT

for one hour, and then washed 3 X 10mins in TTBS. Information regarding antibody dilution, incubation, species and manufacturer can be found in Table 2.7.

Antibody	***Dilution***	***Incubation***	***Molecular weight (kDa)***	***Manufacturer***	***Catalogue No.***	***Species***
Primary						
Beta Actin	1:2000	Overnight	42	Abcam	ab8226	Mouse
Tubulin	1:4000	Overnight	50	Abcam	ab59680	Rabbit
NSE	1:1000	Overnight	47	Abcam	ab180943	Rabbit
14-3-3 Beta	1:500	Overnight	28	Abcam	ab16859	Mouse
14-3-3 Zeta	1:500	Overnight	28	Abcam	ab188368	Mouse
SDHA	1:500	Overnight	70	Abcam	ab14715	Mouse
Porin	1:500	Overnight	39	Abcam	ab14734	Mouse
Tfam	1:500	Overnight	29	Abcam	ab119684	Mouse
Total OXPHOS cocktail	1:1000	Overnight	Multiple	Abcam	ab110411	Mouse
Synaptophysin	1:500	Overnight	42	Abcam	ab32127	Rabbit
Tau	1:500	Overnight	45-65	Cell Signalling	4019	Mouse
α-Synuclein	1:500	Overnight	14	Abcam	ab138501	Rabbit
TrkB	1:500	Overnight	110	Cell Signalling	4603	Rabbit
NeuN	1:500	Overnight	48	Merck	MAB377	Mouse
S100 beta	1:500	Overnight	11	Abcam	ab14849	Mouse
GFAP	1:500	Overnight	48	Abcam	ab10062	Mouse
MBP	1:500	Overnight	33	Abcam	ab62631	Mouse
Secondary						
Anti-Rabbit	1:2000	1h		Agilent	P0399	Swine
Anti-Mouse	1:2000	1h		Agilent	P0260	Rabbit

Table 2.7. List of all antibodies used within the study. Information regarding dilution factor, incubation time, molecular weight of target, species and manufacturer details are included. Overnight refers to 16-20 hour incubation at 4°C on a roller. NSE; neuron specific enolase, SDHA; succinate dehydrogenase, Tfam; mitochondrial transcription factor A, OXPHOS; oxidative phosphorylation, TrkB; Tropomyosin receptor kinase B, NeuN; Neuronal nuclei, GFAP; glial fibrillary acidic protein, MBP; myelin basic protein.

To visualise probed proteins, membranes were exposed with Clarity™ Western ECL Blotting Substrate (Clarity Western peroxide solution: Clarity Western luminol/enhancer solution, 1:1, Bio-Rad) for five minutes in the dark. Blots were then imaged using the Amersham Imager 600 (GE Healthcare Life Sciences) and semi-quantitative protein analysis was carried out using ImageQuant TL 1D v8.1. Protein measurements were normalised to total protein levels.

Protein loading control

Normalisation to total protein levels has emerged as a more reliable alternative to housekeeping proteins (Collins *et al.*, 2015). Following protein transfer to the PVDF membrane, the reversible total protein stain Blot FastStain™ (G-Biosciences) was applied to the membrane as per manufacturer's instructions. Stained blots were imaged and total protein was quantified using the Amersham Imager 600 (GE Healthcare Life Science) and analysed using ImageQuant TL 1D v8.1. An example membrane is shown in Figure 2.2, showing the highly specific binding of Blot FastStain™ to all proteins present on the PVDF membrane.

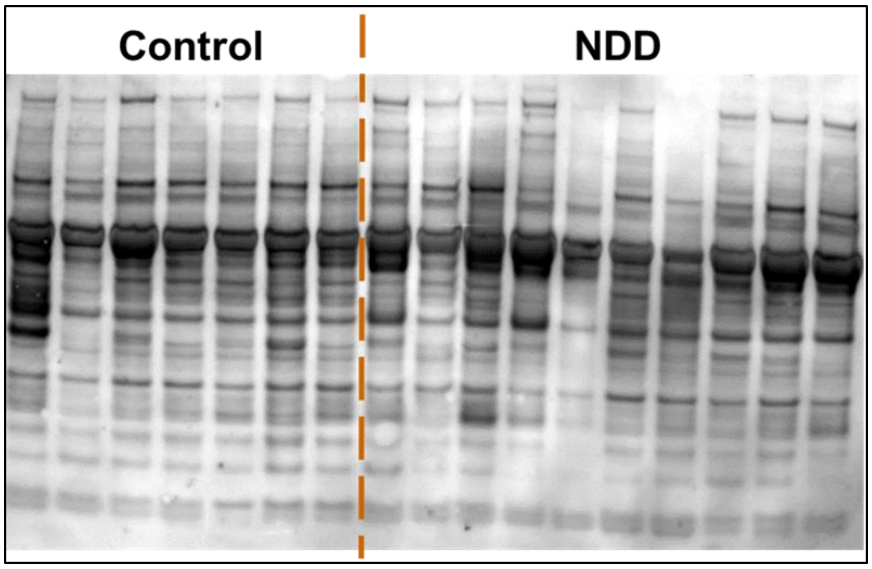

Figure 2.2. Example Blot FastStain™ membrane. All proteins are reversibly bound and can be quantified to give total protein in lane to be used for normalisation of proteins of interest. Orange dashed line separates controls and neurodegenerative disease (NDD) patients.

Membrane was de-stained using warm dH2O as per manufacturers recommendation and membrane was washed with TTBS and then blocked with 5% milk powder TTBS solution for one hour at RT prior to primary antibody incubation.

2.8.2 ELISA

All enzyme-linked immunosorbent assays (ELISAs) were carried out as per manufacturer's instructions, detailed in their respective results chapters. All ELISA plates were read using the Varioskan LUX multimode microplate reader and SkanIT 4.1 software (ThermoFisher Scientific). Concentrations for standards, and dilution factors of samples are loaded into the software prior to measurement. Plates are inserted into the machine and mixed for 10-15 seconds, then optical density is read at a wavelength of 450nm. Standard curves were generated for each plate and unknown sample concentrations were read from this using the equation of the line. Example standard curves are shown in Figure 2.3 below.

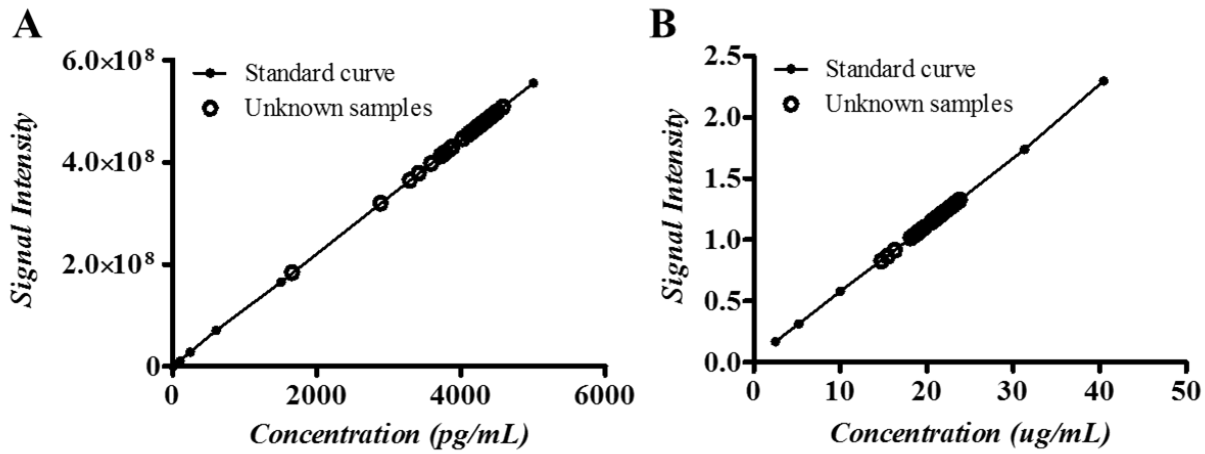

Figure 2.3. Example standard curves from ELISA for alpha synuclein (A) and CHI3L2 (B) proteins. The concentration of the unknown samples are extrapolated from the standard curve.

2.9 DNA extraction

Mitochondrial DNA was extracted from ventricular CSF using UltraPure™ (phenol:chloroform:isoamyl alcohol) optimised from the original manufacturers protocol. Briefly, 800µL Ultrapure solution was mixed with 400µL CSF samples via vortex, and incubated at room temperature for 10 minutes in a 2mL screw-cap graduated conical tube (Starlab). Samples were centrifuged 10,000g for 30 minutes and incubated at -80°C for 48 hours. Samples were thawed and centrifugation was repeated for 30 minutes. The upper aqueous phase (~200µl) was transferred to a new 1.5mL eppendorf (Starlab) for ethanol precipitation. As per manufacturer instructions, 1µL glycogen (20µg/µL), 200µL 7.5M Ammonium Acetate (NH_4OAc) and 1mL 100% ethanol were added to the aqueous fraction and incubated at -80°C overnight to precipitate the DNA from the sample. Samples were centrifuged for 30 mins at 16,000g and supernatant was removed from the pellet. The pellet was washed twice with 70% ethanol and then dried at room temperature for 10 minutes. Pellets were resuspended in 20µL nuclease-free water and stored at -20°C.

2.10 mtDNA enrichment

Rigorous optimisation steps were required to enable successful amplification of the vCSF ccf-mtDNA. These steps are discussed in section 3.3.2, and only finalised protocols are included below.

2.10.1 Long Range PCR

mtDNA was enriched prior to sequencing using three-fragment long-range PCR amplification with Takara PrimeSTAR GXL DNA polymerase (Takara Bio Company). Primers sequences are detailed in Table 2.8, and primer pairs are mapped to show coverage of the whole mitochondrial genome in Figure 2.4.

PCR Fragments	*Product Size (bp)*	*Forward sequence*	*Reverse sequence*
1	6154	CAGCCGCTATTAAAGGTTCG	GTTGTCGTGCAGGTAGAGG
2	6102	ACCACCCAACAATGACTAATC	GGTTGTTTGATCCCGTTTCG
3	4436	ATTCATCGACCTCCCCACC	GGAGGGGGGTTCATAGTAG

Table 2.8. Gene product size and primer sequences for three fragment long range PCR of circulating cell free mtDNA in the CSF.

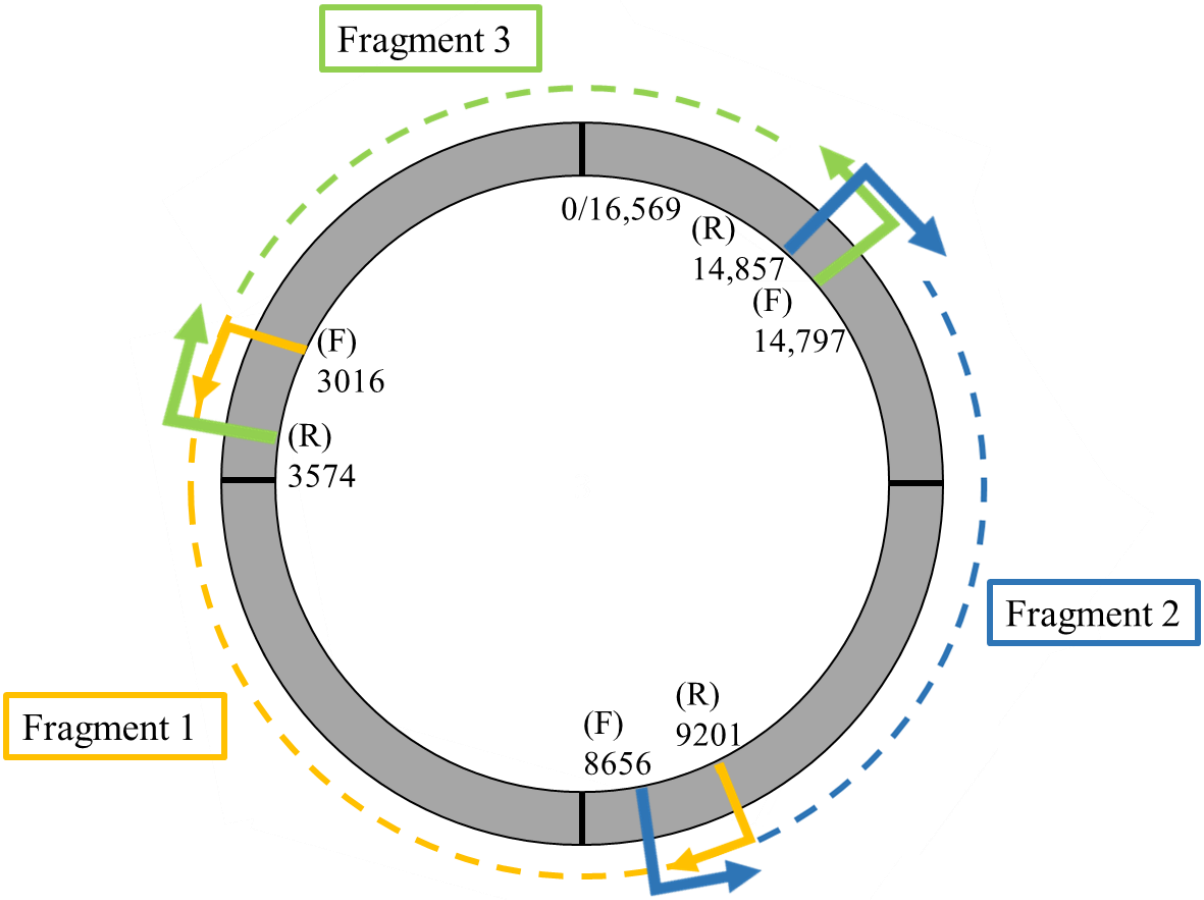

Figure 2.4. Schematic of the mitochondrial genome, mapped with the three mtDNA sequencing primers pairs; 1 (yellow), 2 (blue) and 3 (green). Numeric values indicate the base pair position. (F) indicates forward primer binding site position, (R) indicates reverse primer binding site position.

Reactions were set-up in a DNA-free UV treated hood to minimise contamination. The 23µL total mastermix consisted of 15µL nuclease-free H_2O, 5µL 5X PrimeSTAR GXL Buffer (Mg^{2+} plus), 2µL dNTP Mixture (2.5mM), 0.5µL forward primer (10mM). 0.5µL reverse primer (10mM) and 0.5µL PrimeSTAR GXL DNA Polymerase. 2µL of extracted mtDNA was loaded with the PCR mastermix and the reaction was run on an Applied Biosystems® Veriti® 96 well thermal cycler (Thermo Fisher Scientific) with the following program: 1) activation step for five min at 94°C 2) 35 cycles of; denaturation for 10s at 98°C and annealing for seven min at 68°C 3) extension for 10 mins at 72°C.

2.11 Next Generation Sequencing (NGS)

2.11.1 Product purification and pooling

Following mtDNA enrichment, mtDNA products were purified using AgenCourt AMPure XP bead technology (Beckman Coulter). AMPure XP beads were added in a 1.8µL bead:1µL product ratio in a 96 well PCR plate (Applied Biosystems) and incubated for five minutes to allow PCR amplicons to bind to the paramagnetic beads. The PCR plate was transferred to the magnetic plate (96S Super Magnet Plate, ALPAQUA) for two minutes to separate beads and bound DNA product from contaminants. Liquid was removed from the plate without disturbing the bead ring and beads were washed twice with 70% ethanol. Beads were left to air dry and then eluted into 20µL of nuclease-free H_2O, pipette mixed thoroughly and incubated for two minutes off the magnet. The plate was transferred back to the magnet and eluate was transferred to a new 96 well PCR plate.

Following purification mtDNA concentration was measured via the Qubit™ dsDNA Broad Range Assay kit (ThermoFisher Scientific) on the Qubit 3.0 Fluorometer. DNA standards were used to calculate the DNA concentration of the purified mtDNA products as per manufacturer's instructions. Each individual, purified mtDNA fragment was diluted to 1ng/µL, then all three fragments comprising each sample were pooled together to create a 15nM mtDNA pool, as shown in the schematic below (Figure 2.5).

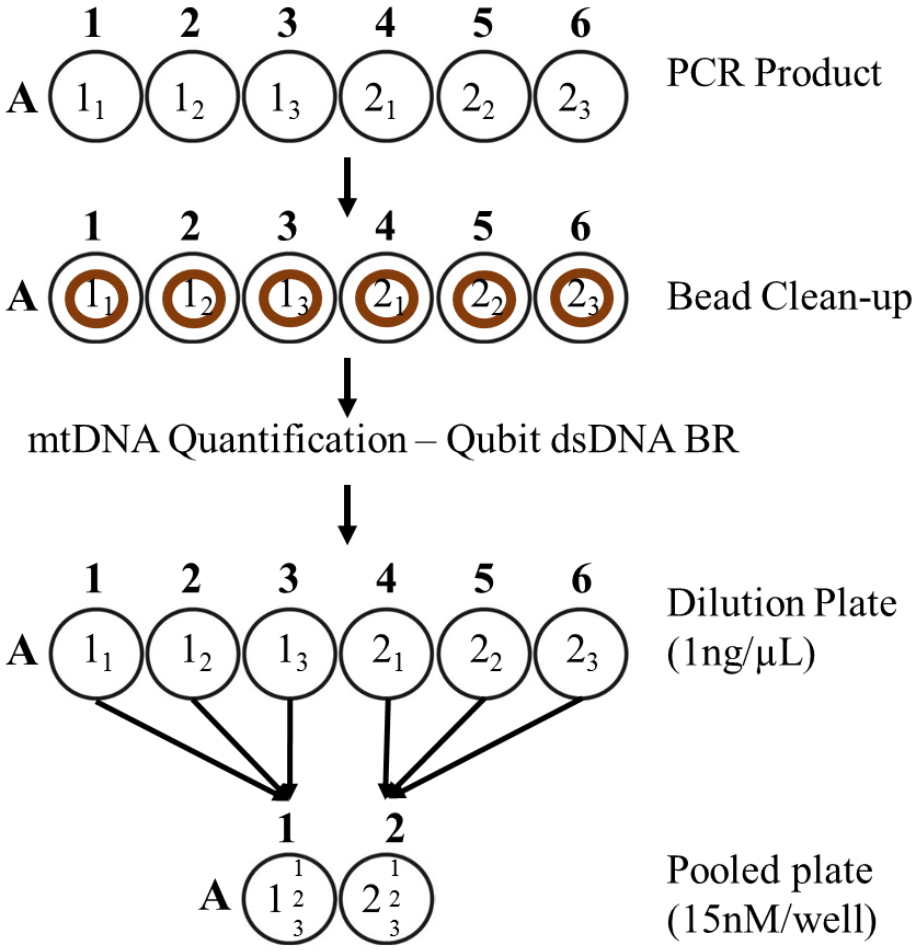

Figure 2.5. Schematic work flow of purification (Bead Clean-up), dilution (Dilution Plate) and pooling (Pooled plate) of amplified circulating cell free mtDNA product. A1-6 represent well positions with samples labelled $1_{1\text{-}3}$ and $2_{1\text{-}3}$ representing the PCR fragments 1-3. Bead clean up was carried out with Ampure XP beads (brown ring), then samples were diluted to 1ng/µl and pooled.

2.11.2 Library Preparation and MiSeq run

Samples in the pooled 15nM plate were further diluted down to 0.2ng/µL. Library preparation was carried out using the Nextera XT DNA Library Preparation Kit (Illumina) according to manufacturer's instructions. Very briefly, DNA was tagmented and subsequently amplified using the Nextera XT Index Kit. The DNA library was cleaned using the AMPure XP beads similarly to methods described previously (in Section 2.11.1) and 20µL of cleaned library was transferred to a new plate and bead based normalisation was carried out. Normalised libraries were then pooled and prepared for sequencing using the MiSeq Reagent Kit and the Illumina MiSeq v3.0 sequencing platform in paired-end, 250bp reads.

2.12 Bioinformatic analysis

Post-run FASTQ files were analysed using an established in-house bioinformatics pipeline. Briefly, reads were aligned to the Hg19 using BWA v0.7.10 invoking –mem (Li and Durbin, 2009). Aligned reads were sorted and indexed using Samtools v0.1.18 (Li *et al.*, 2009), duplicate reads were removed using Picard v1.85 (http://broadinstitute.github.io/picard/). Variant calling was performed using VarScan v2.3.8 (minimum depth =100bp, support reads = 10, base-quality = >30, mapping quality = >10 and variant threshold = 0.05) (Koboldt *et al.*, 2009). Variants were annotated with ANNOVAR v529 (Wang *et al.*, 2010). Heteroplasmic variants are defined as >1% minor allele frequency.

2.13 Electron Microscopy

CSF samples were pelleted by centrifugation at 14,000 rpm for 20 minutes. Supernatant was discarded and pellets were fixed in 2.0% glutaraldehyde in 0.1M cacodylate buffer. The following procedures were carried out by the Electron Microscopy Services (Newcastle University, UK). Samples were rinsed in cacodylate buffer and post-fixed in 1.0% osmium tetroxide for 1 hour. Samples were then dehydrated in graded acetone and embedded into Taab medium epoxy resin. After polymerisation, ultrathin sections (70nm) were collected on copper grids and stained with uranyl acetate and lead citrate and imaged on a Philips CM100 Transmission Electron Microscope (TEM) under the guidance of Dr Kathryn White at the Newcastle University Electron Microscopy Service.

Chapter 3. Ccf-mtDNA copy number variation in broad Neurodegenerative Disease - NDD-vCSF cohort.

3.1 Introduction

Mitochondrial DNA (mtDNA) copy number is found to be associated with neurodegenerative disease (NDD), with vulnerable disease-associated brain regions showing depleted levels of mtDNA compared to neurologically healthy controls (Petersen *et al.*, 2014; Rice *et al.*, 2014; Pyle *et al.*, 2015a; Dölle *et al.*, 2016; Grunewald *et al.*, 2016; Wei *et al.*, 2017). The same significant trend is also reported for lumbar CSF (lCSF) circulating cell free mtDNA (ccf-mtDNA) copy number. A pilot study carried out using the ICICLE-PD cohort (described in Section 2.1.1 (Pyle *et al.*, 2015b)) established that reduced ccf-mtDNA copy number in the lCSF could be potentially utilised as a biomarker for sporadic PD, based upon receiver operating curve (ROC) analysis (area under curve = 0.81 (Pyle *et al.*, 2015b)). Similarly, Podlesniy *et al.* (2013), also reported the same association in AD patients, raising the possibility that reduced ccf-mtDNA copy number could be a biomarker for broader NDD (Podlesniy *et al.*, 2013; Podlesniy *et al.*, 2016a).

As discussed in the introduction (Sections 1.4, 1.5.4, 1.6.4 and 1.7.4), there are very few clinical diagnostic biomarkers for NDDs, and the gold standard for diagnosis is still based on neuropathological examination at autopsy (Rachakonda *et al.*, 2004). Therefore, discovery of a biological marker, such as CSF ccf-mtDNA copy number could have huge implications in the future diagnosis and early-management of these diseases.

Although preliminary studies in PD and AD are promising (Podlesniy *et al.*, 2013; Pyle *et al.*, 2015b; Podlesniy *et al.*, 2016a; Podlesniy *et al.*, 2016b), ccf-mtDNA copy number has never been studied in a diverse range of NDD patients and conclusions from current literature are limited by low population size and lack of large-scale replication.

Previous studies of ccf-mtDNA copy number have focussed on the use of lCSF extracted from participants in-life through lumbar puncture biopsy. However, no studies have investigated ccf-mtDNA in the ventricular portion of the CSF (vCSF). Previous work has indicated differences in the composition of the lCSF and vCSF in both health and disease (Rubalcava and Sotelo, 1995; Sommer *et al.*, 2002) and due to the localisation of the vCSF to the ventricles rather than spinal cord this fluid may be advantageous over lCSF in studies of NDD as this is closer to the lesion site (Jeromin and Bowser, 2017). Additionally, as the vCSF is in direct contact with the

brain parenchyma and near to the main site of CSF production, it may more sensitively reflect biochemical and cellular changes occurring within the brain (Mollenhauer, 2014). Therefore, using a large cohort of vCSF samples obtained from a range of NDD patients at post-mortem, this chapter aimed to investigate the relationship between vCSF ccf-mtDNA copy number and broad NDD, to validate previous work in lCSF (Podlesniy *et al.*, 2013; Pyle *et al.*, 2015b).

In addition, previous literature (reviewed in Aucamp *et al.* (2018) and discussed in Section 1.2.1) has alluded to two main mechanisms of release of ccf-mtDNA, consisting of either cell death or active release, but most have overlooked using omics technologies to support their conclusions. Therefore, to increase the understanding of the mechanisms underlying ccf-mtDNA, this chapter also aimed to correlate specific vCSF proteins to ccf-mtDNA copy number. The proteins investigated were related to four key areas; neuronal survival (neuron specific enolase (NSE), 14-3-3 proteins (beta and zeta), neuronal nuclei (NeuN), tropomyosin receptor kinase B (TrkB)), synaptic vesicles (synaptophysin), mitochondrial integrity (porin, succinate dehydrogenase (SDHA), transcription factor A (TFAM), oxidative phosphorylation complexes (OXPHOS)) and neurodegeneration (tau, α-synuclein) (depicted in Figure 3.1).

Proteins related to these key areas were selected under the assumption that association to ccf-mtDNA copy number could corroborate a mechanism behind ccf-mtDNA export. For example, correlation of neuronal survival and neurodegeneration proteins to ccf-mtDNA could support the cell death theory of ccf-mtDNA release. Correlation of vesicular protein to ccf-mtDNA could instigate active release of ccf-mtDNA confined to vesicles. Whereas, correlation of mitochondrial proteins to ccf-mtDNA could imply the encapsulation of ccf-mtDNA within cell free mitochondria.

Neuronal Survival proteins: NSE (Haque *et al.* (2018), 14-3-3 proteins (Clapp *et al.* (2012) and TrkB (Klein *et al.*, 1991) are neurotrophic factors that are associated with neuronal injury and apoptosis. Previous literature shows that NSE and 14-3-3 proteins can be quantified within the CSF and can be used as a measure of neuronal damage (Tanabe *et al.*, 2001; Foote and Zhou, 2012; Schmidt *et al.*, 2014). In NDDs the levels of NSE, 14-3-3 and TrkB are found to be associated with particular diseases, rather than NDD as a whole. NSE levels in the serum and CSF are found to be unchanged between PD patients (Schaf *et al.*, 2005), AD patients (Nooijen *et al.*, 1997; Chaves *et al.*, 2010) and healthy controls, but conversely are significantly

elevated in Creutzfeldt-Jakob disease (CJD) (Zerr *et al.*, 1995; Kropp *et al.*, 1999). Additionally, 14-3-3 proteins are found to be associated with various NDDs (Foote and Zhou, 2012; Shimada *et al.*, 2013), but are typically only detected in the CSF of CJD patients (Zerr *et al.*, 2000), or other rare rapidly progressing dementia conditions (Burkhard *et al.*, 2001; Huang *et al.*, 2003; Jayaratnam *et al.*, 2008). TrkB protein is associated with NDDs, with reports of differential TrkB protein expression in PD patients (Fenner *et al.*, 2014; Kang *et al.*, 2017) and AD patients (Allen *et al.*, 1999; Ferrer *et al.*, 1999; Michalski *et al.*, 2015) compared to controls, depending on brain region and neuronal population. However, levels of TrkB have never been assessed within the CSF. Similarly, NeuN, a marker for neuronal count (Mullen *et al.*, 1992) is also associated with NDD, with lower NeuN levels being observed in the brains of PD patients and frontotemporal lobar dementia patients compared to controls (Murphy *et al.*, 2014; Yousef *et al.*, 2017). Again, levels of NeuN have never been assessed within the CSF. Due to the strong links between these proteins and neuronal death and NDDs, a correlation of these proteins to ccf-mtDNA copy number could support the theory that ccf-mtDNA is exported in response to cell death.

Synaptic vesicle protein: Synaptophysin is a synaptic vesicle protein (Wiedenmann and Franke, 1985) used as a marker of synaptic content. Levels of this protein are found to be significantly reduced in the brain of AD patients (Sze *et al.*, 1997; Sinclair *et al.*, 2015) and DLB patients (Mukaetova-Ladinska *et al.*, 2013) and in mouse models of PD (Bate *et al.*, 2010). However, current efforts to detect synaptophysin in the CSF have been unsuccessful (Davidsson *et al.*, 1996; Schlaf *et al.*, 1998). Correlating synaptophysin levels to ccf-mtDNA copy number could indicate that mtDNA are packaged into presynaptic vesicles prior to export, suggesting an active mechanism of release.

Mitochondrial integrity proteins: The range of mitochondrial proteins investigated relate to major components of the mitochondria. Porin is an outer mitochondrial membrane protein (Colombini, 1979) commonly used as a marker of mitochondrial mass in proteomic studies (Vincent *et al.*, 2016). SDHA and OXPHOS are used as markers of the inner mitochondrial membrane (Kühlbrandt, 2015) and Tfam is a major constituent of the mitochondrial nucleoid (as reviewed in Istiaq Alam *et al.* (2003)). It is unknown whether ccf-mtDNA circulates encapsulated within cell-free mitochondria, however, previous literature in cell culture (Maeda and Fadeel, 2014), mouse retinal ganglion cell axons (Davis *et al.*, 2014) and CSF (Chou *et al.*, 2017)

suggest this may be true. Additionally, work in substantia nigra neurons of PD patients indicates that expression of mitochondrial proteins NDUFB8, SDHA and TFAM are reduced compared to controls and this coincides with a reduction in mtDNA copy number (Grunewald *et al.*, 2016). Thus, supporting a potential relationship between mitochondrial protein expression and ccf-mtDNA copy number.

Neurodegeneration proteins: Tau and α-synuclein proteins are linked to the pathogenesis of various NDDs (Ross and Poirier, 2004) and previous research in the CSF has shown strong evidence for these proteins as biomarkers for NDD (Hansson *et al.*, 2006; Mollenhauer *et al.*, 2011). Correlation of these current biological markers of NDDs to ccf-mtDNA copy number could provide important comparison to validate the utility of ccf-mtDNA as a novel biomarker.

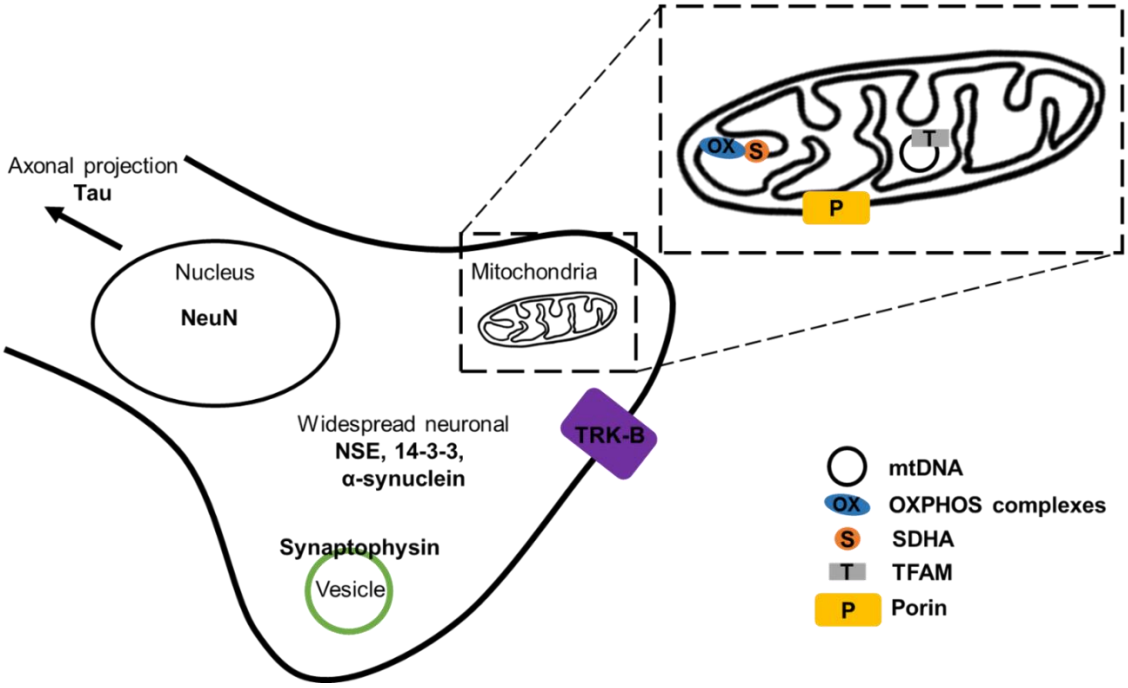

Figure 3.1. Schematic demonstrating the neuronal origin and localisation of the proteins targeted within the vCSF in this chapter where Neuronal Nuclei protein (NeuN) is located in the nucleus, neuron specific enolase (NSE), 14-3-3 beta and zeta (14-3-3) and alpha synuclein are located throughout the neuron, synaptophysin is located in the vesicular membrane, tau is located in the axon, tropomyosin receptor kinase B (TRK-B) is located in the presynaptic membrane and oxidative phosphorylation complexes (OXPHOS), succinate dehydrogenase (SDHA), transcription factor A (TFAM) and Porin are localised to the mitochondria.

Furthermore, high levels of mtDNA deletions (Corral-Debrinski *et al.*, 1992; Bender *et al.*, 2006; Muller *et al.*, 2013) and mtDNA mutations (Coskun *et al.*, 2004; Lin *et al.*, 2012; Coxhead *et al.*, 2016; Hoekstra *et al.*, 2016) have been found within the brains of NDD patients (reviewed in (Keogh and Chinnery, 2015). Therefore, using the mtDNA deletion assay and next generation sequencing (NGS), this chapter aims to assess the integrity of the ccf-mtDNA, comparing this between disease groups and controls. Analysis of ccf-mtDNA integrity could potentially uncover further understanding of the mechanism of ccf-mtDNA export. If ccf-mtDNA is discovered to harbour high mutational levels this may indicate a preferential selection process of ccf-mtDNA export, targeting only faulty mtDNA for removal. Differently, if ccf-mtDNA mutational burden is low and heteroplasmy levels are similar between disease groups and controls, this would suggest that export is not predetermined based on mtDNA integrity, but instead is part of normal turnover.

3.2 Chapter specific Methods

3.2.1 NDD-vCSF cohort

The NDD-vCSF cohort consisted of 136 vCSF samples; made up of 6 neurodegenerative disease (NDD) groups: 8 Parkinson's Disease (PD), 45 Alzheimer's Disease (AD), 15 Dementia with Lewy bodies (DLB), 11 Dementia (including vascular dementia and frontotemporal dementia), 8 Motor Neuron Disease (MND), 5 Progressive Supranuclear Palsy (PSP) and a group of 44 age-matched neurologically healthy controls. Samples were collected directly from hemisected vesicles of the post-mortem brain at the Newcastle Brain Tissue Resource (NBTR) and processed as described in Section 2.2.2. A detailed description of the NDD-vCSF cohort demographics is outlined in the Section 2.1.3.

3.2.2 mtDNA Quantification

Nuclear DNA (nDNA) and ccf-mtDNA were quantified by multiplex Taqman qPCR amplification of the nuclear encoded gene *B2M*, and the mitochondrial genes *MTND1* and *MTND4*, as described in Section 2.3-2.7.

3.2.3 Protein Quantification

VCSF total protein enrichment was measured using Qubit® Protein Assay (Thermo Fisher Scientific). Due to low protein concentration and sample volume limitations only a subset of the cohort were included in western blot protein analysis (14 Controls, 5 PD, 27 AD, 6 DLB, 1 Dementia, 1 MND and 2 PSP). Detailed western blot procedure is described in Section 2.8.

3.2.4 Antibodies

Monoclonal primary antibodies to Neuron specific enolase (NSE, ab180943), 14-3-3 Beta (ab16859), 14-3-3 Zeta (ab188368), Synaptophysin (ab32127), SDHA (ab14715), Porin (ab14734), Tfam (ab119684), Neuronal nuclei (NeuN, MAB377), Tropomyosin receptor kinase B (TrkB, cell signalling 4019), Tau (cell signalling, 4019), total OXPHOS (ab110411) and α-synuclein (ab138501) were used to probe the membrane, followed by HRP-conjugated species-specific secondary antibodies. Further details can be found in Section 2.8. The optimal loading quantity for western blot was 20µg of total protein, therefore, haem contaminated samples and low protein

abundance samples were excluded resulting in a usage of 14/40 controls 42/86 NDD cases.

3.2.5 Alpha synuclein ELISA

Alpha synuclein protein levels were measured in 39 vCSF samples by quantitative sandwich enzyme linked immunosorbent assay (ELISA, BioLegend, 844101). ELISA plates were pre-coated with α-synuclein specific antibody. The α-synuclein standard was prepared as per manufacturer's instructions ranging from 1500pg/mL to 6.1pg/mL and samples were diluted 1:20 in 1x reagent diluent. Plate wells were washed and aspirated then 200µl of standard or sample was added to each well in triplicate or duplicate, respectively. The plate was incubated at 4°C overnight, then wells were washed and aspirated three times. Biotinylated primary antibody was added to each well in 50µl volumes and incubated for 2 hours at room temperature. Aspiration and washes were repeated and then 200µl of HRP Streptavidin was added to the plate and incubated for 1 hour. Wells were washed and aspirated three more times and then 100µl of chemiluminescent substrate was added to each well. Optical density of samples were measured using the microplate reader as described previously in Section 2.8.2.

3.2.6 mtDNA Sequencing

Ccf-mtDNA was extracted and enriched prior to sequencing by methods described in Section 2.9-2.10. Successfully amplified samples (74/126 or 59%: 53% of controls and 62% of NDD cases) were then prepared and sequenced on the Illumina MiSeq v3.0 by methods described in Section 2.11 and analysed using an in-house bioinformatics pipeline to calculate heteroplasmy level for each sample (Section 2.12).

3.2.7 Transmission Electron Microscopy

CSF samples were pelleted by centrifugation at 14000 rpm for 20 minutes. Pellets were processed by methods described in Section 2.13 and ultrathin sections were imaged using a Philips CM100 TEM at the Newcastle University Electron Microscopy Research Service under the guidance of Dr Kathryn White.

3.3 Methods optimisation

3.3.1 WB Loading Control Optimisation

Initially, traditional housekeeping genes; *beta-actin* and *beta-tubulin* were targeted as loading controls in western blot to correct for differences in loaded protein concentration. These proteins are believed to be constitutively and ubiquitously expressed across all tissue types and disease states (Li and Shen, 2013), and hence can be used as an internal control to ensure uniform loading or to correct for differences in protein concentration. However, recent evidence criticises this belief, showing significant variation in these housekeeping genes across cell types, tissue types and in disease states (Li and Shen, 2013; Collins *et al.*, 2015). Additionally, neither of the above housekeeping proteins are secreted proteins which further questions their validity as loading controls in biological fluids (Collins *et al.*, 2015).

In initial optimisation, there was a great degree of variability in beta-tubulin level within the subset of samples tested (n=10, Figure 3.2), which did not match the uniform loading confirmed by the Ponceau stain. Although beta-actin levels were relatively consistent in the trial blot, literature has shown that expression of this housekeeping protein can be modulated by neurodegenerative disease (Eaton *et al.*, 2013; Li and Shen, 2013), as these diseases are typically associated with cytoskeletal pathology (Cairns *et al.*, 2004) and dysfunction of cytoskeletal proteins, including actin (Bamburg and Bloom, 2009). Moreover, the molecular weight of beta-tubulin and beta-actin, 55kDa and 45kDA respectively, correspond to that of three of the proteins of interest, thereby further restricting their utility as loading controls in this study.

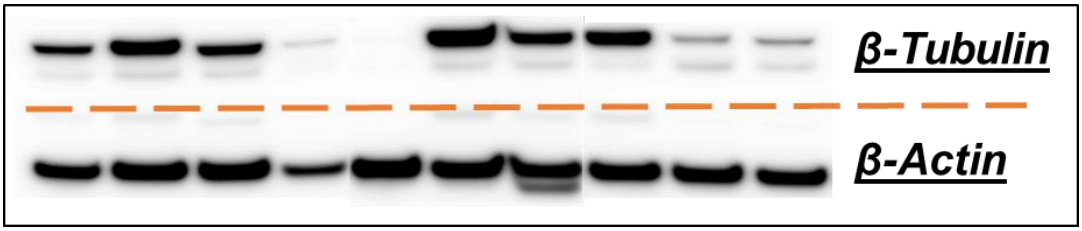

Figure 3.2. Western blot of housekeeping proteins beta-tubulin and beta-actin. Both proteins show variability in their expression and were considered to be unsuitable for use in the study as loading controls. Each band represents an individual sample.

More recently, normalisation to total protein has emerged as a more reliable alternative to using individual housekeeping proteins (Collins *et al.*, 2015). BLOT-FastStain™ (G-Biosciences, USA), a reversible total protein stain (described in Section 2.8) was trialled as a loading control and was found to accurately, reliably and reversibly bind to and allow quantification of all CSF proteins in the sample lane. This was used as the loading control in all subsequent western blot experiments (example blot shown in Section 2.8).

3.3.2 mtDNA Sequencing Optimisation

i) Long range PCR

The low copy abundance of the ccf-mtDNA in the CSF (~3570 copies per µl, typically less than a single cell (Rooney *et al.*, 2015)) presented significant challenges for sequencing. Initially, neat unprocessed CSF ccf-mtDNA was amplified using a two fragment primer set, previously used in whole mtDNA sequencing within our lab (Coxhead *et al.*, 2016), using Takara PrimeSTAR GXL DNA polymerase (Table 3.1, A and B). This generated two ~9Kb products covering the entire mitochondrial genome.

PCR Fragment	Product Size (bp)	Forward sequence	Reverse sequence
A	9966	CCCTCTCTCCTACTCCTG	CAGGTGGTCAAGTATTTATGG
B	9102	CATCTTGCCCTTCATTATTGC	GGCAGGATAGTTCAGACG
6F & 6R	600	CAGCCGCTATTAAAGGTTCG	GGAGGGGGGTTCATAGTAG

Table 3.1. Gene product sizes and forward and reverse primer sequences for primers used in mtDNA long range PCR optimisation.

To test the amount of vCSF required to generate a suitable PCR product, three vCSF samples were loaded with the PCR mastermix in titrated volumes (1, 3, 5, 10 and 20µl) alongside a blood DNA control (BC) and a negative, no DNA, control (neg), to assess the optimal reaction conditions. The blood control amplified effectively and was clearly visible on the agarose gel, however, none of the vCSF samples were detected regardless of loading volume (Figure 3.3).

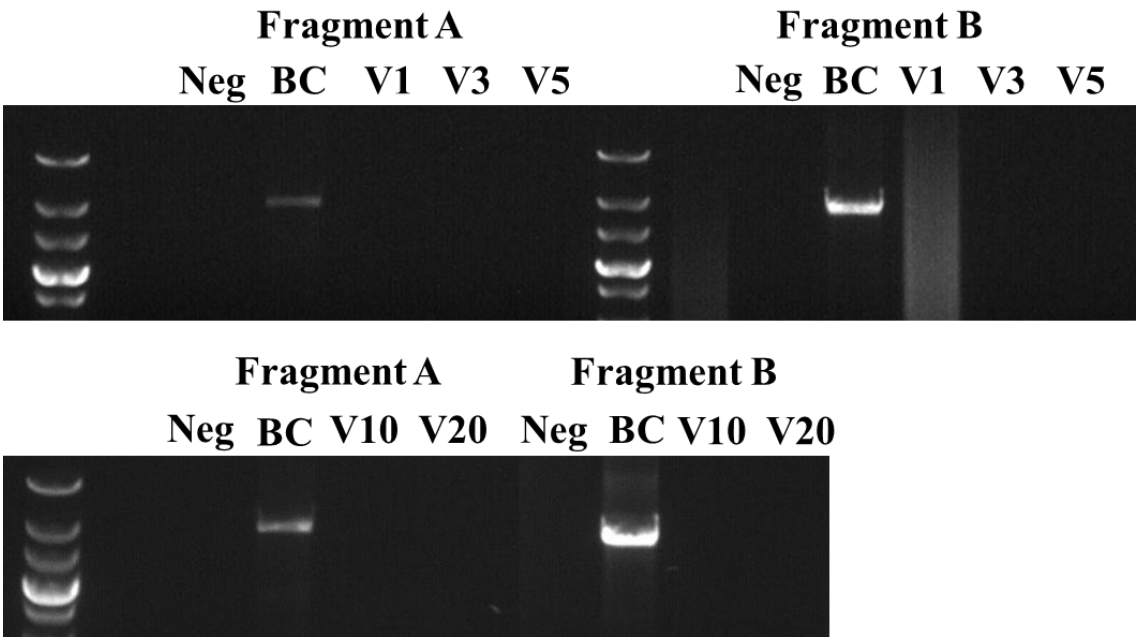

Figure 3.3. Gel electrophoresis image of PCR products generated by two fragment long range PCR of the whole mitochondrial genome. BC: blood control; V:ventricular sample. Numbers 1,3,5,10 and 20 refer to volume of neat CSF in PCR reaction (in µl). Ladder is the GeneRuler™ 1kb Plus DNA ladder (ThermoFisher Scientific™).

Subsequently, it was decided that it may be more effective to extract and purify the ccf-mtDNA from the vCSF samples prior to enrichment; concentrating the vCSF ccf-mtDNA into a smaller elution volume and thus increasing the per µl concentration. To assess this, ccf-mtDNA from 4 vCSF samples and 1 blood control was extracted using UltraPure™ (phenol:cholorofrom:isoamyl alcohol) as described in Section 2.9.

To test the PCR reaction efficacy, a 600bp amplicon was amplified alongside the long range primer pairs (A and B) from the extracted ccf-mtDNA using 6F and 6R primer pairs (Table 3.1). However, whilst the 600bp amplicon appeared to work, the long range PCR primers continued to fail (Figure 3.4).

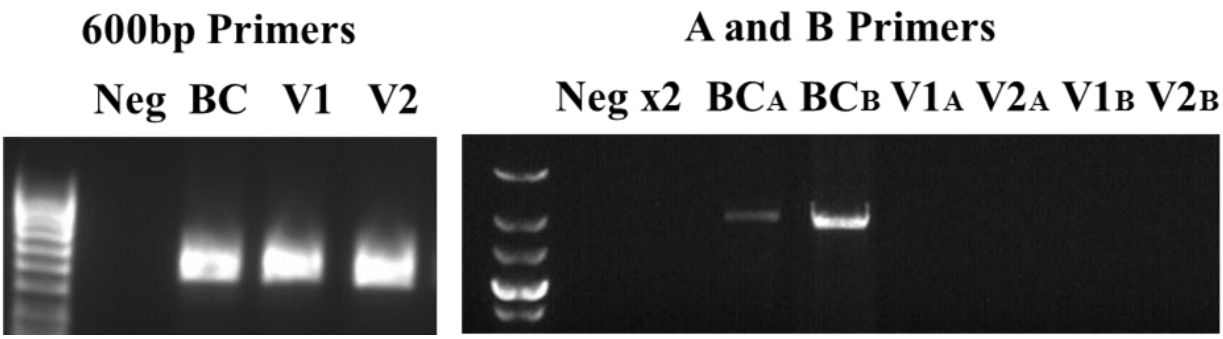

Figure 3.4. Gel electrophoresis image of the 600bp PCR product generated from the ccf-mtDNA extracted from the vCSF. BC: blood control; V: ventricular samples 1 and 2. Ladder is 100bp for 600bp primer set and 1Kb for A and B primers. Bands indicate successful amplification of product.

Template availability is known to affect the efficiency of long range PCR (Jia *et al.*, 2014), and given that the shorter amplicons (600bp) were continually successful, the number of overlapping long range PCR primer pairs was increased to 3, thereby decreasing the amplicon size to 4-6Kb (Table 3.2). Figure 3.5 shows the gel image for two vCSF ccf-mtDNA extracts amplified using these primers and confirms successful enrichment of the whole ccf-mtDNA genome. Amplification of Fragment 3 appeared less efficient than 1 and 2, with all samples requiring a second round of amplification.

PCR Fragment	***Product Size (bp)***	***Forward sequence***	***Reverse sequence***
1	6154	CAGCCGCTATTAAAGGTTCG	GTTGTCGTGCAGGTAGAGG
2	6102	ACCACCCAACAATGACTAATC	GGTTGTTTGATCCCGTTTCG
3	4436	ATTCATCGACCTCCCCACC	GGAGGGGGGGTTCATAGTAG

Table 3.2. Gene product sizes (base pairs (bp)) and forward and reverse primer sequences used in three-fragment PCR of ccf-mtDNA, prior to sequencing.

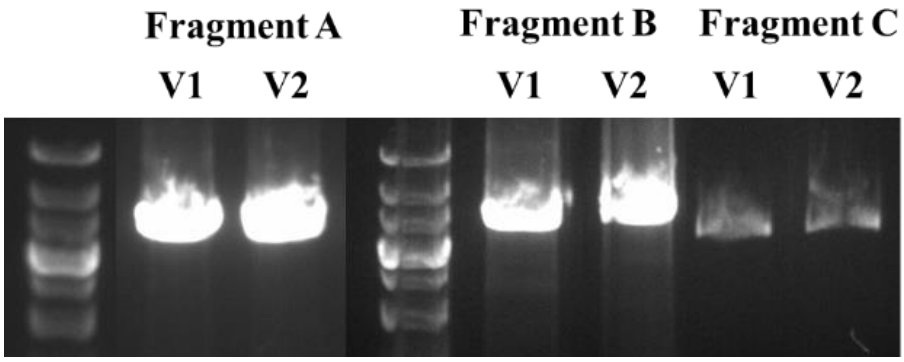

Figure 3.5. Gel electrophoresis image of the PCR products generated for each of the three fragments covering the mitochondrial genome. V: ventricular samples 1 and 2. Ladder is the GeneRuler™ 1kb Plus DNA ladder (ThermoFisher Scientific™). Bands indicate successful amplification product.

ii) *<u>REPLI-g</u>*

In addition to long range PCR, a commercially available multiple displacement amplification kit (REPLI-g Mitochondrial DNA kit (Qiagen)) was also tested to amplify the ccf-mtDNA. This kit can potentially work on very low template concentration (~1pg purified mtDNA), thus providing a solution for samples that fail long range PCR. REPLI-g amplification was performed on 9 CSF samples (4 ventricular CSF, 5 lumbar CSF), using an amplification mix comprised of 13.5µL REPLI-g mt Reaction buffer and 1µL REPLI-g Human mt Primer mix. A total DNA template volume of 10µl (consisting of 3µL extracted mtDNA and 7µL RNase free H_2O) was added to the amplification mix, vortexed, centrifuged and incubated for five minutes at 75°C. Samples were cooled to room temperature and then 1µL REPLI-g Midi DNA Polymerase was added, mixed and centrifuged. Samples were incubated at 33°C for 8 hours and then inactivated at 65°C for 3 mins. Samples were then stored at 2-8°C until use in sequencing.

Next generation sequencing was performed (described in Section 2.11) on 20 long range PCR amplified-samples and 9 REPLI-g amplified-samples, and comparisons were drawn about the efficiency of the different amplification methods. All 20 long range PCR samples were sequenced successfully, whereas 5 of the 9 REPLI-g samples failed. Coverage levels of the REPLI-g amplified samples were significantly lower (61%) than that of the long range PCR amplified samples (95%) (Table 3.3).

Method	***PCR***	***REPLI-g***
n	20	4
Coverage (%)	95	61

Table 3.3. Average coverage (%) of the entire ccf-mtDNA when amplified using PCR compared with REPLI-g.

In addition, 3 samples were amplified using both methods to allow for direct comparisons (Table 3.4). Sample 20110205 was not sequenced effectively. This is evidenced by the low coverage in the long range PCR (66%) and REPLI-g (2%) amplified samples, possibly due to low ccf-mtDNA template prior to amplification and sequencing, thus was excluded from further analysis.

Similar to previous studies (Koref *et al.*, 2018), homoplasmy level was no different between the different amplification methods, however, heteroplasmy level was markedly higher in REPLI-g amplified samples compared to long range PCR samples.

ID	***20101115***		***20110135***		***20110205***	
Method	PCR	REPLI-g	PCR	REPLI-g	PCR	REPLI-g
Coverage (100x) (%)	99.96	80.59	99.97	83.34	66.23	1.87
Homoplasmy	9	9	29	26	24	23
Heteroplasmy	54	46	62	915	31	11

Table 3.4. Comparison of three fragment long range PCR and REPLI-g enrichment of the mtDNA for sequencing. Coverage of the mitochondrial genome at a depth of 100 reads is given as a percentage. Homoplasmy and heteroplasmy counts are displayed.

3.4 Results

3.4.1 NDD-vCSF cohort inclusion criteria and demographics.

An inclusion threshold for ccf-mtDNA copy number was set based on previous literature of mtDNA copy number (1,000-10,000 copies per cell (Rooney *et al.*, 2015)), lCSF ccf-mtDNA copy number (10-1,000 copies per µl (Pyle *et al.*, 2015b; Perez-Santiago *et al.*, 2016; Cervera-Carles *et al.*, 2017; Leurs *et al.*, 2018)), and frequency distribution analysis of the vCSF ccf-mtDNA copy number (Figure 3.6, A). This determined an upper copy number limit of 10,000 copies per µl, leading to the exclusion of 7% (10/136) of the cohort (4 controls, 1 AD, 4 DLB and 1 dementia).

Frequency distribution was also assessed for nuclear DNA (nDNA) level using the *B2M* copy number generated from qPCR (Figure 3.6, B). This showed that more than 85% of samples had less than 2 copies of nDNA. In preliminary studies *B2M* copy number level was considered a measure of possible cellular contamination and used to remove poor quality, contaminated samples (Pyle *et al.*, 2015b). However, recent reports show evidence of ccf-nDNA in the CSF (Adalsteinsson *et al.*, 2017; Connolly *et al.*, 2017), suggesting that nDNA contamination (*B2M)* may not be a result of carryover of cells or cellular debris. Moreover, correlation analysis showed no significant association between nDNA and ccf-mtDNA copy number levels (r^2=0.01, p>0.05), therefore, this threshold was not included as part of the inclusion criteria.

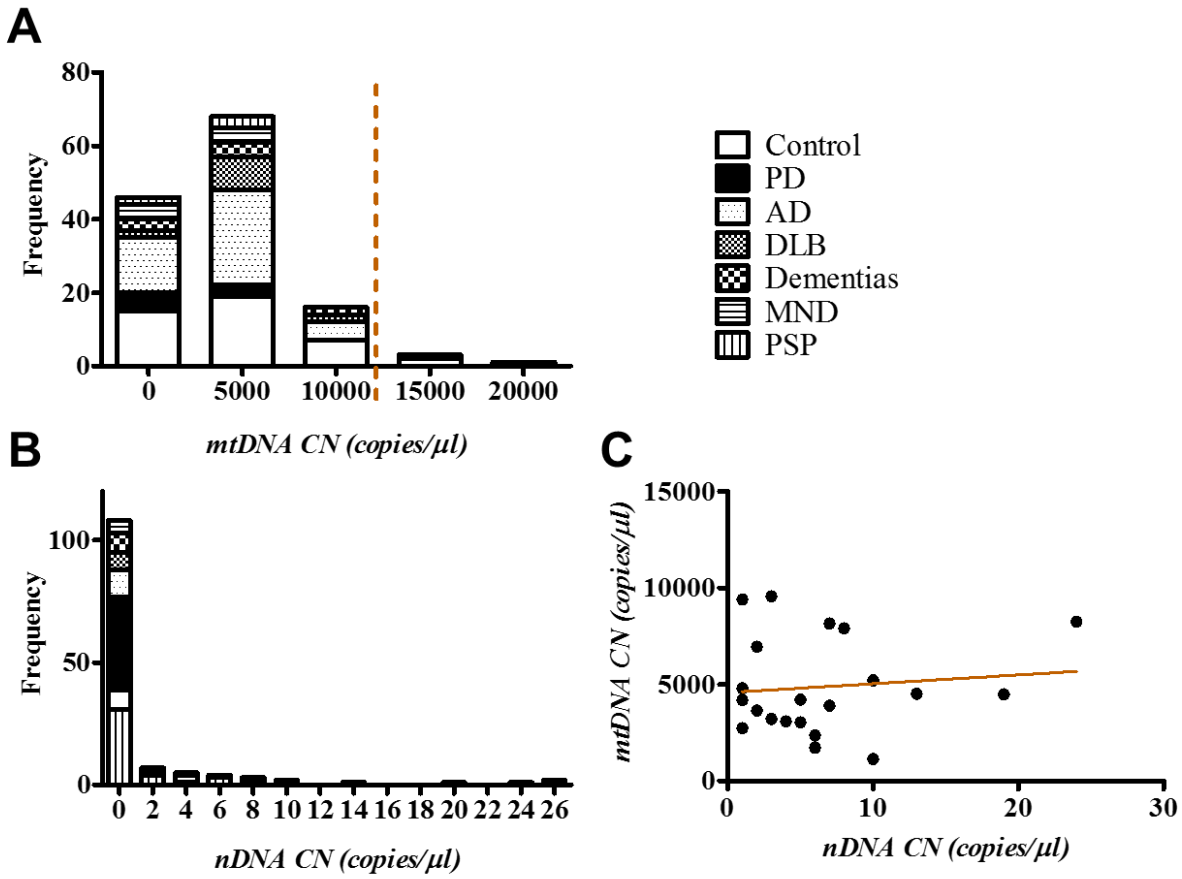

Figure 3.6. Frequency histograms of the circulating cell free copy number measured for mitochondrial DNA (A) and nuclear DNA (B). Orange dotted line indicates the exclusion threshold based on histogram data. C) shows no relationship between ccf-mtDNA copy number and ccf-nDNA copy number suggesting, nDNA 'contamination' or ccf-nDNA copy number does not influence ccf-mtDNA copy number (r^2=0.01, p>0.05).

The final NDD-vCSF cohort consisted of 126 samples, the demographic information for each individual disease group can be found in Table 3.5. In addition, there were no significant differences found in gender, age at death or post-mortem interval between NDD cases or controls (p column in Table 3.5, independent t-test p>0.05).

Disease group	Control (40)	NDD (86)	p	PD (8)	AD (44)	DLB (11)	Dementia (10)	MND (8)	PSP (5)
Gender (M/F)	16/24	49/37	$8.35x10^{-1}$	5/3	23/21	6/5	9/1	2/6	4/1
Age at death (years)	74±14	79±11	$5.83x10^{-1}$	80±8	82±10	79±8	70±12	73±12	77±16
Age of onset (years)	67±21	72±11	-	71±10	74±10	72±9	64±15	71±12	73±16
Disease Duration (years)		7±4	-	10±5	7±4	7±3	6±4	2±1	4±2
Post-Mortem Interval (hours)	36±24	40±26	$6.11x10^{-1}$	57±28	38±27	33±21	44±25	26±14	61±19

Table 3.5. Final inclusion cohort demographic information for the NDD-vCSF cohort, stratified for controls and Neurodegenerative disease (NDD) and each neurodegenerative disease condition alone. PD; Parkinson's Disease, AD; Alzheimer's disease, DLB; Dementia with Lewy bodies, Dementia, MND; Motor neuron disease, PSP; Progressive supranuclear palsy. Gender is displayed as the number of males/females. All other demographic is displayed as mean ± standard deviation. P values generated by independent t-tests are present for comparison of age at death and post-mortem interval between controls and NDD patients, p>0.05. Differences in gender were tested by Pearson's Chi-squared test, p>0.05.

Similarly to previous work (Miller *et al.*, 2003), there was no correlation identified between age (age at death) and ccf-mtDNA copy number when analysing the entire cohort, or when stratified by control or NDD (Table 3.6, Pearson's $p>0.05$). Similarly, there was no correlation between the age of onset or duration of disease and ccf-mtDNA copy number (Table 3.6, Pearson's $p>0.05$).

Furthermore, there was also no relationship between ccf-mtDNA copy number and gender (Pearson's $p>0.05$, Table 3.6) for the whole cohort or when stratified by case or control; control males = 3910±2524 copies/µl, control females = 3701±2108 copies/µl, disease males = 3477±2305 copies/µl and disease females = 3458±2421 copies/µl.

Perhaps most importantly, post-mortem interval was not found to have any association with ccf-mtDNA copy number, implying that CSF extraction, storage and transport had no implication on the outcome or viability of the ccf-mtDNA (Table 3.6)

Variable	*Whole Cohort*	*Control*	*NDD*
Male vs Female	$9.42x10^{-1}$	$7.78x10^{-1}$	$9.70x10^{-1}$
Age at death	$7.75x10^{-1}$	$9.30x10^{-1}$	$7.06x10^{-1}$
Age of onset	-	-	$9.24x10^{-1}$
Disease duration	-	-	$1.4x10^{-1}$
Post-mortem Interval	$1.45x10^{-1}$	$2.88x10^{-1}$	$1.29x10^{-1}$

Table 3.6. P values assessing the relationship between gender, age at death, age of onset, disease duration and post-mortem interval and ccf-mtDNA copy number with Pearson's significance testing.

3.4.2 Clinical diagnosis of NDD and controls can often be conflicting.

Following on from the preliminary study carried out in our lab previously, it was hypothesised that ccf-mtDNA copy number would be different across a wider range of NDDs. In life, the patients included in the NDD-vCSF cohort were given a clinical diagnosis based on symptomatic presentation and controls were assumed to be neurologically normal due to the absence of NDD phenotypes.

Based on clinical categorisation there was no significant difference in ccf-mtDNA copy number between healthy aged controls (n=41) and NDD cases (n=85) (mean

ccf-mtDNA copy number was 3550±398 and 3578±239, respectively, p>0.05, Mann Whitney U, Figure 3.7, A). Consistently, this result remained when using linear regression with nDNA as a covariate (p>0.05). Additionally, sub-stratifying NDD individuals into their respective disease groups (10 PD, 41 AD, 8 DLB, 13 dementia, 8 MND and 5 PSP patients) also failed to highlight any differences in ccf-mtDNA copy number (Figure 3.7, B, Kruskal-Wallis ANOVA p>0.05).

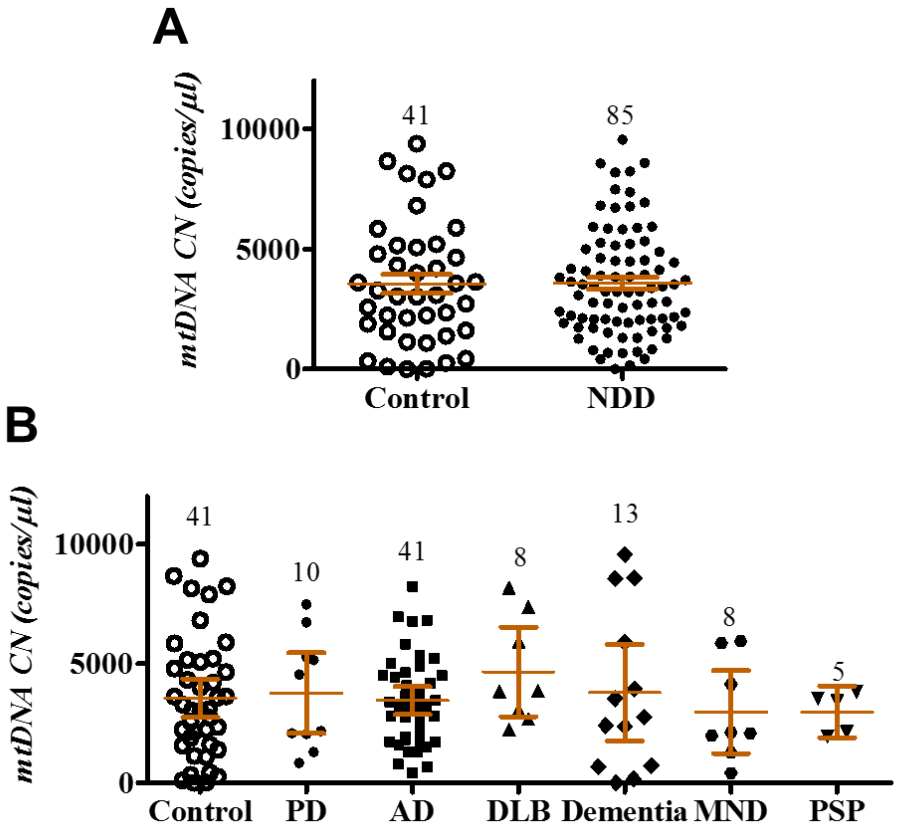

Figure 3.7. Clinical Diagnosis - Ccf-mtDNA copy number (CN) per microliter of CSF for controls and neurodegenerative disease (NDD) cases; when NDDs are grouped as one category (A) or sub stratified into specific disease groups (B). Orange bars show mean+SEM. N numbers are above stacks. PD; Parkinson's disease, AD; Alzheimer's disease, DLB: Dementia with Lewy Bodies, MND; Motor Neuron Disease, PSP; Progressive Supranuclear Palsy.

However, clinical diagnosis has limited accuracy (~70-80% (Love, 2004; Jankovic, 2008; Beach *et al.*, 2012)), and neuropathological assessments post-mortem are often used to confirm diagnosis. As the NND-vCSF samples were post-mortem, neuropathologically confirmed diagnoses were available from the NBTR. Neuropathological assessment led to the reclassification of ~50% (58/126) of the NDD-vCSF cohort (Table 3.7).

n	*Clinical Diagnosis*	*Neuropathological Diagnosis*
7	AD	Control
1	MND	
2	PSP	
2	AD	PD
1	Dementia	
3	Control	
5	PD	AD
3	DLB	
5	Dementia	
2	Control	
4	MND	
2	PSP	
1	PD	PSP
2	AD	
1	DLB	
1	Dementia	
2	PD	DLB
3	AD	
1	Dementia	
2	Control	
2	AD	Dementia
1	Control	
1	PSP	
1	AD	MND
1	Dementia	
2	Control	

Table 3.7. Summary of the number of participants that changed diagnosis after post-mortem neuropathological examination. Clinical diagnosis indicates the original diagnosis, neuropathological diagnosis indicates the new diagnostic group and n indicates the number of samples that changed groups.

3.4.3 Ccf-mtDNA copy number analysis in vCSF of NDD cases compared to controls.

Similar to clinical data (Section 3.4.2), vCSF ccf-mtDNA copy number was not significantly different between NDD cases and controls (mean ccf-mtDNA copy number was 3469±253 in 86 NDD and 3785±356 in 40 controls, Mann Whitney U $p>0.05$) when samples were stratified based on neuropathological diagnosis (Figure, 3.8, A). Additionally, this result was unchanged by the inclusion of nDNA copy number as a covariate in linear regression ($p>0.05$).

Sub-stratifying NDD cases into specific neuropathological disease groups (i.e. 8 PD, 44 AD, 11 DLB, 10 Dementia, 8 MND and 5 PSP) revealed a significant reduction in ccf-mtDNA copy number in PD cases compared to controls (where mean ccf-mtDNA copy number was 1548 in 8 PD and 3785 in 40 controls, Mann Whitney U $p=7.6x10^{-3}$). Additionally, ROC curve analysis of PD cases and controls, generated an area under the curve of 0.81, suggesting that ccf-mtDNA copy number was a strong predictor of PD status. No other NDD group had significantly different ccf-mtDNA copy number level compared to controls (Figure 3.8, B).

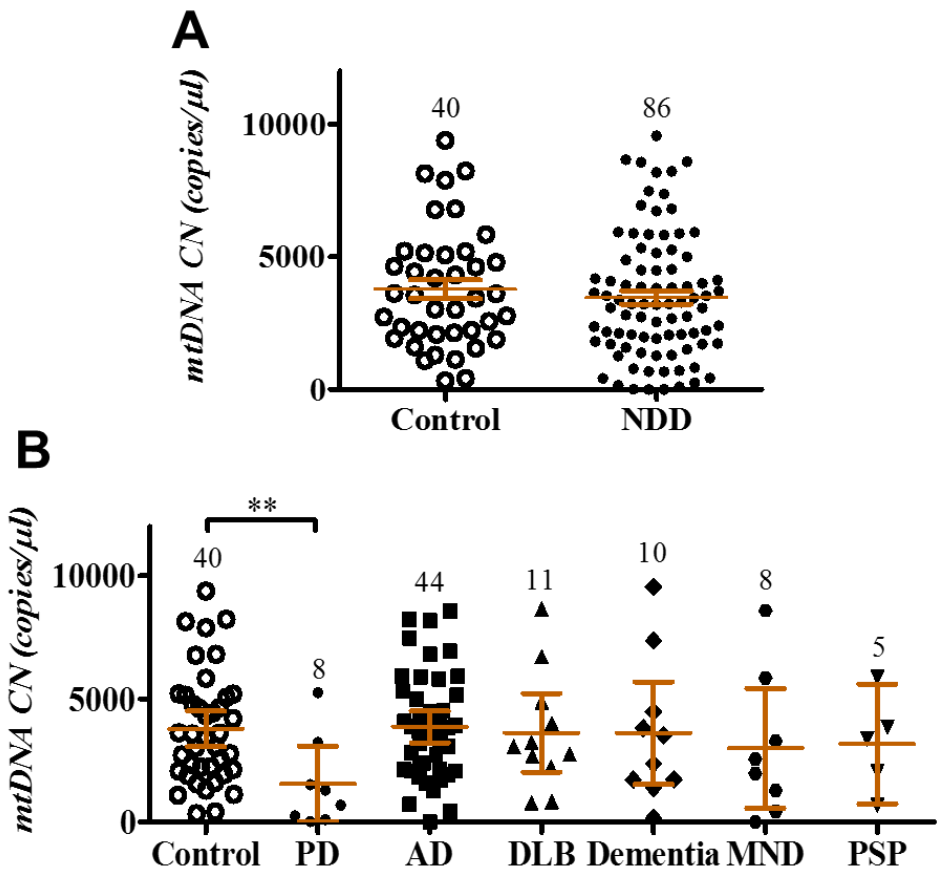

*Figure 3.8. Neuropathological diagnosis - Ccf-mtDNA copy number (CN) per microliter of CSF for controls and neurodegenerative disease (NDD) cases; when NDDs are grouped as one category (A) or sub stratified into specific disease groups (B). Orange bars show mean+SEM. N numbers are above stacks. PD; Parkinson's disease, AD; Alzheimer's disease, DLB: Dementia with Lewy Bodies, MND; Motor Neuron Disease, PSP; Progressive Supranuclear Palsy. ** indicates significant difference from controls at p<0.01.*

3.4.4 Phenotypic correlations to ccf-mtDNA copy number.

The majority of the cohort (99%) underwent cognitive assessment in life (through the mini-mental-state-exam, MMSE), and tau pathology assessment post-mortem (tau Braak staging) to indicate the presence and severity of cognitive impairment or dementia.

The cohort was subdivided into two groups; cognitively normal (MMSE ≥24; 84% controls, 19% NDD) or cognitively impaired (MMSE ≤23; 16% controls, 81% NDD) to investigate if ccf-mtDNA copy number was associated with cognitive impairment. Figure 3.9 shows that cognitively impaired individuals had higher vCSF ccf-mtDNA copy number than cognitively normal individuals, where mean ccf-mtDNA was

3529±364 in 32 cognitively normal controls compared to 4916±1230 in 6 cognitively impaired controls (Mann Whitney U p>0.05), and 2181±591 in 16 cognitively normal NDD compared to 3763±269 in 70 cognitively impaired NDD (Mann Whitney U $p=4.60x10^{-3}$). In addition, ccf-mtDNA copy number was found to be significantly lower in cognitively normal NDD compared to controls (Mann Whitney U $p=1.47x10^{-2}$).

Furthermore, Tau Braak stage was found to be significantly correlated to ccf-mtDNA copy in the entire cohort (Pearson's $r^2=0.2$, $p=4.50x10^{-2}$), but this was largely driven by NDD cases ($r^2=0.2$, $p=4.20x10^{-2}$ for NDD cases and p>0.05 for controls, Table 3.8).

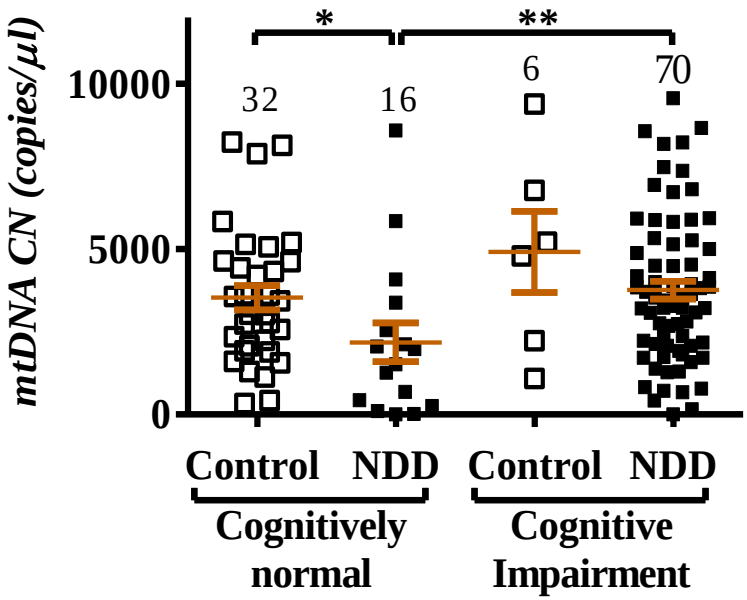

Figure 3.9. Effect of cognitive impairment (measured by MMSE) on ccf-mtDNA copy number (CN) per microliter of CSF in controls and NDD cases. Cognitively normal is defined as an MMSE score ≥24, whereas cognitively impaired is an MMSE score ≤23. Orange bars indicate mean+SEM, n numbers are above stacks.

Variable	Whole Cohort		Control		NDD	
	r^2	p	r^2	p	r^2	p
Braak Stage	0.20	$4.50x10^{-2}$ *	0.25	$2.68x10^{-1}$	0.23	$4.20x10^{-2}$ *

*Table 3.8. Correlation analysis of Tau Braak stage to ccf-mtDNA copy number for NDD cases, controls and the whole cohort. P and r^2 values were generated by Pearson's significance testing. * indicates significance at p<0.05.*

3.4.5 Abundance of targeted vCSF proteins in NDD and controls and correlation to ccf-mtDNA copy number.

Protein abundance was measured in the vCSF using western blot to target a number of specific proteins related to neuronal health and survival, synaptic vesicles, mitochondria and neurodegeneration.

Four neurotrophic factors were investigated in NDD cases and controls including neuron specific enolase (NSE), 14-3-3 zeta and 14-3-3 beta and TrkB (BDNF receptor). Figure 3.10 shows example blots of each protein of interest normalised to their total protein content, calculated from entire BLOT Fast Stain membranes. NSE levels were not significantly different between 42 NDD cases and 14 controls (Figure 3.10, A, Mann Whitney U $p>0.05$), however, the other three proteins (14-3-3 beta, 14-3-3 zeta and TrkB) showed a trend towards lower vCSF abundance in NDD cases compared to controls. This trend was significant for 14-3-3 zeta protein abundance (normalised 14-3-3 zeta expression was 0.39±0.18 in 31 NDD patients and 0.64±0.21 in 8 controls, Mann Whitney U $p=6.7x10^{-3}$, Figure 3.12, B) and TrkB protein abundance (normalised TrkB expression was 0.39±0.42 in 28 NDD and 0.53±0.24 in 13 controls, Mann Whitney U $p=4.1x10^{-2}$, Figure 3.10, D).

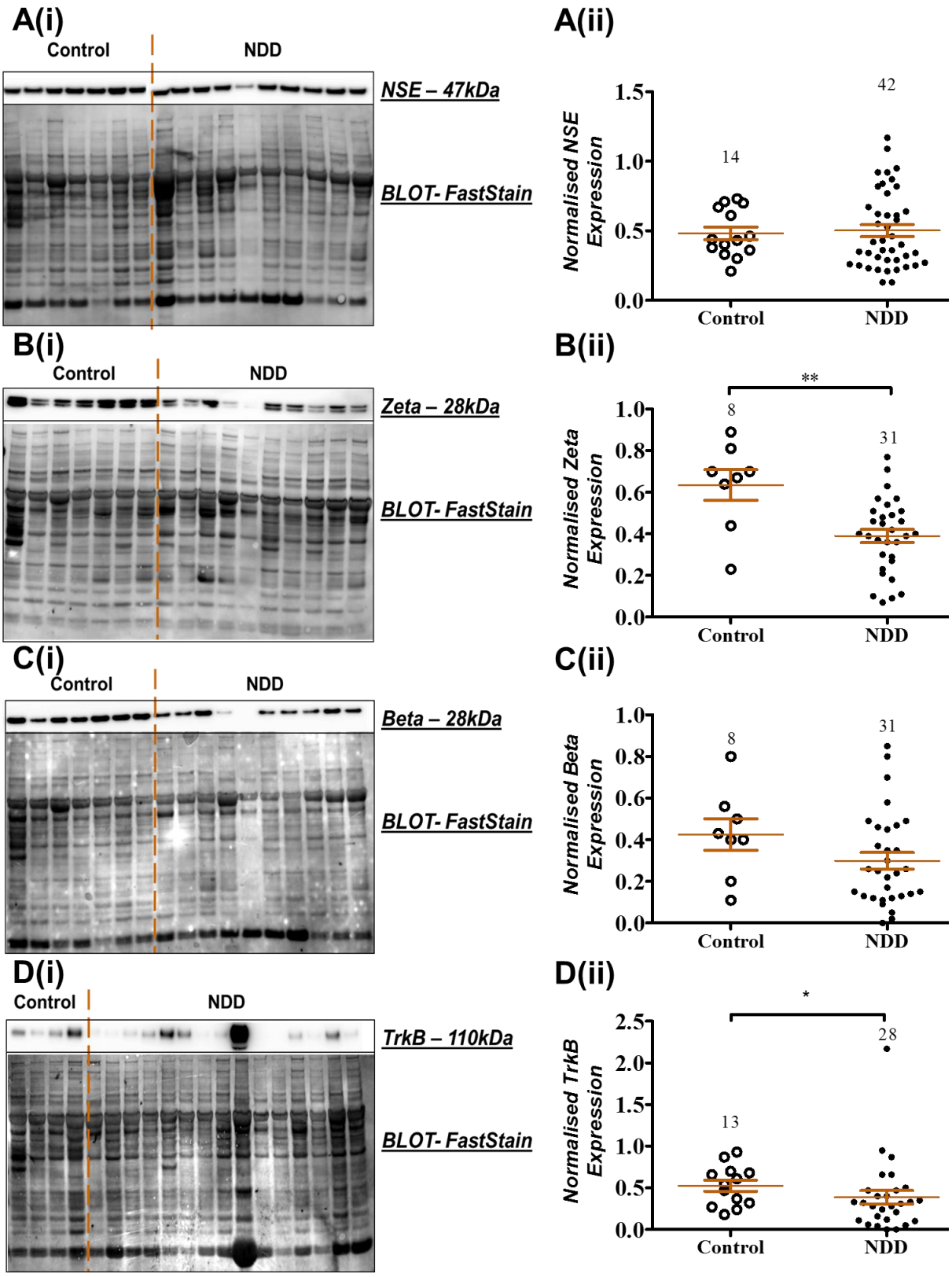

*Figure 3.10. Protein abundance of Neuron specific enolase (NSE) (A), 14-3-3 zeta (B), 14-3-3 beta (C) and Tropomyosin receptor kinase B (TrkB) (D) proteins in the ventricular CSF (vCSF) of controls compared to neurodegenerative disease (NDD) cases. A-Di) show example blots of targeted proteins and total protein stain (BLOT-FastStain) with cases and controls separated with an orange dotted line. A-Dii) show mean normalised protein expression for NDD cases compared to controls. * indicates significance at p<0.05, ** p<0.01. Orange bars indicate mean+SEM, n numbers are above stacks.*

Additionally, synaptophysin; a synaptic vesicle membrane protein, was also measured in the vCSF and levels were found to be elevated (although not significantly) in NDD cases compared to controls (normalised synaptophysin expression was 0.91±0.90 in 31 NDD patients and 0.29±0.24 in 8 controls, Mann Whitney U $p>0.05$, Figure 3.12, A). Consistently, TEM confirmed the presence of abundant synaptic vesicles (~40nm) in the vCSF (Figure 3.11), along with a mixture of exosomes (~100nm) and larger microvesicles (~250-1000nm). These structures were found to be present in the NDD case and control sample.

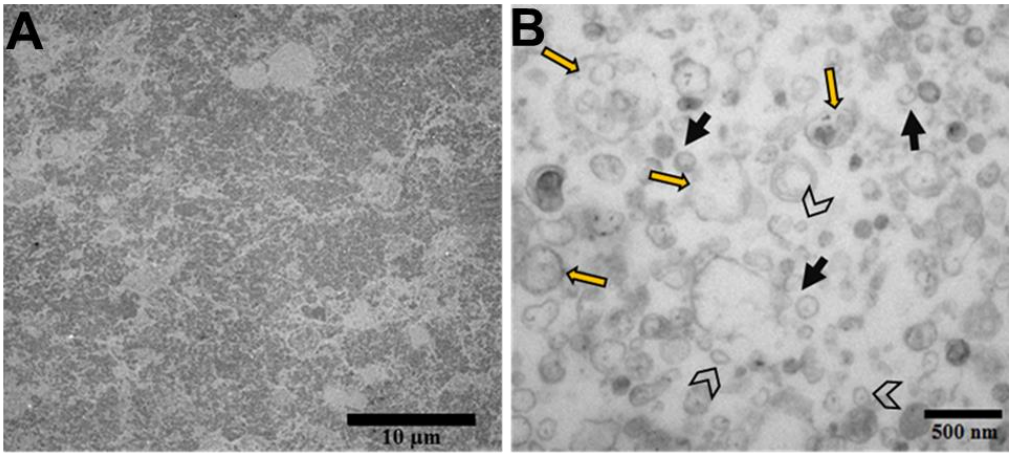

Figure 3.11. Transmission electron microscopy of the vCSF showing abundant proteinaceous material (A, grey staining) and synaptic vesicles (B). Arrows on B point to examples: small synaptic vesicles; black arrow heads, exosomes; open chevrons, large microvesicles; orange arrows.

Mitochondrial proteins Porin (Figure 3.12, B), SDHA (Figure 3.12, C) and Tfam (Figure 3.12, D) were detected in the vCSF by western blot, whereas the OXPHOS complexes were undetectable. However, analysis showed there were no significant differences in the abundance of these proteins in the vCSF between NDD cases and controls (Mann Whitney U $p>0.05$).

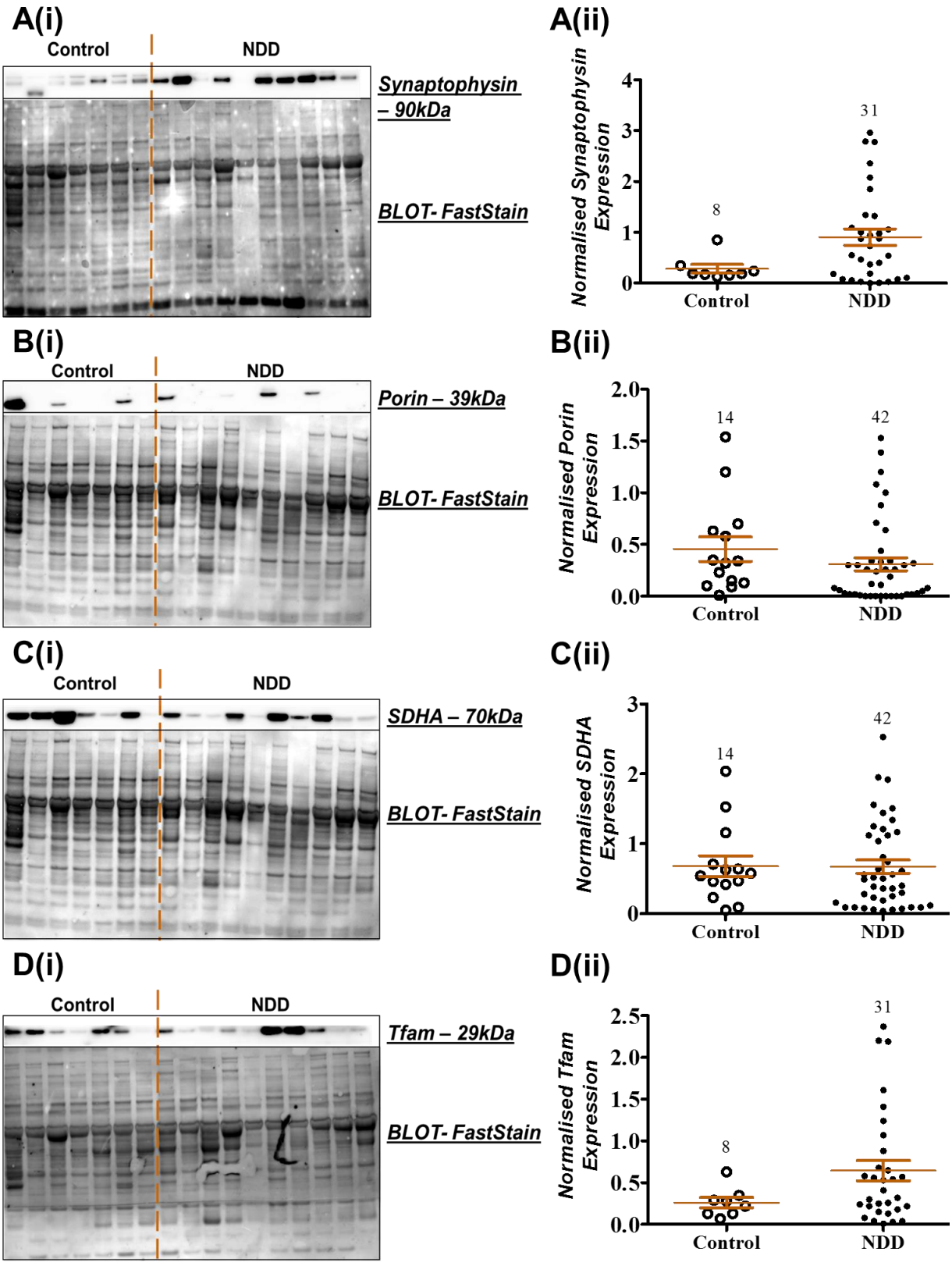

Figure 3.12. Protein abundance of synaptophysin (A) and mitochondrial proteins Porin (B), Succinate dehydrogenase (SDHA) (C) and Transcription factor A (Tfam) (D) proteins in the ventricular CSF (vCSF) of controls compared to neurodegenerative disease (NDD) cases. A-Di) show example blots of proteins of interest and total protein stain (BLOT-FastStain) with cases and controls separated with an orange dotted line. A-Dii) show mean normalised protein expression for NDD cases compared to controls. Orange bars indicate mean+SEM, n numbers are above stacks.

Similar to other work (Chou *et al.*, 2017), TEM of the vCSF could detect mitochondria-like structures measuring ~500nm in size. However, these structures were extremely scarce, and were degraded and fragmented, but still showed remnants of a double-membraned structure and cristae-like formations (Figure 3.13).

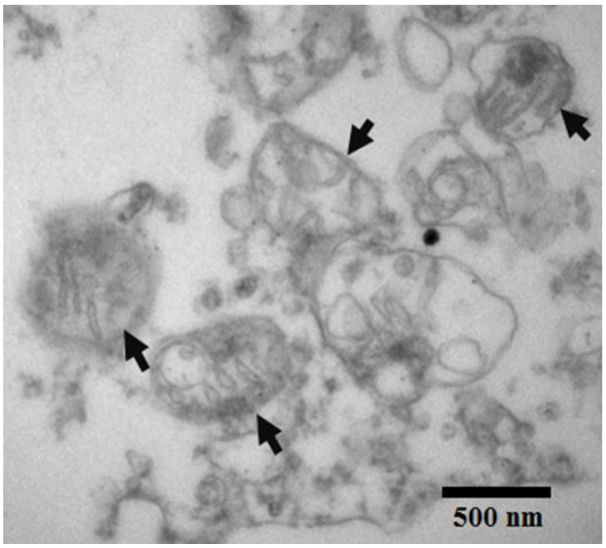

Figure 3.13. Transmission electron microscopy of the vCSF showing examples of the mitochondria-like structures discovered (arrows).

Ccf-mtDNA copy number levels were compared to vCSF protein abundance (Pearson's correlation, Table 3.9). SDHA levels in the vCSF of the entire cohort were found to be significantly associated to ccf-mtDNA copy number ($p=2.70x10^{-2}$), however, the correlation was weak ($r^2=0.29$). No other proteins were found to be correlated to ccf-mtDNA copy number when analysing the entire cohort, or when stratified by NDD cases and controls; indicated by $r^2<0.3$ and $p>0.05$, Table 3.9.

Protein of interest	Whole cohort		Control		NDD	
	r^2	p	r^2	p	r^2	p
NSE	-0.04	$7.67x10^{-1}$	0.30	$2.96x10^{-1}$	-0.11	$4.72x10^{-1}$
14-3-3 Zeta	-0.16	$3.23x10^{-1}$	-0.45	$2.60x10^{-1}$	-0.03	$8.55x10^{-1}$
13-4-4 Beta	-0.27	$9.01x10^{-2}$	-0.45	$2.61x10^{-1}$	-0.22	$2.45x10^{-1}$
SDHA	0.29	$2.70x10^{-2}$ *	0.53	$5.30x10^{-2}$	0.23	$1.52x10^{-1}$
Porin	0.07	$6.14x10^{-1}$	-0.26	$3.63x10^{-1}$	0.18	$2.51x10^{-1}$
Synaptophysin	0.05	$7.43x10^{-1}$	-0.19	$6.47x10^{-1}$	0.03	$8.89x10^{-1}$
Tfam	0.18	$2.85x10^{-1}$	-0.14	$7.46x10^{-1}$	0.17	$3.64x10^{-1}$
TrkB	-0.13	$4.33x10^{-1}$	0.12	$7.04x10^{-1}$	-0.23	$2.38x10^{-1}$
Alpha Synuclein	-0.03	$8.69x10^{-1}$	-0.06	$8.93x10^{-1}$	-0.01	$9.83x10^{-1}$

*Table 3.9. Correlational analysis of vCSF proteins of interest and ccf-mtDNA copy number for NDD cases, controls and the whole cohort. P values are given, generated by Pearsons rank significance testing. * indicates significance at p<0.05.*

VCSF alpha synuclein (α-synuclein) was undetectable by western blot, however, it was detectable by ELISA, which is more sensitive to low protein concentrations. In keeping with previous reports (Berge *et al.*, 2016; Mollenhauer *et al.*, 2017), CSF α-synuclein levels were invariable between controls and NDD cases (mean α-synuclein abundance was 8.5pg/mL and 7.56pg/mL, respectively, Mann Whitney U p>0.05, Figure 3.14, A) and, did not correlate to mtDNA copy number (Pearson's p>0.05 for controls and NDD cases, Table 3.9, Figure 3.14, B).

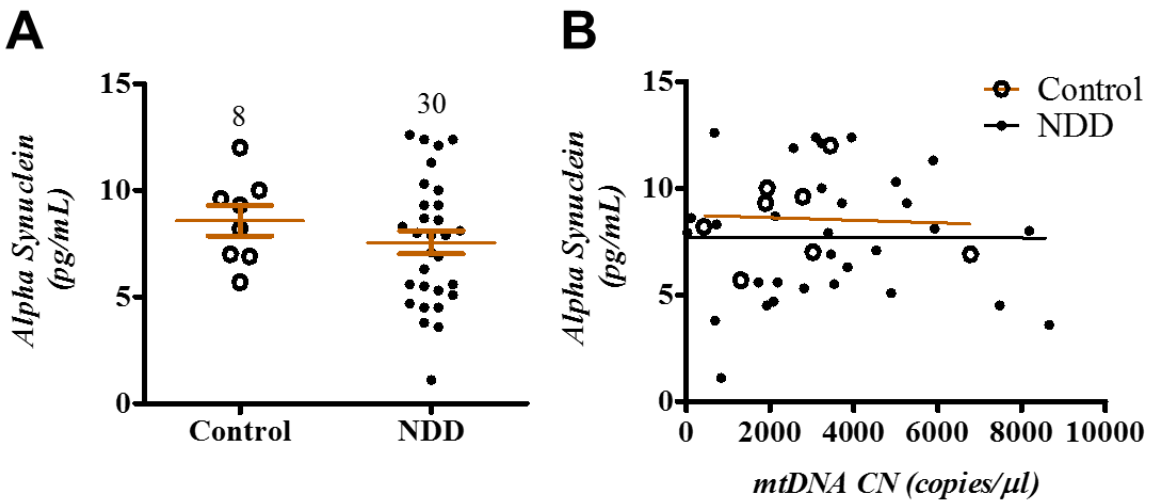

Figure 3.14. Alpha synuclein expression (pg/mL) in the vCSF of NDD cases and controls (A). Orange bars are mean+SEM, n numbers are above stacks. B) Scatter plot of alpha synuclein against ccf-mtDNA copy number for controls (orange regression line) and NDD cases (black regression line).

Tau and NeuN proteins were undetectable in the vCSF by western blot, and more sensitive analysis could not be performed as there were no commercially available ELISA kits.

3.4.6 Ccf-mtDNA integrity in NDD cases compared to controls.

To assess the quality and integrity of the ccf-mtDNA in the vCSF, qPCR was used to calculate ccf-mtDNA deletion level (%) using the ratio of *mtND1* and *mtND4* and next-generation sequencing was carried out to assess heteroplasmy levels.

Approximately 23% of controls and 25% of cases were shown to have deleted portions of ccf-mtDNA in the major deletion arc which exceeded 10% (accepted detection threshold for qPCR (Grady *et al.*, 2014)). However, there was no difference between cases or controls in the deletion level observed (Figure 3.15, A; mean deletion level was 20.3% in 8 controls and 20.2% in 23 NDD cases, $p>0.05$, Mann Whitney U). Furthermore, there was no association between ccf-mtDNA copy number level and ccf-mtDNA deletion level (Figure 3.15, B).

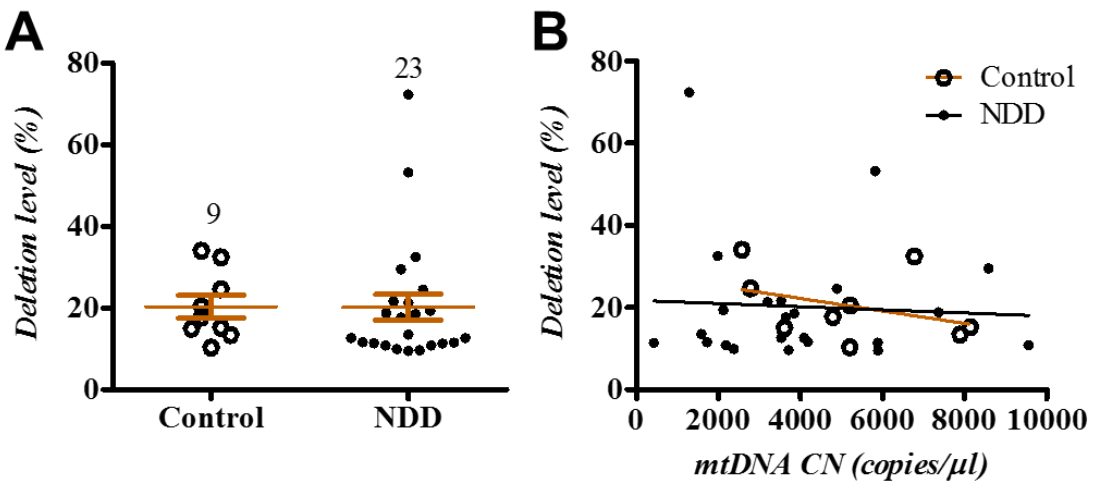

Figure 3.15. Ccf-mtDNA deletion level in the vCSF of controls and neurodegenerative disease (NDD) patients. A) Ccf-mtDNA deletion level (%) of NDD cases compared to controls. Orange bar indicates mean+SEM, n numbers are above stacks. B) Ccf-mtDNA deletion level plotted against ccf-mtDNA copy number for controls (orange regression line) and NDD cases (black regression line).

In addition, correlation analysis showed no association between ccf-mtDNA deletion level (%) and cohort demographics i.e. gender, age and PMI, or dementia severity scores (Table 3.10, Pearson's p>0.05).

Variable	*Whole Cohort*	*Control*	*NDD*
Male vs Female	$2.66x10^{-1}$	$1.60x10^{-1}$	$9.20x10^{-2}$
Age at death	$8.99x10^{-1}$	$4.75x10^{-1}$	$8.37x10^{-1}$
Age of onset	-	-	$9.62x10^{-1}$
Disease duration	-	-	$8.40x10^{-2}$
Post-mortem interval	$6.78x10^{-1}$	$4.25x10^{-1}$	$5.52x10^{-2}$
Braak Stage	$5.20x10^{-2}$	$8.84x10^{-0}$	$5.00x10^{-2}$
Dementia Severity	$9.57x10^{-1}$	$4.59x10^{-1}$	$7.38x10^{-1}$

Table 3.10. Correlation analysis of patient demographics compared to ccf-mtDNA deletion level. Values are p values generated by correlation analysis with Pearson's significance testing.

Using an established NGS methodology and bioinformatics pipeline (Section 2.12), ccf-mtDNA heteroplasmy levels were calculated for NDD cases versus controls. Total variant frequency was not significantly different (Mann Whitney p>0.05) between NDD cases and controls and there was no association between total variant frequency and ccf-mtDNA copy number (Figure 3.16, A and B).

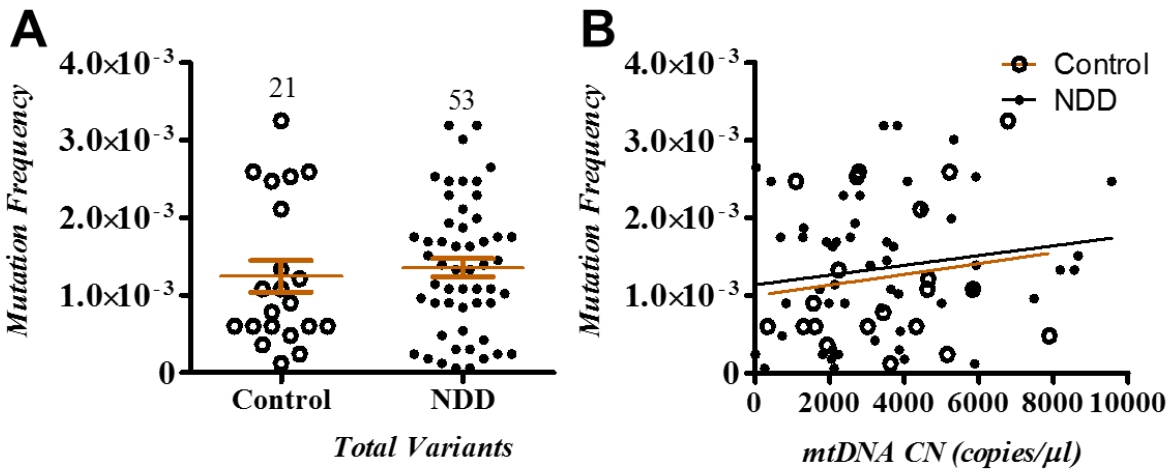

Figure 3.16. Ccf-mtDNA heteroplasmy levels for controls and NDD cases. A) Total ccf-mtDNA mutation frequency (per genome) of NDD cases compared to controls. Orange bars indicate mean+SEM, n numbers are above stacks. B) Total ccf-mtDNA mutation frequency plotted against ccf-mtDNA copy number for controls (orange regression line) and NDD cases (black regression line).

Furthermore, no significant differences were reported in mutation frequency of NDD cases compared to controls when ccf-mtDNA variants were stratified by type (i.e. non-coding, protein-coding, rRNA, tRNA) (Figure 3.17, A-D). In addition, there was no difference between NDD cases and controls when mutation frequency was stratified into synonymous and nonsynonymous mutations (Figure 3.17, E-F).

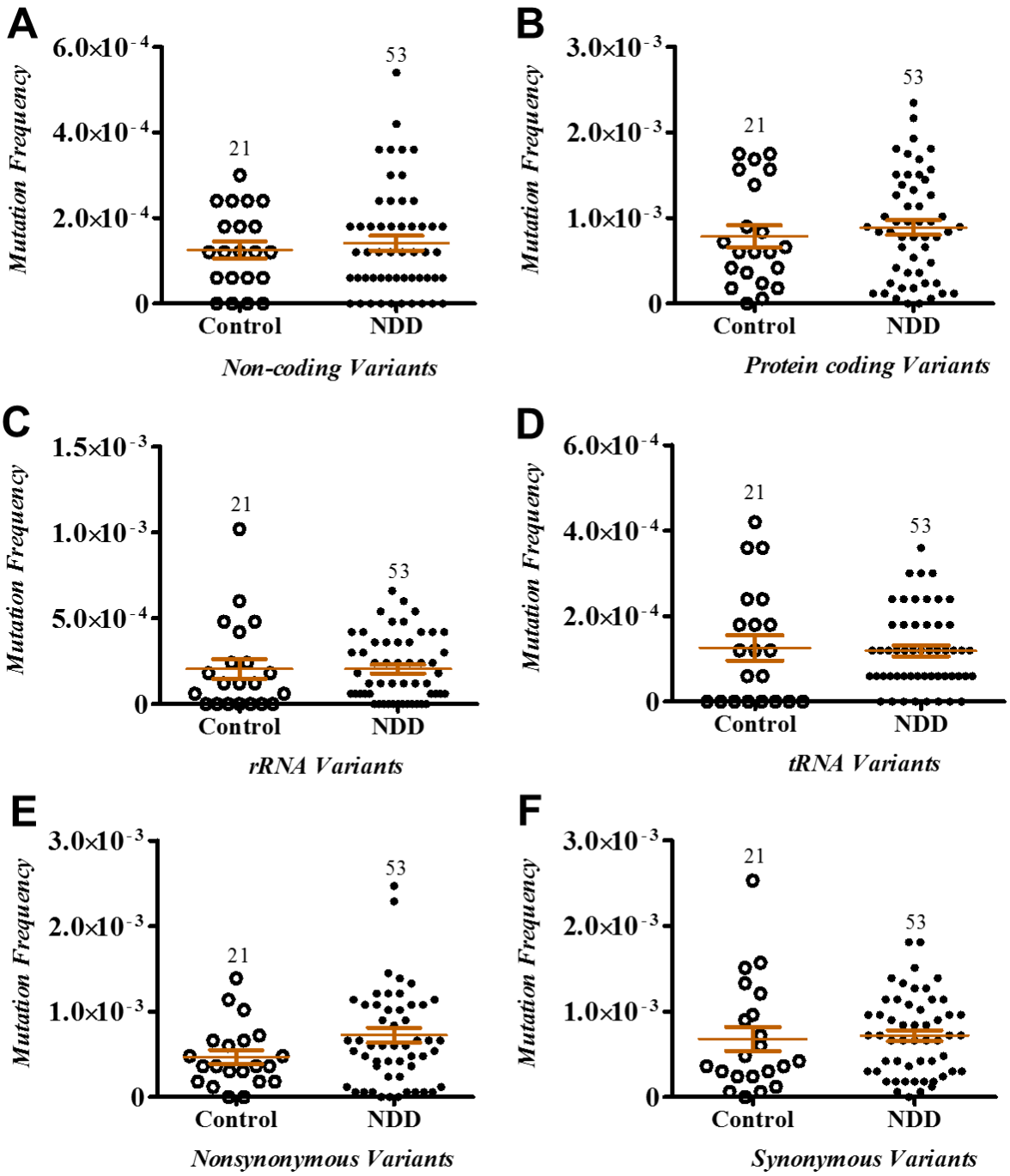

Figure 3.17. Mean ccf-mtDNA mutation frequency per genome compared between NDD cases and controls. Each graph is a different variant type; non-coding (A), protein coding (B), rRNA (C), tRNA (D), nonsynonymous (E), synonymous (F). Orange bars indicate mean+SEM, n numbers are above stacks.

In addition, there was no difference in the ratio of nonsynonymous (Ka) to synonymous (Ks) mutations (Ka/Ks) between NDD cases and controls (Figure 3.18, A) and this did not correlate to ccf-mtDNA copy number in cases or controls (Figure 3.18, B, Pearson's p>0.05).

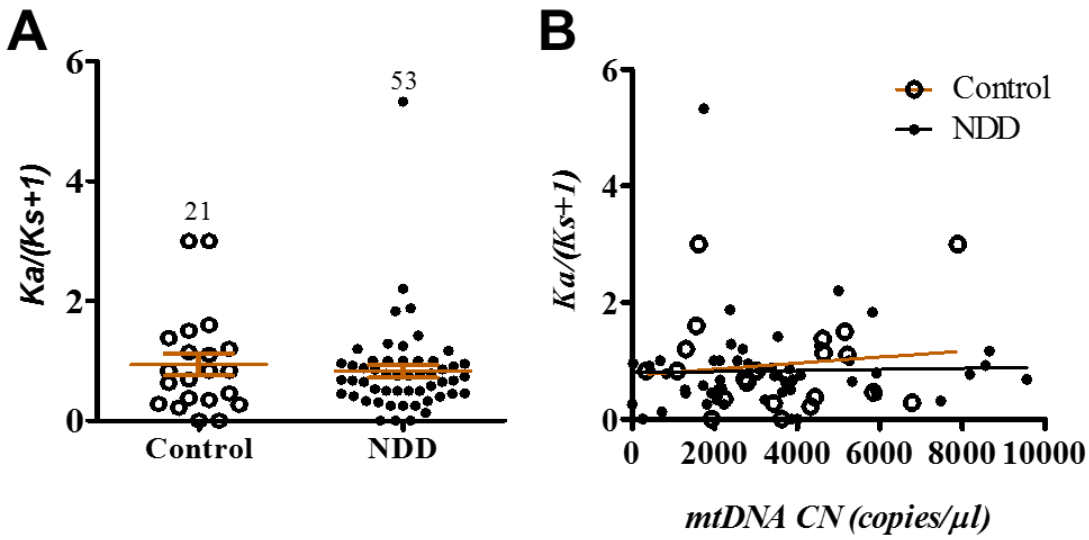

Figure 3.18. Mean ratio of nonsynonymous (Ka) to synonymous (Ks) ccf-mtDNA mutations (A) compared between NDD cases and controls (orange bars indicate mean+SEM, n numbers above stacks) and (B) compared against ccf-mtDNA copy number for controls (orange regression line) and NDD cases (black regression line).

NGS data was also stratified by individual NDD group (Table 3.11), however, there were no remarkable differences between the NDD groups and controls in mutation frequency of any of the variant types (Kruskal-Wallis p>0.05).

Mutation frequency (per mtDNA)	Control (21)	PD (5)	AD (31)	DLB (5)	Dementia (6)	MND (2)	PSP (3)	p
Non coding	1.26×10^{-4}	1.44×10^{-4}	1.43×10^{-4}	4.80×10^{-5}	2.60×10^{-4}	1.40×10^{-4}	4.00×10^{-3}	1.68×10^{-1}
Protein coding	7.86×10^{-4}	1.13×10^{-3}	8.81×10^{-4}	7.60×10^{-4}	1.26×10^{-3}	5.80×10^{-4}	3.40×10^{-4}	3.10×10^{-1}
rRNA	2.06×10^{-4}	2.52×10^{-4}	2.25×10^{-4}	9.60×10^{-5}	2.80×10^{-4}	1.40×10^{-4}	0	1.97×10^{-1}
tRNA	1.26×10^{-4}	1.32×10^{-4}	1.30×10^{-4}	4.80×10^{-5}	1.30×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	7.14×10^{-1}
Total	1.24×10^{-3}	1.66×10^{-3}	1.38×10^{-3}	9.52×10^{-4}	1.93×10^{-3}	9.63×10^{-4}	4.40×10^{-4}	1.69×10^{-1}
Non synonymous	4.69×10^{-4}	7.70×10^{-4}	6.01×10^{-4}	4.80×10^{-4}	8.32×10^{-4}	4.80×10^{-4}	1.60×10^{-4}	2.23×10^{-1}
Synonymous	6.80×10^{-4}	8.92×10^{-4}	7.74×10^{-4}	4.68×10^{-4}	7.53×10^{-4}	4.80×10^{-4}	2.80×10^{-4}	4.87×10^{-1}
Ka/Ks	0.70	0.86	0.78	1.03	1.11	1.00	0.57	7.36×10^{-1}

Table 3.11. Mean ccf-mtDNA mutation frequency per mitochondrial genome for individual neurodegenerative diseases and controls. PD; Parkinson's disease, AD; Alzheimer's disease, DLB' Dementia with Lewy bodies, MND; Motor Neuron disease, PSP; Progressive supranuclear palsy. n numbers are in brackets. P column is the p value generated by Kruskal-Wallis comparing mean mutation frequency across all disease groups.

3.5 Discussion

This is the first study to investigate both the abundance and integrity of circulating cell free mtDNA (ccf-mtDNA) copy number within the ventricular CSF (vCSF) of a broad range of neurodegenerative diseases (NDDs). Despite previous reports in Alzheimer's disease (AD) and Parkinson's disease (PD) (Podlesniy *et al.,* 2013; Pyle *et al.,* 2015), there was no significant difference in ccf-mtDNA copy number between other NDD patients compared to controls, suggesting that ccf-mtDNA copy number is not a useful biomarker for broad NDD. Measurement of targeted protein abundance and ccf-mtDNA integrity in the vCSF gave insight into the mechanism behind ccf-mtDNA release, suggesting that ccf-mtDNA are mostly intact, mitochondria-free molecules released during normal cell turnover processes and not as a consequence of neurodegeneration.

3.5.1 Low ccf-mtDNA copy number is specifically associated with PD, not broad NDD.

In keeping with work by Pyle *et al.,* (2015), stratification of NDDs into subtypes revealed a significant reduction in ccf-mtDNA copy number in PD patients compared to controls, suggesting that low ccf-mtDNA copy number persists through early stage to end stage PD. In addition, further analysis supported the validity of low ccf-mtDNA as a potential biomarker of PD, showing >80% specificity and sensitivity in distinguishing PD from controls.

Alzheimer's Disease (AD), Dementia with Lewy Bodies (DLB), non-AD Dementia, Motor Neuron Disease (MND) and Progressive Supranuclear Palsy (PSP) patients showed no significant difference in ccf-mtDNA copy number compared to controls, indicating that vCSF ccf-mtDNA copy number is not associated with broad NDD.

Initial studies in PD (Pyle *et al.*, 2015b) and AD (Podlesniy *et al.*, 2013) and the heterogeneity of clinical features of NDDs (Noe *et al.*, 2004; Ravits *et al.*, 2013; Lee *et al.*, 2014; Li and Wu, 2016; Takemoto *et al.*, 2016) led to the hypothesis that ccf-mtDNA copy number could have potential as a biological marker for broad NDD. However, subsequent research has been conflicting and has highlighted challenges associated with the validation of ccf-mtDNA copy number as a biological marker in this heterogeneous group of disorders.

Podlesniy *et al.,* (2016) further validated low ccf-mtDNA copy number as a biomarker for preclinical AD, by proving its specificity in distinguishing AD from other forms of dementia (non-AD Dementia and a rapidly progressing form of Dementia (Crutzfeldt Jakob disease, CJD)) and non-demented NDD controls. This indicates that despite phenotypic similarities, ccf-mtDNA copy number differences are present between different forms of dementia, and therefore suggests that ccf-mtDNA copy number may behave differently depending on the type of NDD. However, work by Podlesniy *et al.,* (2016) did not include a group of non-NDD controls, therefore, conclusions are limited as comparison of ccf-mtDNA copy number between NDD patients and healthy controls were not evaluated. A more recent study (Cervera-Carles *et al.,* 2017) replicated the previous work by Podlesniy *et al.* (2013) in a larger cohort including preclinical AD, mild cognitive impairment (MCI), AD and healthy controls. Cervera-Carles *et al*., (2017) reported the converse to Podlesniy *et al*. (2013) observing that ccf-mtDNA copy number was higher in AD patients compared to controls, but supported findings from the NDD-vCSF cohort as they established no signficant difference in ccf-mtDNA copy number between preclincal AD, MCI and controls. Furthermore, work by Cervera-Carles *et al.,* (2017) highlighted significant dispersion in ccf-mtDNA copy number within disease groups and controls, likely owing to the biological and clinical heterogenity of the cohort, concluding that due to this variance, ccf-mtDNA was not robust enough to distinguish AD patients from controls (ROC curve, ~70% sensitivity and specificity (Cervera-Carles *et al.*, 2017)). Similarly, work in the NDD-vCSF cohort also indicated large interindividual variability; shown by coefficient of variance of 60% for control ccf-mtDNA copy number and 68% for NDD ccf-mtDNA copy number. This greatly limits the biomarker potential of ccf-mtDNA as it indicates that it lacks the sensitivity, specificity and replicability needed to be a valid biomarker.

Overall, ccf-mtDNA copy number analysis in the NDD-vCSF cohort has disproved the hypothesis that ccf-mtDNA copy number is a biomarker for broad NDD. However, it is important to recognise that findings reported here are specific to post-mortem vCSF, rather than lCSF taken in-life, and therefore may be influenced by differences in CSF composition (Gerber *et al.*, 1998; Sommer *et al.*, 2002) or by effects of end-stage disease.

Comparison of previously published lCSF ccf-mtDNA copy number (Pyle *et al.*, 2015b) and vCSF ccf-mtDNA copy number highlights a significant difference in ccf-

mtDNA copy number level (Figure 3.19, Mann Whitney U p<0.0001). As mtDNA is possibly released in response to cell death (Aucamp *et al.*, 2018), the significant elevation in ccf-mtDNA copy number in the vCSF may be a reflection of the late-disease stage and old age of these participants, as these factors are associated with increased neuronal death (Morrison and Hof, 1997; Gorman, 2008). Alternatively, this could be a factor relating to the post-mortem nature of the vCSF samples as studies have demonstrated significant differences in composition, cell count and protein level of ante-mortem and post-mortem CSF (Wyler *et al.*, 1994; Arroyo *et al.*, 2005). This is possibly as a result of lysing neurons, breakdown of the blood brain barrier and cessation of CSF turnover. This could imply a major limitation of the use of vCSF in biomarker discovery, potentially suggesting that ccf-mtDNA copy number is an indirect measure of dead cell count in the vCSF. However, as *B2M* (nDNA) level was low and in most samples completely absent, cells are unlikely to be present. To ensure this, however, it would be appropriate to assess whether vCSF ccf-mtDNA copy number correlates to vCSF cell count in an independent cohort.

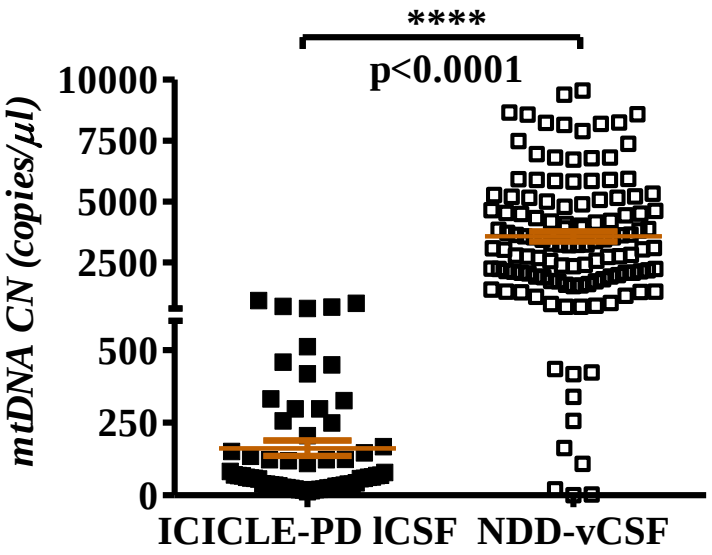

*Figure 3.19. Comparison of ccf-mtDNA copy number (CN) per microliter of CSF in the ante-mortem lumbar CSF (ICICLE-PD cohort) and the post-mortem ventricular CSF (NDD-vCSF cohort). Orange bars indicate mean+SEM. **** indicates significant difference calculated by Mann Whitney U at p<0.0001.*

3.5.2 Cognitive impairment is associated with increased ccf-mtDNA copy number.

Unexpectedly, ccf-mtDNA copy number was found to be significantly increased in individuals with cognitive impairment compared to those who were cognitively normal. This coincided with a significant positive correlation between ccf-mtDNA copy number and tau Braak stage. Importantly, tau Braak stage and cognitive impairment also correlated to one another showing that increased tau pathology was associated with cognitive impairment (Pearson's correlation, $r^2= 0.55$, $p<0.01$).

This suggests that vCSF ccf-mtDNA copy number may be useful for monitoring tau-associated cognitive decline in healthy and diseased individuals, and is supported by previous work (Lee *et al.*, 2010; Kim *et al.*, 2013) in leukocytes reporting similar potential for mtDNA copy number as a marker of cognitive decline and dementia in healthy elderly individuals. In addition, previous reports indicate a significant positive correlation between leukocyte mtDNA copy number and MMSE score (Lee *et al.*, 2010; Kim *et al.*, 2013), whereas this study indicated a positive correlation between ccf-mtDNA copy number and tau-associated cognitive decline. This suggests that 'good' cognitive function (MMSE >24) may promote retention of mtDNA within the cell. Whereas cognitive impairment and tau pathology, which is associated with increased mitochondrial dysfunction (Bishop *et al.*, 2010; Cheng and Bai, 2018), may promote the export of mtDNA from cells into circulation, hence the elevation of vCSF ccf-mtDNA, but the depletion of cellular mtDNA (Lee *et al.*, 2010; Kim *et al.*, 2013) in cognitively impaired individuals. Additionally, as cognitive decline and tau pathology is also associated with widespread neuronal and synaptic loss (Gendron and Petrucelli, 2009; Bishop *et al.*, 2010), owing to the neurotoxic effects of Tau accumulation, mtDNA may also be exported out of neurons as a result of increased cell death. To assess this fully, it would interesting to measure and compare ccf-mtDNA copy number in the CSF to mtDNA copy number in peripheral blood leukocytes and neuronal tissue in matched participants to assess if there is a correlation between these two levels.

3.5.3 VCSF targeted protein assessment shows disease association but not an association to ccf-mtDNA copy number.

A large targeted proteomic screen was carried out to better understand the mechanism of ccf-mtDNA export and to establish proteomic links to ccf-mtDNA abundance. However, although proteomic differences were present between NDD cases and controls, there were no significant associations to ccf-mtDNA.

i) *Neuronal Proteins*

The vCSF levels of NSE, 14-3-3 beta and 14-3-3 zeta observed in the diseased individuals and controls were contrasting to previous reports (Nooijen *et al.*, 1997; Siman *et al.*, 2009; Schmidt *et al.*, 2014). These proteins are typically shown to be upregulated in the CSF in response to neurodegeneration and neuronal damage (Siman *et al.*, 2009; Foote and Zhou, 2012; Schmidt *et al.*, 2014), and are rarely detected in healthy controls (Shiga *et al.*, 2006). However, there was no difference observed in NSE levels, and there was a downregulation of 14-3-3 and TrkB protein levels in NDD patients compared to controls. The discrepancy between these findings and those reported previously may be explained by differences in CSF sampling. Previous work used lCSF samples obtained in-life whereas samples in the NDD-vCSF cohort were vCSF obtained post-mortem, suggesting differences may be attributed to effects associated with death and end-stage disease or differences in composition of the two portions of CSF (Gerber *et al.*, 1998; Sommer *et al.*, 2002). Based on this, these findings suggest that in the latter stages of NDDs, where neuronal loss is almost complete, neurotrophic factors are downregulated; either because their actions are no longer successful, or their synthesis and release is no longer possible, due to the gross neurodegeneration that has preceded (Shiga *et al.*, 2006). Furthermore, the detection of these proteins in the vCSF of healthy controls may be explained by the onset of neuronal degeneration associated with aging (Morrison and Hof, 1997).

Although CSF α-synuclein level has been suggested as a potential biomarker for NDDs (Mollenhauer, 2014), its diagnostic utility has been questioned by discrepant findings with some studies reporting significant elevation in total α-synuclein in AD (Majbour *et al.*, 2017), PD with dementia (Hansson *et al.*, 2014) and DLB (Kapaki *et al.*, 2013), whereas others report a significant reduction in AD (Ohrfelt *et al.*, 2009; Korff *et al.*, 2013), PD (Tokuda *et al.*, 2006; Mollenhauer *et al.*, 2008; Hong *et al.*,

2010; Mollenhauer *et al.*, 2011) and DLB (Mollenhauer *et al.*, 2008; Gao *et al.*, 2015; Eusebi *et al.*, 2017). Findings from the NDD-vCSF cohort show that there were no differences in vCSF α-synuclein level across the different NDDs, and reported a non-significant reduction in NDD cases compared to controls, further questioning the validity of this protein as a marker of NDD. However, it is important to recognise that the α-synuclein ELISA assay was not specific to different forms of α-synuclein, and therefore may not have captured oligomeric or phosphorylated forms, which may contribute to the result that there was no difference between NDD cases and controls.

As one of the main theories of ccf-mtDNA release is related to cell death (Aucamp *et al.*, 2018) one could assume that levels of protein markers of neuronal death (NSE, 14-3-3 proteins and TrkB) and neurodegeneration (α-synuclein) may be associated with ccf-mtDNA copy number. However as this was not observed, my findings may imply that ccf-mtDNA export is a far more complex process that is not simply coupled to cell death. However, it is important to recognise that the findings reported in this study are subject to low population validity and post-mortem effects and are greatly limited by bias associated with targeted protein investigation.

ii) *Mitochondrial proteins*

It is unknown whether ccf-mtDNA circulates as a free entity or encapsulated within the mitochondria. In keeping with previous research (Chou *et al.*, 2017), this work confirmed the presence of mitochondrial-like structures (TEM) and important mitochondrial proteins (Porin, SDHA, Tfam) within the vCSF. However, mitochondrial protein levels were no different between cases and controls and largely did not correlate to ccf-mtDNA copy number, suggesting that ccf-mtDNA does not circulate within the mitochondria or bound in nucleoids with Tfam. The weak correlation between ccf-mtDNA copy number and SDHA may suggest that in some instances, ccf-mtDNA are released with other mitochondrial components, or perhaps within the mitochondria. However, these protein components may be degraded more quickly than ccf-mtDNA in different individuals, explaining the variation between subjects and the subsequent weak correlation between the two variables. Due to the novelty of this finding and the vast degree of interindividual protein level variation, it is imperative that this research is replicated in a large cohort using more robust proteomic assessment methods, such as ELISA or mass spectrometry.

iii) *Synaptic Proteins*

Previous work indicates that ccf-mtDNA may be contained within microvesicles, exosomes and exophers (Guescini *et al.*, 2010; Balaj *et al.*, 2011; Waldenstrom *et al.*, 2012; Fernando *et al.*, 2017; Melentijevic *et al.*, 2017; Wang *et al.*, 2017), however, this had never been investigated in human body fluids. Synaptotagmin, a synaptic vesicle protein had previously been described in the human CSF, however, until now synaptophysin had not been detected (Davidsson *et al.*, 1996; Davidsson *et al.*, 1999). TEM and protein work indicated the presence of a variety of extracellular vesicles in the human CSF, however, due to the qualitative nature of these techniques, an association to ccf-mtDNA could not be confirmed. Thus, further work is warranted to assess this relationship more robustly, focussing on the extraction of extracellular vesicles from the CSF (Witwer *et al.*, 2013) to allow subsequent mtDNA copy number analysis and sequencing specifically in these fractions.

iv) *Limitations of protein work*

A major limitation of this study was the use of candidate targeted protein investigation, involving the selection of a number of proteins based on existing relationships to neuronal survival, neurodegeneration, mitochondrial integrity and synaptic vesicle localisation, to assess novel relationships between these areas of research and ccf-mtDNA copy number by western blot. However, the targeted nature of this method greatly limits the number of proteins that can be investigated and the selection of these proteins based on prior knowledge introduces bias to the data that prevents other novel avenues being explored. A more fruitful method to establish proteomic links to ccf-mtDNA copy number and develop understanding of the mechanism of this biological phenomenon would be to screen as many possible proteins simultaneously, without *a priori* assumptions about their potential associations with ccf-mtDNA (Chen-Plotkin, 2014). Therefore, future work could employ the use of mass spectrometry, to carry out an unbiased proteomic screen of the CSF. This technique has massive coverage of the proteome and is far more sensitive, accurate and has higher-throughput than western blot, however, involves a more expensive and complicated process (Aebersold *et al.*, 2013; Chen-Plotkin, 2014).

3.5.4 Ccf-mtDNA is largely intact and integrity is similar between NDD cases and controls.

Assessment of ccf-mtDNA integrity aimed to investigate a potential mechanism of ccf-mtDNA export, based on the preferential selection and removal of mutant, damaged mtDNA into the body fluid in a bid to promote cell survival by retention of 'wild type' mtDNA (Melentijevic *et al.*, 2017). However, measures of ccf-mtDNA integrity suggested that the ccf-mtDNA was largely intact and mutational burden was similar between NDD cases and controls. This may suggest that export of ccf-mtDNA is not related to preferential selection, but instead is released during normal cell turnover. Furthermore, studies of mtDNA in brain tissue of patients with PD (Lin *et al.*, 2012; Coxhead *et al.*, 2016), DLB (Lin *et al.*, 2012) and AD (Coskun *et al.*, 2004) have shown significantly elevated levels of heteroplasmic mtDNA mutations implying these mutated molecules are not preferentially removed. Differently, the levels of ccf-mtDNA in the vCSF may directly reflect the levels of mtDNA in the brain. This hypothesis holds true for PD, where CSF ccf-mtDNA levels and brain mtDNA levels are reduced compared to controls (Pyle *et al.*, 2015a; Pyle *et al.*, 2015b). However, this is opposed by studies in AD which suggest that brain mtDNA copy number is lower in AD than controls (Rice *et al.*, 2014), whereas ccf-mtDNA copy number was no different between AD and controls in the NDD-vCSF cohort.

3.6 Conclusion

Overall, investigation of ccf-mtDNA in the NDD-vCSF cohort has revealed that ccf-mtDNA copy number is not a biomarker of broad NDD, but could still be a useful biomarker for PD, supporting previous work by Pyle *et al.,* (2015). Due to the low cohort size used in the NDD-vCSF (8 PD versus 40 controls) and Pyle *et al.,* (2015) study (53 PD versus 10 controls), a further large-scale replication study is needed to validate these findings; see Chapter 5, PPMI-PD cohort.

Furthermore, protein abundance analysis of neuronal survival and neurodegenerative proteins in the vCSF has questioned the cell-death theory of ccf-mtDNA export, whilst investigation of mitochondrial integrity has shown a non-selective export of generally intact mtDNA.

Chapter 4. Ccf-mtDNA copy number variation in Progressive Multiple Sclerosis – PMS-vCSF cohort

4.1 Introduction

Similar to Parkinson's disease (PD), Alzheimer's disease (AD) and other neurodegenerative diseases (NDDs), Multiple Sclerosis (MS) also has established links to mitochondrial dysfunction (Mao and Reddy, 2010). At a cellular level, the demyelination that is characteristic of MS pathology has been linked to mitochondrial deficiency, with lesions demonstrating mitochondrial depletion (Dutta *et al.*, 2006) and mitochondrial respiratory chain complex deficiencies (Lucchinetti *et al.*, 2000). Additionally, at a genetic level, inherited mtDNA variation has been found to modulate the risk of MS (Andalib *et al.*, 2013), with mtDNA haplogroup J increasing (Tranah *et al.*, 2015) and haplogroup K decreasing (Hudson *et al.*, 2014) the risk of developing MS. Furthermore, multiple deletions in the mtDNA can be detected in the gray matter of MS patients, which could be indicative of primary mitochondrial disease (Campbell *et al.*, 2011). This is further supported by evidence in the mitochondrial disease Leber's Hereditary Optic Neuropathy, as generally these patients go on to develop demyelinating disease, clinically indistinguishable from MS (Ban *et al.*, 2008).

MS is classically stratified into two subgroups; relapsing-remitting (RRMS) or progressive (PMS). Major progress has been made in understanding the disease mechanisms of RRMS, leading to effective anti-inflammatory and immunomodulatory treatments to reduce the severity and frequency of new demyelinating episodes (Section 1.7.4) (Lassmann *et al.*, 2012). Conversely, the mechanisms underlying PMS are much less understood and subsequently, therapeutics at this stage are greatly limited to symptomatic relief. Interestingly, PMS has been likened to neurodegenerative disease due to the extensive axonal and synaptic loss within the CNS (Bjartmar et al., 2003; Jurgens et al., 2016) following initial inflammation and white matter demyelination. Furthermore, unlike RRMS, PMS involves extensive cortical demyelination, however, these cortical plaques are not associated with increased inflammation (Peterson *et al.*, 2001; Bø *et al.*, 2003; Kutzelnigg *et al.*, 2005). This has led to the widely accepted hypothesis that MS is initiated by an inflammatory process, but following years of chronic inflammation, develops a neurodegenerative component (PMS) that progresses independently from inflammation (Trapp and Nave, 2008; Lassmann, 2010). Currently, there is no single diagnostic test for MS and instead diagnosis is made using clinical, imaging and body fluid biomarkers (Section 1.7.4). In addition, there are no validated prognostic

biomarkers for MS which could reflect the disease severity or predict the conversion from a clinically isolated syndrome (CIS) to RRMS to PMS.

Recently, studies have investigated the potential of ccf-mtDNA copy number as a prognostic biomarker within the lumbar CSF (lCSF) of RRMS (Varhaug *et al.*, 2017) and PMS (Leurs *et al.*, 2018) patients. Initial work focussed on RRMS only, reporting significantly increased ccf-mtDNA copy number levels compared to controls (Varhaug *et al.*, 2017). This was postulated to be due to the acute inflammatory nature of RRMS as ccf-mtDNA copy number was observed to decline with duration of disease (potentially relating to conversion to a more neurodegenerative state with lower inflammation later in disease stage), but conclusions were limited by low population size (21 RRMS cases and 23 controls). Subsequently, to assess whether ccf-mtDNA copy number could indicate the conversion from primarily inflammatory (RRMS) to primarily neurodegenerative MS (PMS), Leurs *et al.*, (2018) measured ccf-mtDNA copy number in a larger cohort of RRMS, PMS and controls (50 RRMS, 27 secondary PMS, 13 primary PMS, 7 controls and 23 non-inflammatory neurological disease controls (NINDC)). Primary PMS cases (n=13) were observed to have significantly higher ccf-mtDNA copy number than NINDC (n=23) but not healthy controls (n=7), however, large dispersion in ccf-mtDNA copy number between and within disease groups made it difficult to reliably distinguish cases from controls (Leurs *et al.*, 2018). Furthermore, Leurs *et al*., (2018) failed to reproduce findings from Varhaug *et al*., (2017) suggesting that previous results for RRMS were limited by low sample size. Importantly, Leurs *et al.*, (2018) indicated a potential difference in ccf-mtDNA copy number level of PMS compared to RRMS cases and controls, however, validation in an independent larger PMS cohort is needed.

In Chapter 3, ccf-mtDNA copy number was only found to be associated with PD patients, suggesting that ccf-mtDNA copy number is specific to particular forms of neurodegenerative disease. Given the existing links between PMS and neurodegeneration and building upon previous literature (Leurs *et al.*, 2018), this chapter utilised a large cohort of neurodegenerative end-stage PMS cases to explore ccf-mtDNA copy number differences between cases and controls and validate the biomarker potential of ccf-mtDNA copy number in PMS. Similar to Chapter 3, vCSF ccf-mtDNA copy number, integrity and targeted protein abundance were analysed and compared between a large cohort of PMS cases and matched controls.

Due to the inflammatory, neurodegenerative nature of PMS, the CSF proteins chosen to be investigated were specifically related to neurodegeneration and the glial response. These included S100 calcium-binding protein B (S100β); an astrocytic marker, glial fibrillary acidic protein (GFAP); a glial marker, myelin basic protein (MBP); a constituent of the myelin sheath and chitinase 3 like 1 (CH3L1) and chitinase 3 like 2 (CHI3L2) proteins; two members of the chitinase family that lack chitinase enzymatic activity. Elevated levels of S100β, GFAP and MBP have been associated with neurodegeneration (Petzold *et al.*, 2002; Rothermundt *et al.*, 2003), whilst an elevation in all five of these proteins has been associated with the progression of MS, and in some cases can predict patient prognosis (Norgren *et al.*, 2004; Thouvenot *et al.*, 2014; Martínez *et al.*, 2015; Mollgaard *et al.*, 2016). Therefore, correlation of ccf-mtDNA copy number to these proteins of interest could potentially improve the prognostic accuracy of these protein biomarkers for PMS and could reveal a mechanistic link between neurodegenerative processes and the export of ccf-mtDNA.

4.2 Materials and methods

4.2.1 PMS-vCSF cohort

The PMS-vCSF cohort consisted of 81 ventricular CSF (vCSF) samples; 44 controls and 37 PMS, details of which are present in Section 2.1.4. Samples were collected and processed as outlined in Section 2.2.2.

4.2.2 mtDNA Quantification and Sequencing

Ccf-mtDNA copy number was quantified by triplex qPCR as described in Sections 2.3 to 2.7. Ccf-mtDNA was extracted, sequenced and analysed as in the NDD-vCSF cohort, using NGS MiSeq technology outlined in Sections 2.9 to 2.12.

4.2.3 Protein Quantification and Antibodies

Protein expression was measured in a subset of the vCSF samples in the PMS-vCSF cohort (12 control and 26 PMS) due to sample volume limitations and low total protein concentration (measured by broad range Qubit 3.0). Western blots were carried out by methods described previously in Section 2.8. Antibodies specifically used in this section were mouse monoclonal primary antibodies targeted to glial fibrillary acid protein (GFAP; ab10062, Abcam), S100 calcium binding protein B (S100β; ab14849, Abcam) and myelin basic protein (MBP; ab62631, Abcam) in 1:500 dilution.

4.2.4 Chitinase protein ELISA

Chitinase 3 like 1 (CHI3L1) and chitinase 3 like 2 (CHI3L2) proteins were measured in the same 38 samples (12 controls and 26 PMS) by quantitative sandwich ELISA (Cusabio, YKL-40 and R&D systems DC3L10, respectively). ELISA plates were pre-coated in antigen specific antibody. For CHI3L1 ELISA 100µl standard or sample was added to each well and incubated for 2 hours. Wells were aspirated and washed three times, then 100µl conjugate was added and incubated for 1 hour. Aspiration and washes were repeated. 90µl substrate solution was added to each well and incubated for 15 minutes, then stopped with 50µl stop solution. Optical density of samples was measured using the microplate reader (as described in Section 2.8.2).

For CHI3L2 ELISA 50µl of standard or sample was diluted in 100µl assay diluent in each well and incubated for 2 hours. Wells were aspirated and washed four times, and 200µl conjugate solution was added to each well and incubated for 2 hours. Aspiration and washes were repeated. 200µl substrate solution was added to each well for 30 mins and stopped with 50µl stop solution. Samples were read using the Varioskan LUX multimode microplate reader (as described in Section 2.8.2).

4.3 Results

4.3.1 PMS-vCSF cohort inclusion criteria and demographics

Frequency distribution data of ccf-mtDNA copy number in PMS-vCSF cohort was similar to the NDD-vCSF cohort (Chapter 3), with ~95% of samples having a ccf-mtDNA copy number below 10,000 copies/µl (Figure 4.1, A). Furthermore a minority of samples had *B2M* levels >2 copies/µl but this did not correlate to ccf-mtDNA copy number (Figure 4.1, C, r^2<0.3, Pearsons p>0.05). In light of the difficulty in obtaining matched control vCSF samples, the controls used in this chapter are the same as those used in the NDD-vCSF chapter (thus, the previous inclusion criteria, Chapter 3, was reapplied).

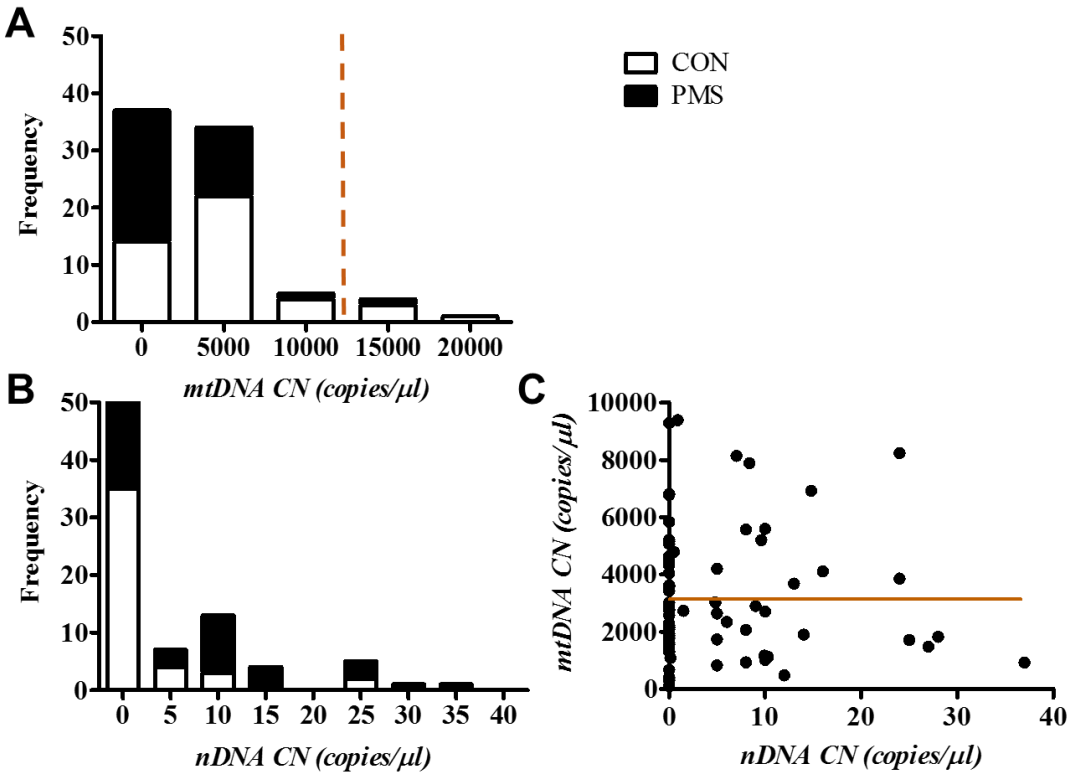

Figure 4.1. Frequency distribution (n) of (A) ccf-mtDNA copy number and (B) ccf-nDNA copy number; controls are in white, PMS cases are in black, orange dashed line represents the upper limit inclusion criteria threshold for ccf-mtDNA copy number. C) shows no relationship between ccf-mtDNA copy number and nDNA copy number, solid orange line represents linear regression (r^2=0.00 and p>0.05).

After removing samples with ccf-mtDNA >10,000 copies/µl (n=5), the final cohort consisted of 76 samples; 40 controls and 36 PMS cases. Demographic information, including gender, mean age at death and post-mortem interval is presented in Table 4.1, stratified by PMS cases and controls. Gender, age at death and PMI were significantly different between cases and controls (Table 4.1). However, no differences were present between PMS cases and controls in brain weight or CSF pH (Table 4.1, Mann Whitney U p>0.05).

Variable	*Control (40)*	*PMS (36)*	*p*
Gender (M/F)	22/18	10/26	1.61×10^{-2} *
Age at death (years)	78±11	63±12	1.00×10^{-3} **
Age of onset (years)	-	33±10	-
Disease duration (years)	-	30±12	-
Post-mortem interval (hours)	37±25	21±13	1.00×10^{-3} **
CSF pH	6.5±1	7±1	1.21×10^{-1}
Brain weight (g)	1295±162	1211±147	5.40×10^{-2}

*Table 4.1. Demographic information for the PMS-vCSF cohort stratified by progressive MS (PMS) case and control (n in brackets), showing mean age, disease duration, post-mortem interval, CSF pH and brain weight ± standard deviation. T tests were used to compare mean age, PMI, CSF pH and brain weight between PMS cases and controls. Pearson's chi square was used to statistically compare gender. * indicates significance at p<0.05, ** at p<0.01.*

In keeping with previous results (Chapter 3), correlation analysis showed there were no significant associations (p>0.05) between ccf-mtDNA copy number and age at death, age of onset, disease duration or post-mortem delay, in cases, controls or the entire cohort (Table 4.2). Additionally, there was no significant association (p>0.05) with gender in PMS cases or controls alone, however, in the whole cohort there was a gender effect for increased ccf-mtDNA copy number in males (Mann Whitney U $p=3.00 \times 10^{-2}$). Furthermore, ccf-mtDNA copy number did not correlate to brain weight or CSF pH (Table 4.2, Pearson's p>0.05).

Variable	Whole Cohort	Control	PMS
Male vs Female	$3.00x10^{-2}$ *	2.68x10-1	2.98x10-1
Age at death	1.98x10-1	7.23x10-1	9.96x10-1
Age of onset	-	-	1.10x10-1
Disease duration	-	-	1.90x10-1
Post-mortem interval	4.69x10-1	4.96x10-1	4.27x10-1

*Table 4.2. Pearson's correlation analysis of gender, age, disease duration, post-mortem interval, CSF pH and brain weight to ccf-mtDNA copy number for PMS cases, controls and the entire cohort. Values are p. * indicates significance at p<0.05.*

4.3.2 Ccf-mtDNA copy number analysis in PMS cases compared to controls.

Similarly to results reported in PD (Pyle *et al.*, 2015b) and AD (Podlesniy *et al.*, 2013), but differently to those reported in RRMS (Varhaug *et al.*, 2017; Leurs *et al.*, 2018) and PMS patients (Leurs *et al.*, 2018), ccf-mtDNA copy number was found to be significantly lower in PMS cases compared to controls (mean ccf-mtDNA copy number was 2413±328 in 36 PMS cases and 3785±356 in 40 controls, Mann Whitney U $p=2.30x10^{-3}$) (Figure 4.2, A). Linear regression, with *B2M* level as a covariate (to ensure this was not confounding the data), further supported this significant association ($p=3.00x10^{-3}$) and proved that ccf-nDNA copy number did not directly influence ccf-mtDNA copy number. Furthermore, due to differences in gender, age and PMI between PMS cases and controls, linear regression was performed with these variables as covariates, however, none were found to significantly effect ccf-mtDNA copy number between PMS cases and controls ($p<0.05$).

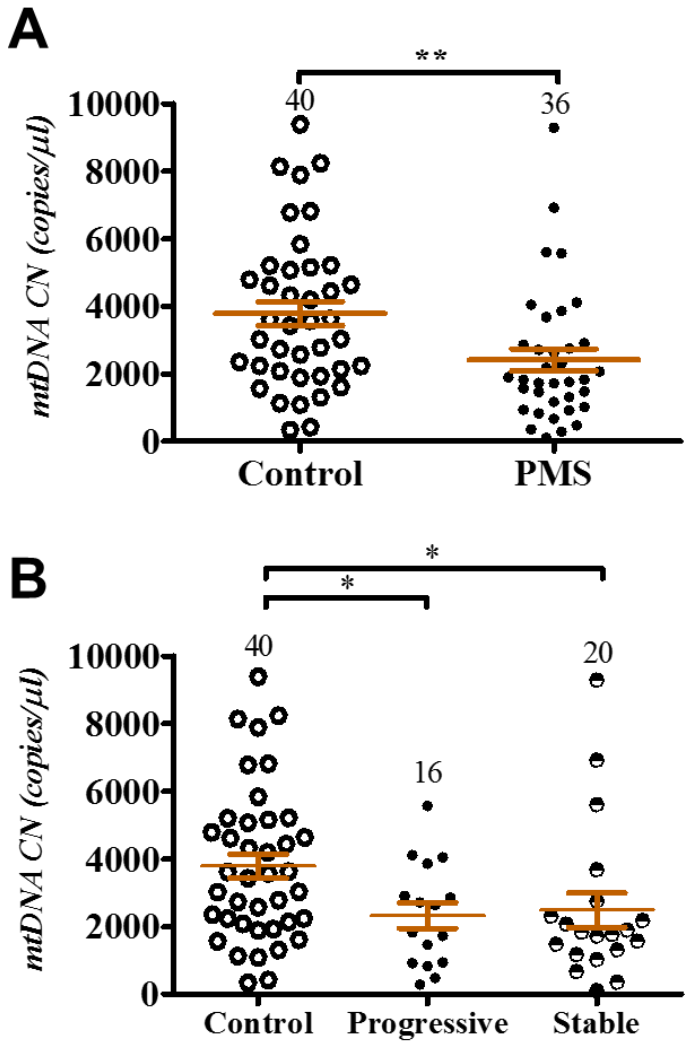

*Figure 4.2. Ccf-mtDNA copy number for (A) PMS cases compared to controls. In (B) PMS cases are stratified into progressive or stable PMS. Orange bars indicate mean +SEM. Numbers denote n. * indicates significance at p<0.05, ** indicates significance at p<0.01.*

PMS was sub-stratified into actively progressing PMS or stable PMS (Lublin, 2014), however, there was no difference between the ccf-mtDNA copy number of these sub-groups (mean ccf-mtDNA copy number was 2323±382 in 16 progressive PMS and 2484±513 in 20 stable PMS) and ccf-mtDNA copy number was still significantly reduced compared to controls, in both groups (Mann Whitney U $p=2.18x10^{-2}$ and $4.06x10^{-2}$, respectively) (Figure 4.2, B).

4.3.3 Abundance of targeted vCSF proteins in PMS cases and controls and correlation to ccf-mtDNA copy number.

Western blot was used to measure levels of specific neurodegenerative and neuro-inflammatory proteins with previous links in PMS. Similar to Chapter 3, the optimal protein concentration loaded onto the blot was 20µg of protein, therefore haem contaminated samples and low protein abundance samples were excluded, resulting in the usage of 12/40 control samples and 26/36 PMS samples.

S100β and GFAP proteins were detected in the vCSF via western blot, however, not all samples expressed these proteins. Only 15% of controls and 46% of PMS cases had detectable S100β levels and 42% of controls and 36% of PMS cases had detectable GFAP levels (Figure 4.3, Ai and Bi, respectively) resulting in large variation. Both S100β and GFAP were elevated in PMS cases compared to controls (Figure 4.3 Aii and Bii, Mann Whitney U $p>0.05$), however neither were found to be associated with ccf-mtDNA copy number (Pearson's $p>0.05$, Table 4.3). MBP could not be detected in the vCSF by western blot, and no commercial ELISA kits were available.

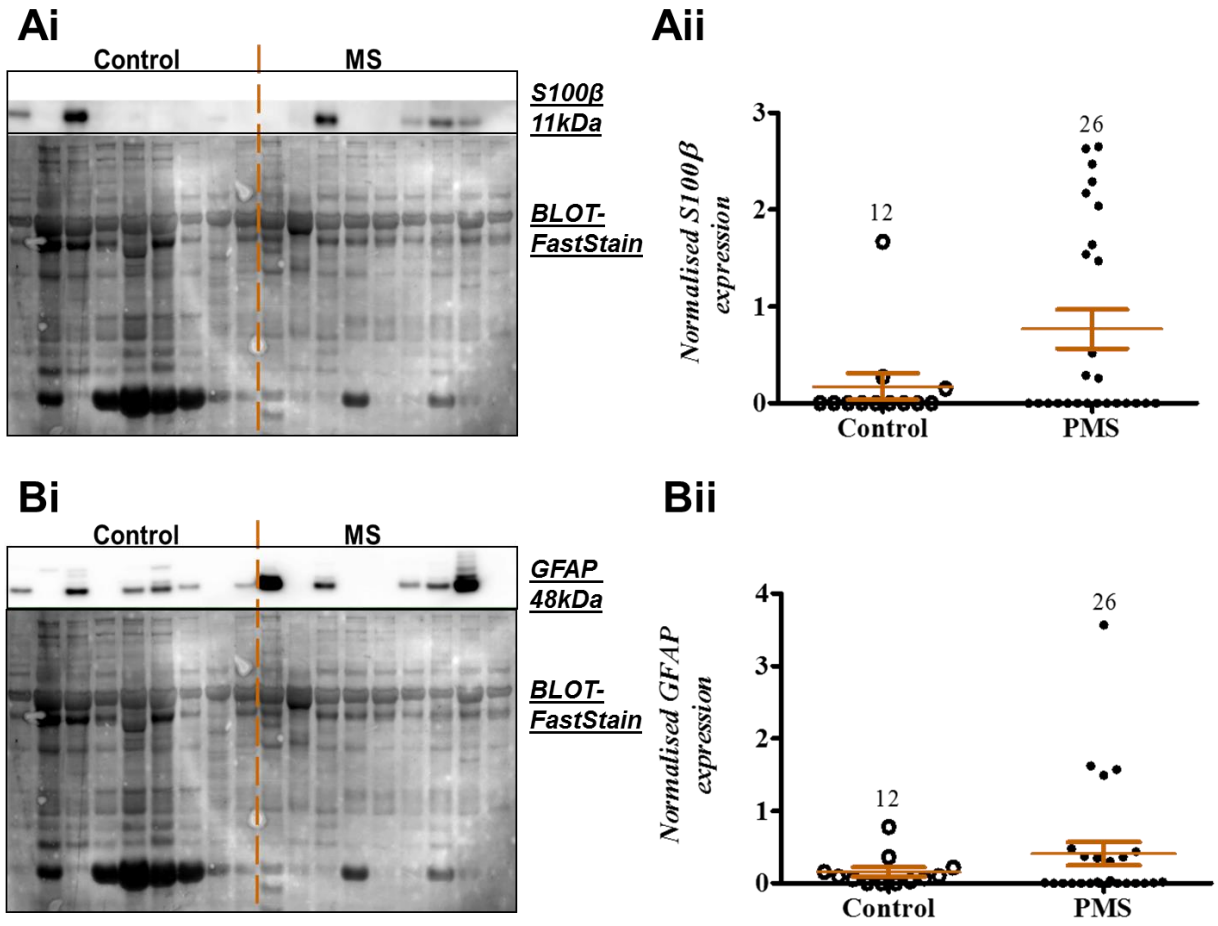

Figure 4.3. Ai and Bi are example blots of S100β and GFAP compared to the total protein stain, BLOT Faststain. Aii and Bii show the mean level and standard error of the mean (orange bar) of these proteins normalised to total protein content. Individual protein levels are also shown as open circles for controls and closed dots for PMS.

CHI3L1 and CHI3L2 are prognostic markers for MS (Thouvenot *et al.*, 2014; Mollgaard *et al.*, 2016). However, in the PMS-vCSF cohort, neither CHI3L1, CHI3L2 nor the ratio of CHI3L1:CHI3L2 were significantly different between PMS cases and controls (Figure 4.4, A-C). Interestingly, CHI3L1 vCSF protein level was found to significantly negatively correlated to ccf-mtDNA copy number in PMS cases (Pearson's r^2=-0.19, p=2.60x10^{-2}, Table 4.3, Figure 4.4, D), but not in controls or the entire cohort (Pearson's p>0.05). Neither CHI3L2 protein level nor the ratio of CHI3L1:CHI3L2 displayed a correlation in PMS cases, controls or the entire cohort (Pearson's p>0.05).

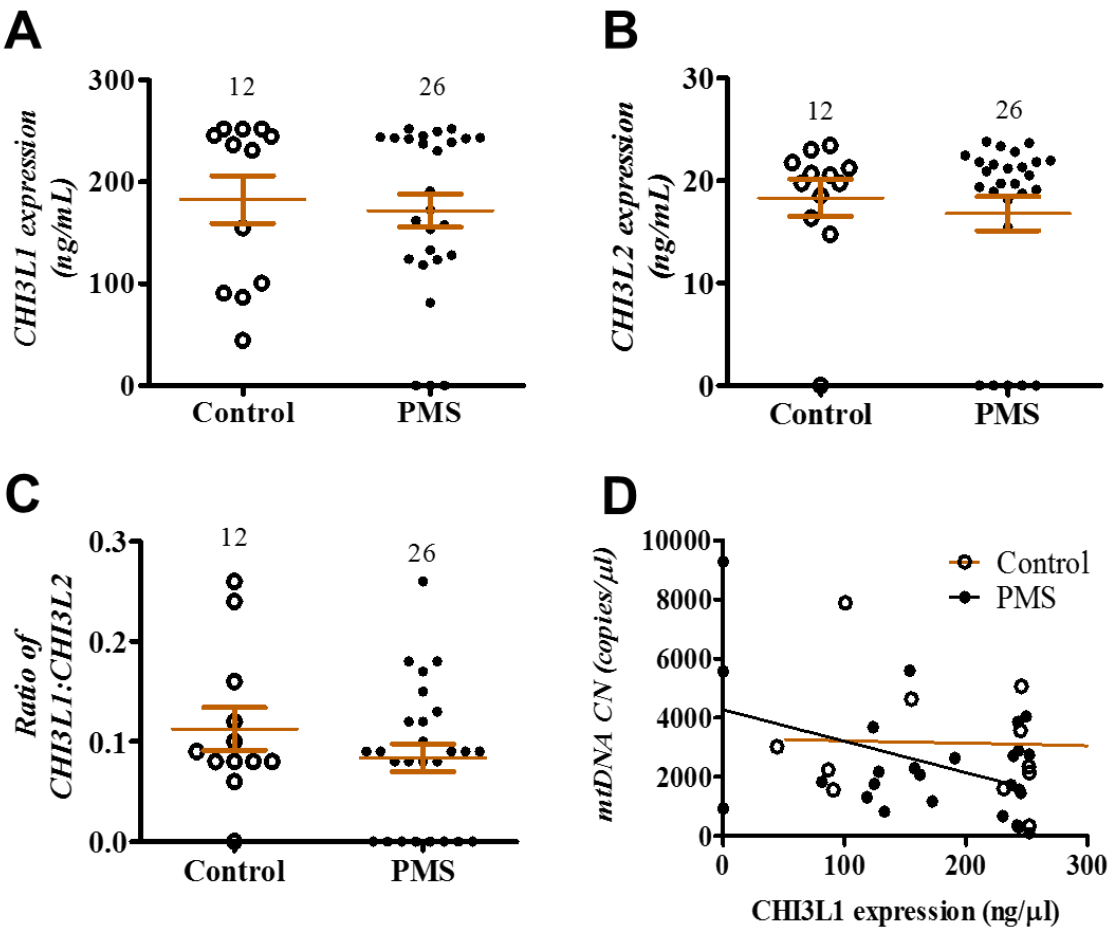

Figure 4.4. Abundance levels of (A) CHI3L1, (B) CHI3L2 and (C) the ratio of CHI3L1:CHI3L2 compared between PMS cases and controls. Orange bars are mean +SEM. N numbers are above stacks. (D) Correlation analysis of CHI3L1 against ccf-mtDNA copy number in PMS cases (black closed circles and black line; r^2=-0.19 and p=2.63x10^{-2}) and controls (black open circles and orange line r^2=0.02 and p>0.05).

Protein of interest	Whole cohort		Control		PMS	
	r^2	p	r^2	p	r^2	p
S100β	-0.10	5.47x10^{-1}	-0.13	6.94x10^{-1}	0.06	7.77x10^{-1}
GFAP	-0.01	9.49x10^{-1}	-0.20	5.35x10^{-1}	0.12	5.56x10^{-1}
CHI3L1	-0.16	3.46x10^{-1}	-0.02	6.66x10^{-1}	-0.19	2.60x10^{-2} *
CHI3L2	-0.12	4.91x10^{-1}	-0.17	6.01x10^{-1}	-0.12	5.57x10^{-1}
Ratio CHI3L1:CHI3L2	-0.18	2.84x10^{-1}	0.03	9.23x10^{-1}	-0.33	1.05x10^{-1}

*Table 4.3. Correlation of vCSF protein levels to ccf-mtDNA copy number in PMS cases, controls and the entire cohort, by Pearson's correlation. * indicates uncorrected significance at the p<0.05 level.*

4.3.4 Ccf-mtDNA integrity in PMS cases compared to controls.

Similar to Chapter 3, ccf-mtDNA deletion level and ccf-mtDNA heteroplasmy levels were calculated for PMS cases using the qPCR deletion assay and NGS sequencing and compared to controls. Ccf-mtDNA deletions (above the 10% threshold) were detected in a minority of the samples; 5 out of 36 (13%) PMS cases and 9 out of 40 (23%) of controls (Figure 4.5, A), indicating that ccf-mtDNA was intact in the majority of the samples.

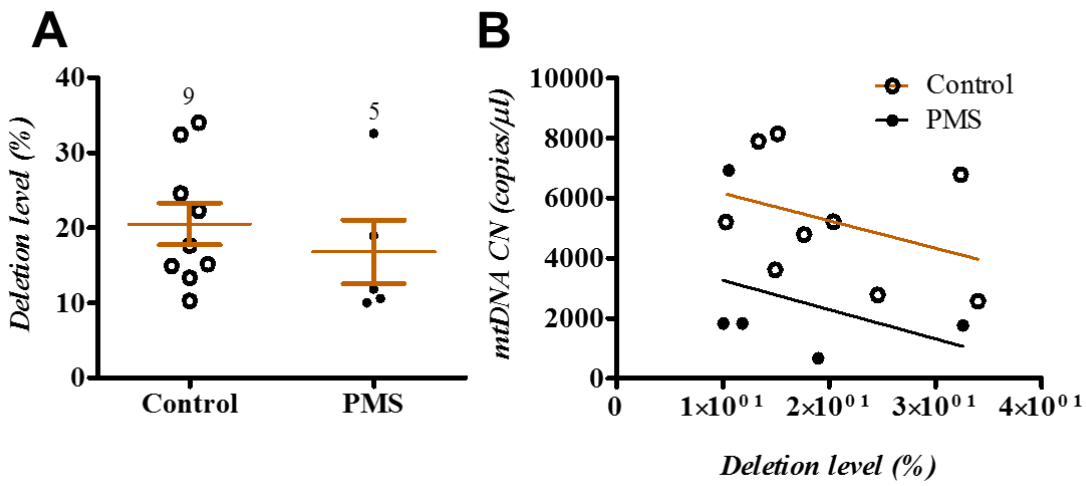

Figure 4.5. Ccf-mtDNA integrity compared between PMS cases and controls using ccf-mtDNA deletion level (A) (%), orange bars are mean deletion % +SEM. B shows the relationship between ccf-mtDNA deletion level and ccf-mtDNA copy number stratified by PMS case (black line) and control (orange line).

Comparison of ccf-mtDNA deletion level between PMS cases and controls showed no significant difference (Mann Whitney U $p>0.05$) and although there appeared to be a negative trend (Figure 4.5, B), there was no significant correlation to ccf-mtDNA copy number in PMS cases (Pearson's $r^2=0.13$, $p>0.05$), controls (Pearson's $r^2=0.14$, $p>0.05$) or when analysed as the whole cohort (Pearson's $r^2=0.14$, $p>0.05$, Figure 4.5). Furthermore, correlation analysis showed no association between ccf-mtDNA deletion level and gender, age at death, age of onset, disease duration, post-mortem delay, CSF pH or brain weight (Table 4.4).

Variable	Whole Cohort	Control	PMS
Male vs Female	$7.21x10^{-1}$	$6.17x10^{-1}$	$5.46x10^{-1}$
Age at death	$1.02x10^{-1}$	$1.74x10^{-1}$	$6.76x10^{-1}$
Age of onset	-	-	$7.53x10^{-1}$
Disease duration	-	-	$9.40x10^{-1}$
Post mortem interval	$3.13x10^{-1}$	$9.86x10^{-1}$	$8.60x10^{-2}$
CSF pH	$3.88x10^{-1}$	$1.00x10^{-0}$	$1.15x10^{-1}$
Brain weight	$6.17x10^{-1}$	$4.88x10^{-1}$	$5.71x10^{-1}$

Table 4.4. Correlation analysis of patient demographics to ccf-mtDNA deletion level in the entire cohort and stratified by PMS cases and controls. Values are p generated by Pearson's significance testing.

NGS identified low level heteroplasmies in all controls (21) and PMS cases (12) tested. Total mutation frequency was elevated in PMS cases compared to controls, but this was not significant (mean mutation frequency in 12 PMS cases was $1.73x10^{-3}$ and in 21 controls was $1.36x10^{-3}$, Mann Whitney U p>0.05) (Figure 4.6, A). Furthermore, total variant frequency did not correlate to ccf-mtDNA copy number in cases, controls or the whole cohort (Figure 4.6, B, Pearson's p>0.05).

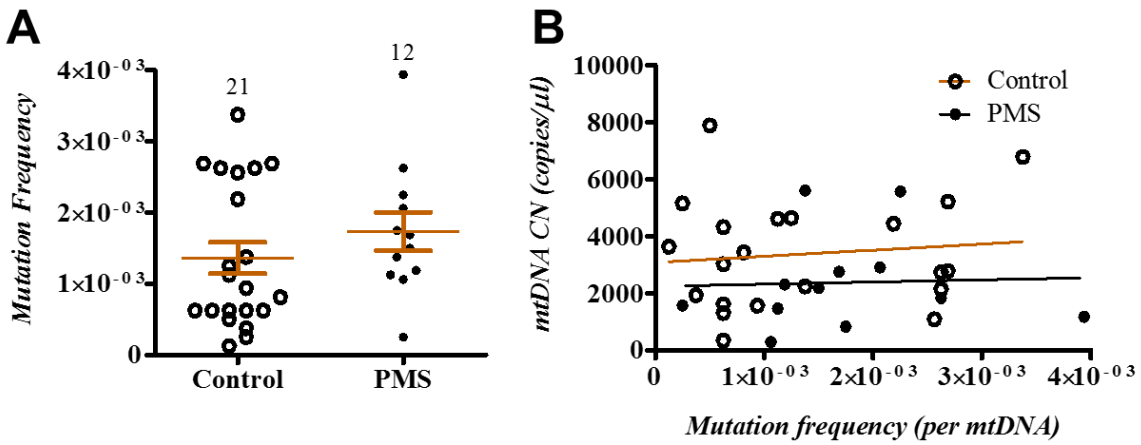

Figure 4.6. Ccf-mtDNA integrity compared between PMS cases and controls, using total mutational burden calculated by NGS (A) orange bars are mean +SEM. B shows the relationship between ccf-mtDNA mutation frequency and ccf-mtDNA copy number for PMS cases (black line) and controls (orange line).

Each mutation type was then investigated separately (Figure 4.7). Non-coding, protein coding, and tRNA variant levels were shown to be elevated in PMS cases compared to controls, though not significantly (Figure 4.7, A, B and D, respectively, Mann Whitney U $p>0.05$), whilst rRNA levels were no different between the two disease groups (Figure 4.7, C). Synonymous variants were less abundant in the PMS cases, whilst non-synonymous variants were more common with these patients compared to controls, however, again this did not reach statistical significance (Figure 4.7 E and F, respectively, Mann Whitney U $p>0.05$).

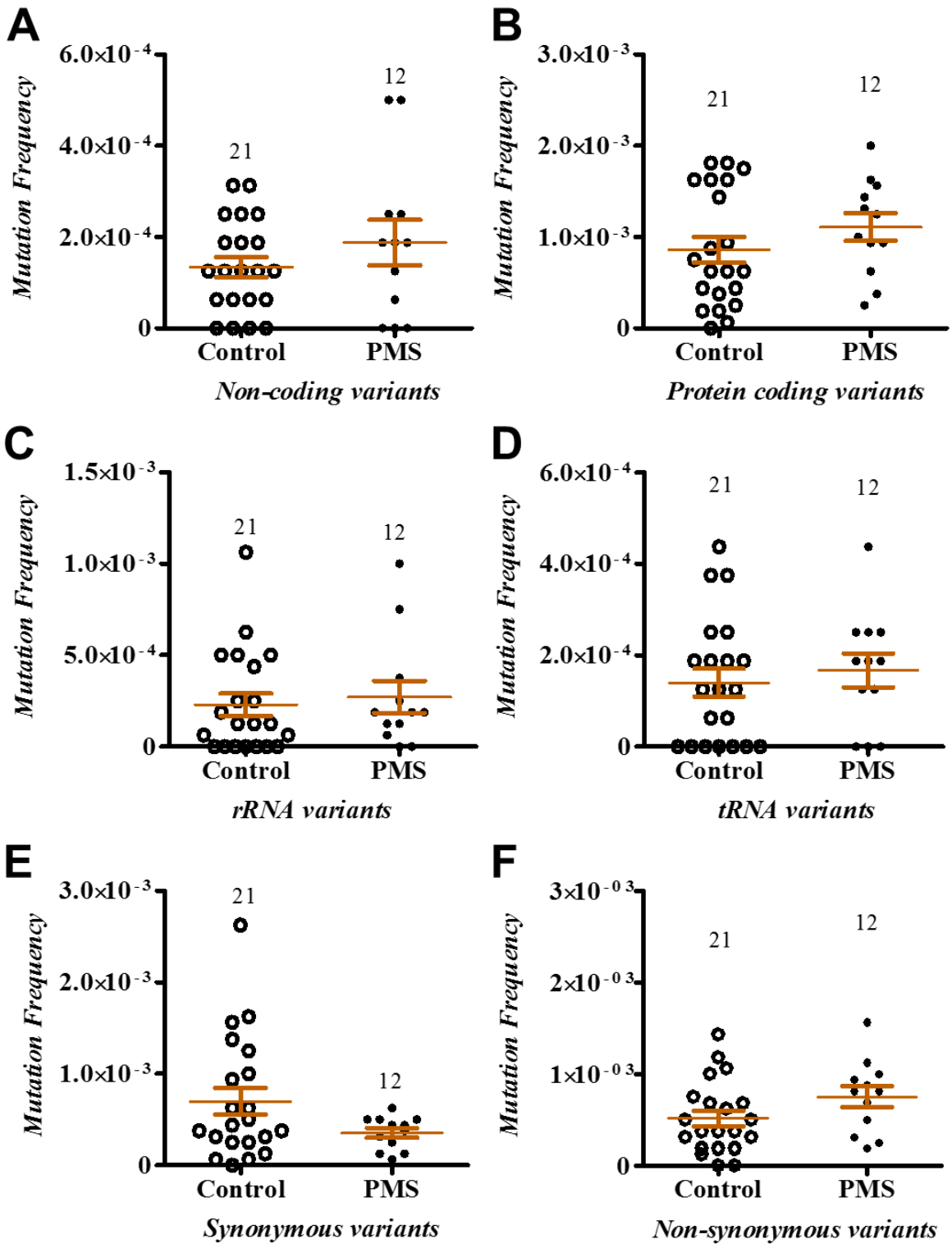

Figure 4.7. Total mutation frequency stratified into respective variant types and compared between PMS cases and controls; (A) Non-coding variants, (B) protein coding variants, (C) rRNA variants, (D) tRNA variants, (E) synonymous variants and (F) non-synonymous variants. Orange bar indicates mean+SEM. N numbers are above stacks. Mutation frequency is per mitochondrial genome.

The ratio of synonymous to nonsynonymous variants (Ka/Ks ratio) can be used as an estimate of the degree of evolutionary constraint of the mtDNA (Li *et al.*, 1985). Analysing this ratio identified a significant difference between PMS cases and controls, with PMS cases having a significantly higher proportion of nonsynonymous to synonymous variation compared to controls (mean Ka/Ks for 12 PMS cases was 1.9 and in 21 controls was 1.0, Mann Whitney U $p=3.70x10^{-3}$) (Figure 4.8, A). However, this ratio did not correlate with ccf-mtDNA copy number in PMS cases, controls or the whole cohort (Figure 4.8, B, Pearson's $p>0.05$).

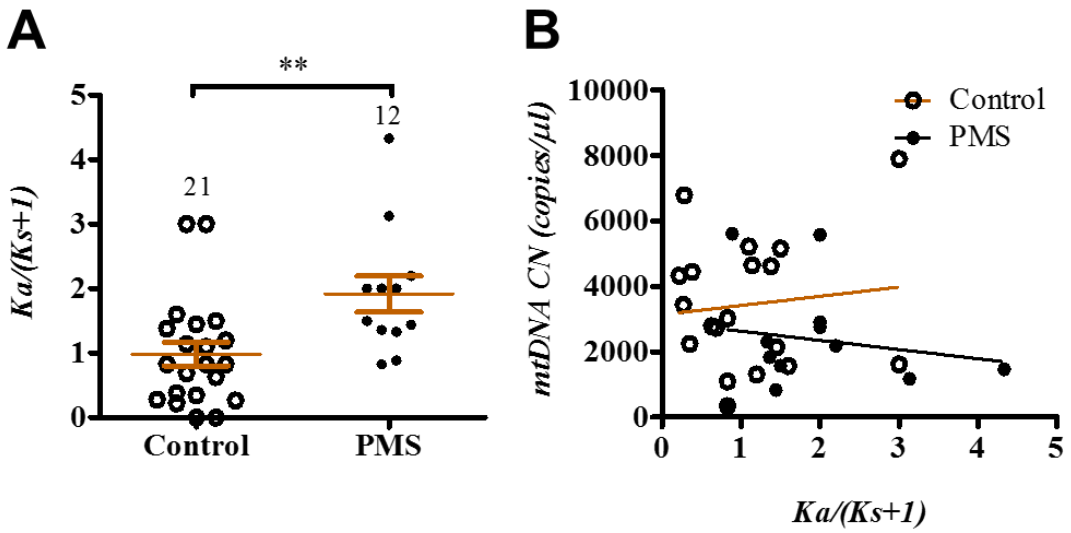

*Figure 4.8. Comparison of the Ka/Ks ratio between PMS cases and controls, to investigate a difference in the number of deleterious or beneficial mutations accumulated in each group (A). B shows the relationship between Ka/Ks ratio and ccf-mtDNA copy number for PMS cases (black line) and controls (orange line). ** indicates significance at p<0.01.*

4.4 Discussion

Investigation of ccf-mtDNA in the PMS-vCSF cohort indicates that ccf-mtDNA copy number is significantly reduced in post-mortem progressive multiple sclerosis (PMS) cases compared to controls. Similar to Chapter 3, protein analysis was limited by the post-mortem nature of the vCSF. Nonetheless, correlation between CHI3L1 and ccf-mtDNA copy number in PMS cases only may support a link between ccf-mtDNA export and inflammation. Assessment of ccf-mtDNA integrity supported results from Chapter 3, showing that ccf-mtDNA is largely intact, with few PMS cases and controls harbouring mtDNA deletions. Conversely to Chapter 3, there was a significant shift in the proportion of non-synonymous to synonymous variation in ccf-mtDNA in the PMS cases compared to controls, which may be related to a mechanism of ccf-mtDNA export.

4.4.1 Low ccf-mtDNA copy number is associated with PMS cases

Ccf-mtDNA was found to be significantly reduced in the ventricular CSF (vCSF) of PMS cases compared to controls. This finding, taken together with findings from the previous chapter (Chapter 3) and from current literature in PD (Pyle *et al.*, 2015b) suggests that ccf-mtDNA could be an indicator of neurodegeneration in specific neurodegenerative conditions that have a strong, established link to mitochondrial dysfunction.

However, similar to other neurodegenerative diseases, the literature surrounding ccf-mtDNA copy number in PMS is inconsistent, with previous reports observing increased levels of ccf-mtDNA copy number in RRMS (Varhaug *et al.*, 2017) and PMS (Leurs *et al.*, 2018). This is perhaps unsurprising with regard to RRMS, as this is an acute inflammatory condition preceding the neurodegenerative processes associated with PMS (Kingwell, 2009). Ccf-mtDNA is a highly immunogenic molecule (Pérez-Santiago *et al.*, 2017) and specific characteristics, such as its susceptibility to oxidative damage, and relative hypomethylation, contribute to make it a potent damage-associated molecular pattern (DAMP) (Nakayama and Otsu, 2018), known to induce inflammation (Collins *et al.*, 2004) through activation of neutrophils (Zhang *et al.*, 2010). Furthermore, literature has shown that high levels of ccf-mtDNA copy number in the CSF and plasma correlates with high levels of inflammatory factors such as monocyte chemoattractant protein-1, tumour necrosis factor-α and various interleukins (Cao *et al.*, 2014; Pérez-Santiago *et al.*, 2017). Therefore, it is possible

that the elevation in ccf-mtDNA copy number observed in these RRMS patients is a direct reflection of the widespread inflammation and upregulation of inflammatory cells associated with this disease, and it is likely that the ccf-mtDNA is acting as a pro-inflammatory factor.

Conversely, PMS is characterised by less inflammation and more neurodegeneration (Trapp and Nave, 2008; Lassmann, 2010) in the form of neuronal and axonal damage and therefore assumingly would show similar ccf-mtDNA copy number levels as neurodegenerative diseases i.e. low ccf-mtDNA copy number in PMS compared to controls. Leurs *et al.* (2018), provided a direct comparison, published just a few months before this work. They investigated lumbar CSF (lCSF) ccf-mtDNA copy number levels in 40 PMS cases (27 secondary PMS and 13 primary PMS) compared to 23 non-inflammatory neurological disease controls (NINDC), and reported a significant increase in ccf-mtDNA copy number levels in their primary PMS patients compared to NINDC (the direct converse to the findings observed here). This discrepancy between results is unlikely to be related to statistical power or population validity and sample size, given the similarity in cohort sizes (40 PMS cases vs 23 NINDC in Leurs *et al*., (2018) compared to 36 PMS cases vs 40 controls in the PMS-vCSF cohort). It is possible that the difference in observations may be explained by the CSF sampling as (i) the composition of the vCSF and lCSF are markedly different in health and disease (Rubalcava and Sotelo, 1995; Gerber *et al.*, 1998; Sommer *et al.*, 2002) and (ii) the CSF composition is likely to be different in living PMS cases compared to end-stage post-mortem PMS cases. Therefore it is possible that the contrasting findings suggest that ccf-mtDNA levels rise in-life in response to disease onset and inflammation and then decrease as neurodegeneration increases and overtakes inflammation. However, longitudinal assessments would be needed to test this hypothesis.

Alternatively, discrepancies between reports may be attributed to the use of non-inflammatory neurological controls in the Leurs *et al*., (2018) study as ccf-mtDNA copy number is known to be affected by other neurological conditions (i.e. depression (Lindqvist *et al.*, 2018), stroke (Tsai *et al.*, 2011), AD (Podlesniy *et al.*, 2013; Podlesniy *et al.*, 2016a) and PD (Pyle *et al.*, 2015b; Podlesniy *et al.*, 2016b)). Therefore, PMS ccf-mtDNA copy number may be elevated compared to neurological controls (Leurs *et al.*, 2018), but decreased compared to healthy controls (PMS-vCSF). In addition, as discussed in Section 4.1, there was large dispersion in ccf-

mtDNA copy number between and within disease groups in the Leur's *et al.*, (2018) study. Furthermore, the significant result observed in their study was limited to a small proportion of PMS cases (~30%) with the majority of PMS cases and controls exhibiting ccf-mtDNA copy numbers below the levels typically observed (typically 20-1000 copies/µl (Podlesniy *et al.*, 2013; Pyle *et al.*, 2015b; Cervera-Carles *et al.*, 2017; Melentijevic *et al.*, 2017), but in Leurs *et al*., (2018) the majority were below 100 copies/µl with a large proportion exhibiting <10 copies/µl, which is below the accurate detection threshold of digital droplet PCR (Jones *et al.*, 2014; Lowes *et al.,* 2018).

4.4.2 VCSF targeted protein assessment revealed no differences between PMS cases and controls and did not correlate to ccf-mtDNA copy number.

CSF protein abundance investigations were conducted to assess the relationship between a number of PMS-related proteins and PMS status. In-keeping with previous literature (Rosengren *et al.*, 1995; Petzold *et al.*, 2002; Norgren *et al.*, 2004; Axelsson *et al.*, 2011; Barateiro *et al.*, 2016), S100β and GFAP protein levels were found to be elevated in PMS cases compared to controls, however, this did not reach statistical significance. This is perhaps not surprising due to the end-stage, largely inactive nature of the PMS-vCSF cohort as generally, inflammation (Kuhlmann *et al.*, 2017) and astrocyte activity (Ponath *et al.*, 2018) is most prominent in early, highly active MS .

VCSF CHI3L1 and CHI3L2 protein levels did not support findings from current literature (Hinsinger *et al.*, 2015), showing no significant difference between PMS cases and controls. Previous reports indicate that CHI3L1 levels are elevated in the lumbar CSF of relapsing-remitting MS (RRMS) and PMS patients compared to controls (Hinsinger *et al.*, 2015; Mollgaard *et al.*, 2016), whereas CHI3L2 levels are only significantly elevated in RRMS but not PMS compared to controls (Hinsinger *et al.*, 2015). However, CHI3L proteins are secreted by important inflammatory factors such as activated macrophages and neutrophils (Di Rosa *et al.*, 2013), therefore, the reduction in inflammation towards the end-stages of PMS could result in less CHI3L protein release, thereby explaining the lack of difference in CHI3L proteins levels between PMS cases and controls in this study. Therefore, similar to investigations of S100β and GFAP levels in PMS, the discrepancies between literature on CHI3L proteins and the results presented here are likely the result of the end-stage nature of

the PMS-vCSF cohort. Furthermore, CHI3L protein levels currently only have clinical utility in predicting the conversion to MS after a clinically isolated syndrome (CIS) (Teunissen *et al.*, 2009a; Canto *et al.*, 2015; Hinsinger *et al.*, 2015; Martinez *et al.*, 2015; Mollgaard *et al.*, 2016) and the ratio of CHI3L1 to CHI3L2 distinguishes RRMS and PMS patients from one another (Hinsinger *et al.*, 2015), rather than distinguishing PMS cases from controls. Therefore, the lack of difference between CHI3L levels in PMS cases and controls may not be entirely surprising and may highlight a limitation regarding the use of 'selected' proteins of interest.

Alternatively, MS treatment (interferon beta and natalizumab) has been shown to reduce CHI3L proteins levels in the CSF (Novakova *et al.*, 2017). Novakova *et al.* (2017) reported that MS patients receiving first (interferon beta) and second (natalizumab) line MS treatments (administered for up to 1 year) had reduced CHI3L1 levels in the CSF compared to no treatment, but elevated CHI3L1 compared to controls. Interestingly, these treatments target the immune response and exert anti-inflammatory effects (Bermel and Rudick, 2007; Kappos *et al.*, 2011) which could possibly reduce CHI3L protein levels. Therefore, it is possible that prolonged usage of these treatments could diminish CHI3L levels to that of controls. As the PMS-vCSF cohort is end-stage (with an average disease duration of 30±12 years) it is extremely likely that all PMS patients enrolled were receiving treatment prior to death. This could, therefore potentially explain why there is no difference in CHI3L protein levels between PMS cases and controls. However, detailed treatment history for these patients was not available, thus could not be analysed.

Similar to Chapter 3, these proteins were investigated as measures of disease severity (S100β and GFAP) and progressive neurodegeneration (using the CHI3L1:CHI3L2 ratio) and were compared to ccf-mtDNA copy number to investigate potential mechanisms related to the release of ccf-mtDNA.

Interestingly, analysis revealed a significant negative correlation between CHI3L1 protein levels and ccf-mtDNA copy number which was present only for PMS cases. CHI3L1 is released from activated inflammatory cells (Di Rosa *et al.*, 2013) and has been shown to possess anti-inflammatory and neuroprotective functions in recent in vivo studies (Bonneh-Barkay *et al.*, 2012; Wiley *et al.*, 2015). As ccf-mtDNA is potentially exported due to cell death, this may suggest that in late-stage PMS samples with high levels of CHI3L1 may have lower ccf-mtDNA copy number due to increased neuronal survival. However, there were no other correlations observed

between any other protein markers of PMS neurodegeneration (S100β, GFAP and CHI3L2) and ccf-mtDNA copy number, showing that ccf-mtDNA export is generally not associated with protein markers of neurodegeneration in the CSF.

As discussed in Chapter 3 (Section 3.5.3), a major limitation of this work is the use of targeted protein investigation and an unbiased proteomic screen using mass spectrometry may be more informative than western blotting in this analysis, due to higher through-put, sensitivity and accuracy (Aebersold *et al.*, 2013). This means a larger proportion of the cohort could have been processed and a far greater number of proteins could have been investigated. Additionally, as discussed throughout this section and highlighted in Chapter 3 (Section 3.5.1 and 3.5.3), proteomic investigations are likely limited by the post-mortem nature of these samples. Post-mortem CSF is reported to have a different proteomic composition to ante-mortem CSF, with protein abundance exceeding normal levels after death (Arroyo *et al.*, 2005; Morris *et al.*, 2012), potentially due to breakdown of the blood brain barrier and infiltration of phagocytes. Indeed, comparison of total post-mortem vCSF protein abundance against ante-mortem lCSF protein abundance reveals a significant increase in protein expression in the post-mortem vCSF samples, regardless of health status (Figure 4.9). Therefore, because post-mortem effects may influence the protein expression profile of the CSF, future work utilising ante-mortem lCSF would be beneficial.

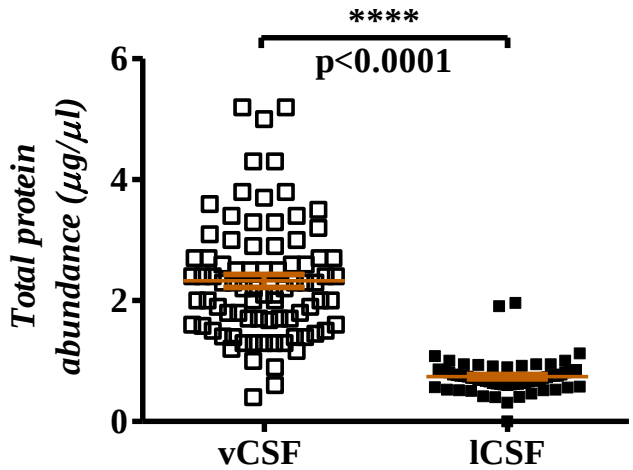

*Figure 4.9. Comparison of total protein abundance (μg/μl) in the post-mortem ventricular CSF (vCSF) (open squares) and ante-mortem lumbar CSF (lCSF) (closed squares), with mean+SEM (orange lines). Post-mortem vCSF consists of 26 PMS-vCSF cohort PMS samples and 58 NDD-vCSF cohort samples. Ante-mortem lumbar CSF consists of 66 ICICLE-PD cohort lCSF samples. **** indicates statistical difference, Mann Whitney U p<0.0001.*

4.4.3 Ccf-mtDNA is largely intact and integrity is similar between PMS cases and controls.

Similar to the previous chapter, ccf-mtDNA integrity was assessed to investigate if ccf-mtDNA export was selective for mutant, damaged mtDNA, in a bid to promote cell survival and health by retaining the 'wild-type' mtDNA molecules (Melentijevic *et al.*, 2017). However, ccf-mtDNA was largely intact in the vCSF of PMS cases and controls; with only a minority of samples showing deletions (5 out of 36 (13%) of PMS cases and 9/40 (23%) of controls) and all samples showing low-level ccf-mtDNA heteroplasmy. Importantly, there were no significant differences observed in deletion or heteroplasmy levels between PMS cases and controls.

The only significant difference was observed as an imbalance of the Ka/Ks ratio. This ratio calculates the number of nonsynonymous to synonymous mutations to estimate the balance between neutral and consequential (beneficial or detrimental) mutations (Mishmar *et al.*, 2003), where a K_a/K_s of 1 indicates neutral selection, whereas an increase or decrease from 1 implies evolutionary selection. The significantly higher ratio in PMS cases, therefore, indicates the presence of more consequential mutations compared to controls, possibly suggesting a selective mechanism resulting in the export of mutant mtDNA into the CSF and retention of the 'wild-type' copies. This hypothesis is supported by previous work in MS, which indicates that MS neuronal tissues harbour relatively few mtDNA mutations (Blokhin *et al.*, 2008), possibly because these mutant mtDNA are preferentially exported. Conversely, it may be more likely that ccf-mtDNA release is part of normal cellular processing and the lower levels seen in PMS cases is a direct consequence of the pre-existing mtDNA depletion in the brain, as shown in previous literature (Mao and Reddy, 2010). However, further work in a larger cohort is needed to investigate this, ideally with matched patient brain tissue, to allow comparison of ccf-mtDNA copy number in the CSF to mtDNA copy number within different brain regions.

4.5 Conclusions

In conclusion, these findings support the hypothesis that low ccf-mtDNA copy number may be an indicator of neurodegeneration, and is associated with PMS. However, due to conflicting reports in the literature, and the limitations associated with using post-mortem vCSF, a large scale, longitudinal validation study is warranted to confirm this result. Furthermore, it would be beneficial to employ a more diverse cohort of RRMS and early stage PMS patients to gain understanding of how ccf-mtDNA copy number may change over the disease course and if ccf-mtDNA copy number can predict the conversion from inflammatory MS (RRMS) to neurodegenerative MS (PMS). Additionally, findings regarding the integrity of the ccf-mtDNA suggest that ccf-mtDNA export is not simply selective for removing mtDNA with mutations or deletions, but instead may be selective only for functionally altered mutant mtDNA, however, large scale replication is necessary to confirm this.

Chapter 5. Validation of ccf-mtDNA copy number as a biomarker for Parkinson's disease using a large-scale longitudinal cohort - PPMI-PD cohort.

5.1 Introduction

Parkinson's Disease (PD) currently affects around 2-3% of the population over 65 years of age (Poewe *et al.*, 2017). However, with an increasingly aged population, the incidence of PD is estimated to rise by >20% in the next seven years (Dorsey and Bloem, 2018), presenting PD as a huge socio-economic burden. To overcome this burden, better clinical management of PD is needed, specifically regarding the identification and development of disease-modifying therapies that could slow or reverse the degeneration occurring in PD. However, to enable the development of new therapeutics, novel discoveries of sensitive and specific biomarkers are crucial to facilitate early diagnosis, monitor disease progression and assess the response to existing and future therapeutics (Sharma *et al.*, 2013).

Potential biomarkers for PD are diverse and can be categorised into four main groups; clinical, biochemical, genetic and imaging (Section 1.5.4) (Figure 5.1).

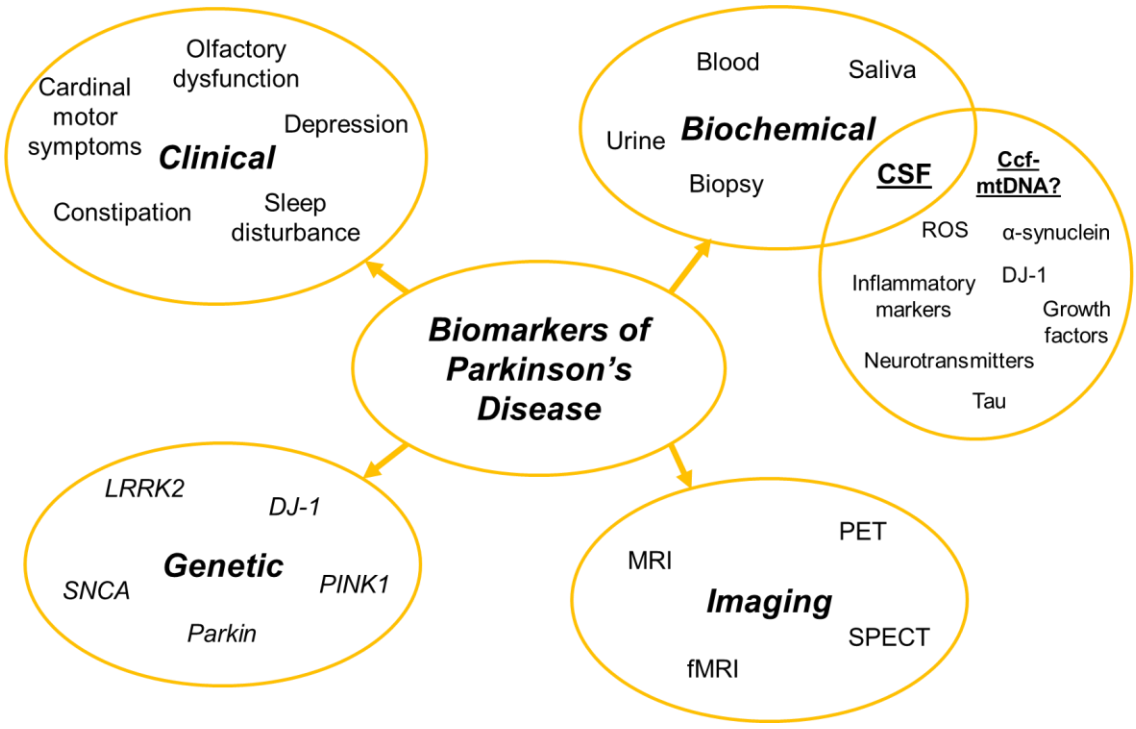

Figure 5.1. Potential biomarkers for PD categorised into four subgroups; Clinical, Genetic, Imaging and Biochemical, with specific emphasis on CSF biochemical markers.

A number of clinical signs often precede the classical motor symptoms of PD, which could potential predict the risk of developing PD (reviewed in (Wu *et al.*, 2011)), including olfactory dysfunction (Haehner *et al.*, 2007; Ross *et al.*, 2008), sleep disturbances (dos Santos *et al.*, 2015) and constipation (Stirpe *et al.*, 2016). However, these preclinical signs have limited specificity and sensitivity as they are

not specific to PD pathology and generally rely on subjective questionnaires. Genetic biomarkers also have limited utility as they are generally specific to familial forms of PD (accounting for <15% of all PD cases) (Deng *et al.*, 2018) and although neuroimaging techniques are efficient and accurate in providing high resolution information about structural and functional brain changes (Section 1.5.4), they are cost-prohibitive for presymptomatic screening (Politis *et al.*, 2017).

Biochemical biomarkers are the most promising avenue for PD as these are typically less-invasive markers present within the saliva, blood or CSF and are specific to pathological processes occurring within PD patients. The CSF is regarded as the most useful source for identifying biomarkers for PD and other neurodegenerative diseases (NDDs) as this is the closest fluid to the affected tissue and is in direct contact with the CNS (Jeromin and Bowser, 2017). Generally, identification of potential biomarkers for PD is guided by genetics and neuropathology studies, highlighting protein abnormalities implicated in disease aetiology or pathology as potential targets (i.e. α-synuclein, DJ-1, oxidative stress markers, BDNF, Tau). However, unlike Alzheimer's disease (AD), where CSF amyloid beta and tau levels can accurately distinguish AD from controls (Olsson *et al.*, 2016), no such biochemical biomarker has been discovered for PD (Andersen *et al.*, 2017). CSF α-synuclein (reviewed in (Parnetti *et al.*, 2013)), oxidative stress proteins (Ilic *et al.*, 1999) and DJ-1 (Hong *et al.*, 2010) have a tendency to be associated with PD, but due to inconsistencies and discrepant findings, none can robustly distinguish PD from other NDDs or cognitively normal individuals.

Work in Chapter 3 which is supportive of preliminary data generated using the ICICLE-PD cohort (Pyle *et al.*, 2015b) have identified a potential PD-specific biomarker in the ventricular and lumbar CSF. This previous work indicates the potential for circulating cell free mtDNA (ccf-mtDNA) as an accurate and specific biomarker of PD, with a predictive ability of ~80%. However, for CSF ccf-mtDNA copy number to be accepted as a valid biomarker of PD, subsequent replication in a larger independent cohort is required to validate the observed effect (Chen-Plotkin, 2014; Smith, 2017).

In addition, the use of small cohorts in preliminary work (7 PD versus 40 Controls in Chapter 3 (NDD-vCSF cohort) and 53 PD versus 10 Controls in ICICLE-PD cohort (Pyle *et al.*, 2015b)) greatly precluded a more detailed analysis, such as correlating ccf-mtDNA to clinical PD outcome measures, PD-associated protein levels, or PD

therapeutics. Therefore, to address these issues and expand upon the initial findings from Pyle *et al.,* (2015) and Chapter 3, a large-scale replication study was performed utilising the Michael J Fox Foundation Parkinson Progression Markers Initiative (PPMI) cohort. The PPMI is a collaborative multi-centre study involving over 400 recently diagnosed PD patients and 200 healthy controls, followed longitudinally for clinical, imaging and biospecimen biomarker assessments (Parkinson Progression Marker Initiative, 2011). The overarching goal of the PPMI is to serve as a large replication cohort for validation of PD biomarkers discovered in other cohorts (Chen-Plotkin, 2014). All study data is integrated into the PPMI online database and is publicly available to collaborators. Therefore, investigation also aimed to establish associations between ccf-mtDNA copy number and disease severity, CSF protein levels and PD treatment.

5.2 Materials and methods

5.2.1 PPMI-PD cohort

Lumbar puncture cerebrospinal fluid samples (lCSF) obtained as part of the PPMI study (PPMI-PD cohort) were used throughout this chapter, comprising 541 lCSF samples at baseline (169 controls and 372 PD cases) and 364 lCSF samples at 36 months (114 controls and 250 PD cases). Details of this cohort are described in Section 2.1.2 and details of sample collection and preparation are outlined in Section 2.2.1.

5.2.2 mtDNA Quantification

Ccf-mtDNA and nDNA copy number was measured by multiplex Taqman qPCR of the mitochondrial genes *MTND1* and *MTND4* and the nuclear gene *B2M*, as described in Section 2.3-2.7. Inter-plate variation of ccf-mtDNA copy number was calculated by coefficient of variance, using a subset (3%) of the cohort population. These samples were run in triplicate and repeated three times on different plates and the coefficient of variance was calculated from the cq values generated. Mean coefficient of variance for all repeated samples was 1.54%, showing cq values were consistent for each sample run-to-run, suggesting low inter-plate variance.

5.2.3 PPMI Phenotyping Data

The PPMI study database provided a myriad of demographic, phenotypic, clinical proteomic and genetic biospecimen data. *Demographic data:* Age, gender, age of onset, disease duration. *Phenotypic data:* Movement Disorder Society – Unified Parkinson's Disease Rating Scale (MDS-UPDRS), Hoehn and Yahr, Self-Reported Autonomic Symptoms in PD (SCOPA-AUT), Montreal Cognitive Assessment (MoCA), Geriatric Depression Scale (GDS), Physical Activity in Early PD (PASE), Sleep assessments and Olfactory testing (UPSIT). *Clinical data:* Family history, medical history, comorbidities, PD medication history. *Proteomic data:* CSF alpha synuclein, amyloid beta, total tau, phosphorylated tau. *Genetic data:* Illumina based Immunochip (Nalls *et al.*, 2016) and NeuroX (Nalls *et al.*, 2015) genotyping.

5.2.4 Genotyping and Quantitative Trait Locus analysis

Case versus control genetic association was carried out with PLINK 1.9 using the two gene arrays provided by the PPMI study online database (NeuroX (267,607 original variants; 55,053 post-QC) (Nalls *et al.*, 2015) and Immunochip (196,525 original variants; 146,185 post-QC) (Cortes and Brown, 2011)). Stringent quality control was incorporated into the association to exclude single nucleotide polymorphisms (SNPs) on the basis of a low call rate (<10%), minor allele frequency <5% and failure of Hardy-Weinberg equilibrium test. A quantitative trait analysis (QTL) was then carried out to specifically identify associations between the most common PD-related risk variants and ccf-mtDNA copy number (a comprehensive table of these variants is included below, Table 5.1)

Following this, QTL was carried out to investigate whether genetic loci could modulate ccf-mtDNA copy number in PD cases and controls. P values were generated to evaluate the relationship between each SNP and ccf-mtDNA copy number. IGV 2.3.97 and Manhattan Plotter were used to create Manhattan and QQ plots.

Reference	dbSNP ID	CHR	BP	Gene
Nalls et al., 2014	rs11724635	4	15737101	*BST1*
	rs11060180	12	123303586	*CCDC62*
	rs6812193	4	184327401	*FAM47E/SCARB2*
	rs591323	8	16697091	*FGF20*
	rs12637471	3	182762437	*MCCC1*
	rs34016896	3	160992864	*NMD3*
	rs823118	1	205723572	*RAB7L1/NUCKS1*
	rs356182	4	90626111	*SNCA*
	rs34311866	4	951947	*TMEM175/GAK/DGKQ*
	rs6430538	2	135539967	*ACMSD/TMEM163*
	rs14235	16	31121793	*BCKDK/STX1B*
	rs3793947	11	83544472	*DLG2*
	rs11158026	14	55348869	*GCH1*
	rs199347	7	23293746	*GPNMB*
	rs7077361	10	15561543	*ITGA8*
	rs115185635	3	87520857	*KRT8P25/APOOP2*
	rs76904798	12	40614434	*LRRK2*
	rs17649553	17	43994648	*MAPT*
	rs12456492	18	40673380	*RIT2*
	rs10797576	1	232664611	*SIPA1L2*
	rs11868035	17	17715101	*SREBF/RAI1*
	rs2823357	21	16914905	*USP25*
	rs2414739	15	61994134	*VPS13C*
	rs329648	11	133765367	*MIR4697*
	rs8118008	20	3168166	*DDRGK1*
	rs35749011	1	155235252	*GBA/SYT11*
	rs9275326	6	32666660	*HLA-DQB1*
	rs117896735	10	121536327	*INPP5F*
	rs60298754	8	89373041	*MMP16*
	rs62120679	19	2363319	*SPPL2B*
	rs1474055	2	169110394	*STK39*
	rs1555399	14	67984370	*TMEM229B*

Table 5.1. List of Parkinson's disease (PD) related single nucleotide polymorphisms (SNPs). Table includes the reference to the original research paper citing a link between these SNPs and PD, the SNP database ID (dbSNP ID), the chromosome (CHR) and base pair (BP) location of the SNP and the gene in which the SNP resides.

5.2.5 Statistical Analysis

Data was analysed using SPSS V22 and Prism V5 using data appropriate tests. Non parametric statistical testing was carried out due to the non-normal distribution of mtDNA copy number (Kolmogorov Smirnov normality test, $p<0.0001$). Statistical significance was set at $p<0.05$ due to the replicative nature of this study, thus Bonferroni multiple significance testing was deemed too stringent and rigorous to be applied as it could in fact mask important statistical differences present within this dataset (Perneger, 1998). Categorical data was assessed using Pearson chi-square tests, all other statistical comparison was performed using non-parametric Mann Whitney U tests or Kruskal Wallis tests, and correlation analysis was assessed with Pearson's correlation coefficient.

5.3 Results

5.3.1 PPMI cohort inclusion criteria and demographics.

Previous literature (Perez-Santiago *et al.*, 2016; Cervera-Carles *et al.*, 2017; Leurs *et al.*, 2018) and the ICICLE-PD cohort (Pyle *et al.*, 2015b) indicate a normal range of 1-1000 copies per µl for ccf-mtDNA copy number in the lCSF. Furthermore, pilot work in the ICICLE-PD cohort showed that lCSF typically does not contain nDNA, and samples with a *B2M* copy number of ≥2 were excluded (Pyle *et al.*, 2015b). Therefore, to ensure accurate replication, these inclusion criteria were applied to the PPMI-PD cohort, supported by frequency histogram data (Figure 5.2) which indicates the suitability of these criteria (i.e. majority of the cohort had a ccf-mtDNA copy number (83%) of ≤1000 copies/µl (Figure 5.2, B) and a nDNA copy number (89%) of ≤1 copy/µl (Figure 5.2, A)).

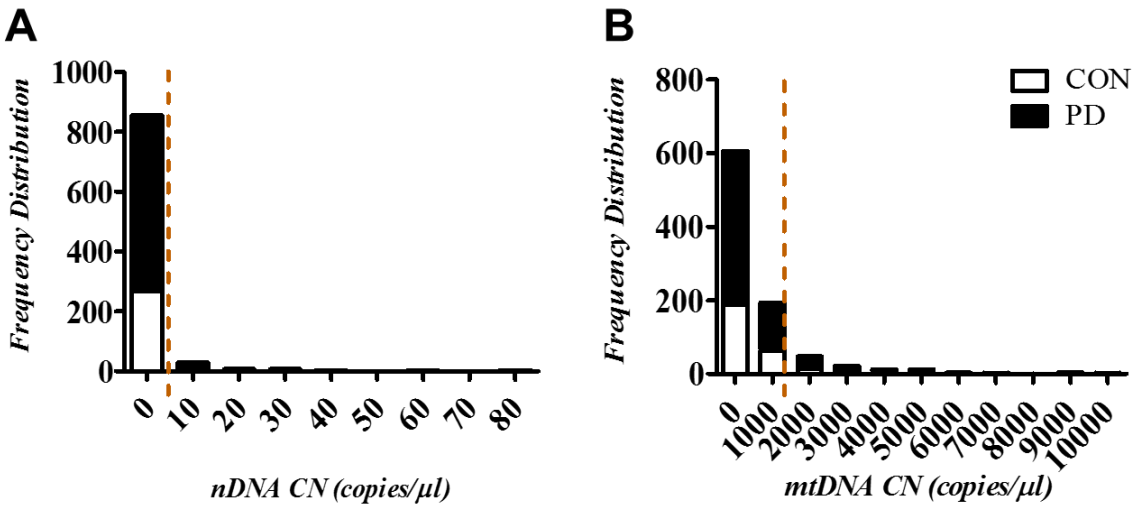

Figure 5.2. Frequency distribution of ccf-mtDNA copy number and nDNA copy number for the entire cohort (BL and V08 combined, n=905). Controls (CON) are in white and PD in black. Orange dashed line indicates the inclusion threshold for nDNA and mtDNA copy number; <1 and <1000, respectively.

Demographic information for the cohort meeting the requirements of the inclusion criteria is presented in Table 5.2. After quality control, the total baseline cohort consisted of 423 lCSF samples; 132 controls and 291 PD cases and the 36 month follow-up (V08) consisted of 263 lCSF samples; 87 controls and 176 PD cases.

In line with previous estimates (Wooten *et al.*, 2004; Miller and Cronin-Golomb, 2010; Gillies *et al.*, 2014; Moisan *et al.*, 2015), women were slightly underrepresented in PD cases, with matched frequency in controls (35%). However, this had no effect on mean ccf-mtDNA copy number at BL (where mean ccf-mtDNA copy number was 283 in 273 males and 253 in 150 females, Mann Whitney U $p>0.05$) or 36 months (where mean ccf-mtDNA copy number was 233 in 171 males and 243 in 92 females, Pearson's Chi-square test $p>0.05$) (Table 5.2 and 5.3). Additionally, mean age was no different for cases and controls (Mann Whitney U $p>0.05$, Table 5.2) and in keeping with previous results in ventricular CSF (Chapter 3 and Chapter 4), there was no correlation between age and ccf-mtDNA copy number at either time point (Pearson's $p>0.05$, Table 5.3).

Time point	***Baseline***			***36 months (V08)***		
Disease group	***Control (132)***	***PD (291)***	***p***	***Control (87)***	***PD (176)***	***p***
Gender (M/F)	82/50	191/100	4.85×10^{-1}	55/32	116/60	6.67×10^{-1}
Age (years)	60±11	61±10	4.43×10^{-1}	64±11	64±10	1.74×10^{-1}
Disease duration (months)	-	7±7	-	-	43±7	-

Table 5.2. Cohort demographic information (including frequency in brackets), gender (male n /female n), age (years) and disease duration (months). Age and disease duration data is mean ± standard deviation. Statistical comparison was carried out between Parkinson's disease (PD) cases and controls by Pearson's chi-square test for gender and Mann Whitney U for age.

Time point	***Baseline***			***36 months (V08)***		
Variable	***Whole cohort***	***Control***	***PD***	***Whole cohort***	***Control***	***PD***
Male vs Female	2.58×10^{-1}	1.07×10^{-1}	7.72×10^{-1}	7.36×10^{-1}	7.45×10^{-1}	3.10×10^{-1}
Age	4.59×10^{-1}	7.43×10^{-1}	4.27×10^{-1}	8.06×10^{-1}	8.28×10^{-1}	6.69×10^{-1}
Disease duration	-	-	7.10×10^{-1}	-	-	2.30×10^{-1}

Table 5.3. Pearson's significance testing showed no significant relationships between gender, age or disease duration and ccf-mtDNA copy number. P values are given for the entire cohort and controls and PD cases separately, $p>0.05$.

5.3.2 Large-scale replication of ccf-mtDNA levels in PD cases compared to controls.

Similar to previous investigations (Pyle *et al.* (2015a) and Chapter 3), comparison of ccf-mtDNA copy number between PD cases to controls revealed a reduction at BL (where mean ccf-mtDNA copy number in 291 PD cases was 261±257 copies/µl versus 297±270 copies/µl in 132 controls, Mann Whitney U $p>0.05$) and 36 months (V08) (where mean ccf-mtDNA copy number was 219±222 copies/µl in 76 PD patients and 273±250 copies/µl in 87 controls, Mann Whitney U $p=5.6x10^{-2}$, Figure 5.3, A), although this did not reach statistical significance. Additionally, receiver operating curve (ROC) analysis at BL and 36 months (V08) yielded an area under the curve of 0.55 and 0.57, respectively, indicating that ccf-mtDNA copy number could not accurately predict the status (PD or control) of the individuals (Hajian-Tilaki, 2013).

Similar to Chapter 3 and 4, the qPCR deletion assay was used to measure ccf-mtDNA integrity. Over 50% of the cohort were found to possess ccf-mtDNA deletions exceeding 10% (the accepted detection threshold (Grady *et al.*, 2014)), however, there was no significant difference in the deletion percentage of control ccf-mtDNA compared to that of PD cases at BL or V08 (Figure 5.3, B). Interestingly, all participants were observed to accumulate ccf-mtDNA deletions over time, increasing from 33% to 40% in PD cases and 32% to 35% in controls (Figure 5.3, B).

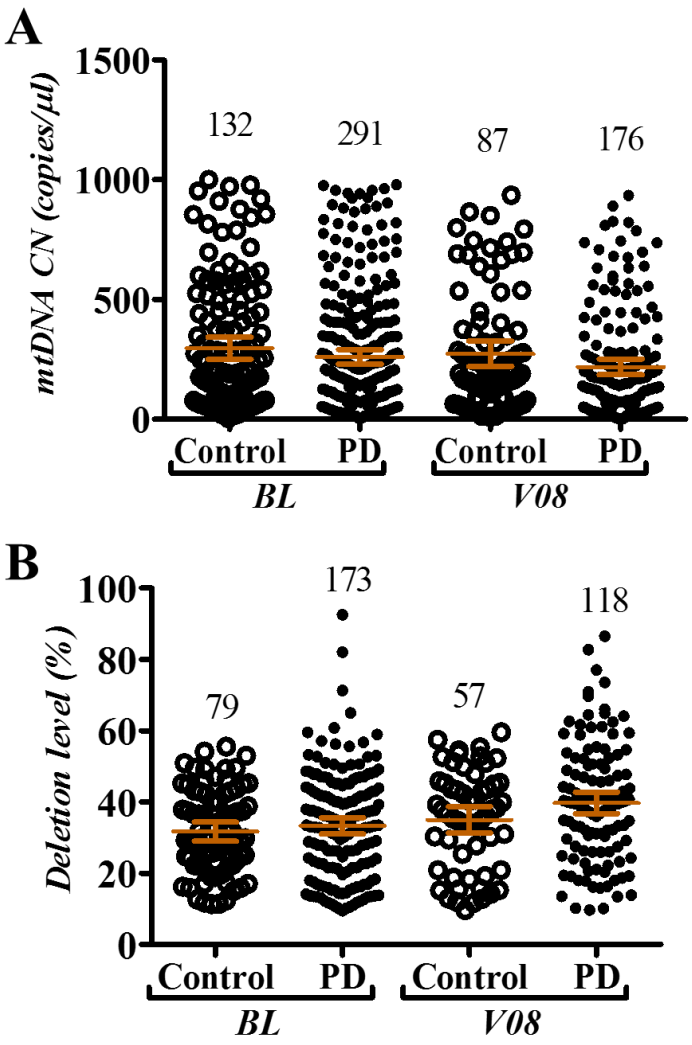

Figure 5.3. Ccf-mtDNA copy number and deletion level (%) of controls and PD patients at baseline (BL) and 36 months (V08). Dot plots with mean and SEM (orange bars) of (A) ccf-mtDNA copy number (copies/µl) and (B) deletion level (%) for PD cases and controls. N numbers are above stacks.

5.3.3 Change in ccf-mtDNA copy number and deletion level over time.

A subset of samples; 58 controls and 130 PD cases, have matched CSF biopsies at BL (0 months) and 36 months (V08). CSF ccf-mtDNA copy number appeared to reduce over time in matched CSF samples, where mean change in ccf-mtDNA copy number was -20±434 copies/µl and -59±290 copies/µl for controls and PD cases, respectively (calculated as 36 month ccf-mtDNA copy number minus BL ccf-mtDNA copy number, Figure 5.4, A). However, this was not significantly different between cases and controls (Mann Whitney U p>0.05) and there was large dispersion in the change in ccf-mtDNA copy number ranging from -965 to +805 copies/µl for controls

and -862 to +796 copies/µl for PD cases (Figure 5.4, A). Moreover, a change in ccf-mtDNA deletion level was also calculated for matched samples from BL to 36 months (V08) (Figure 5.4, B). This indicated no significant difference in the change in ccf-mtDNA deletion level for PD cases (4.6% increase) or controls (1.5% increase) (Mann Whitney U p>0.05, Figure 5.4, B).

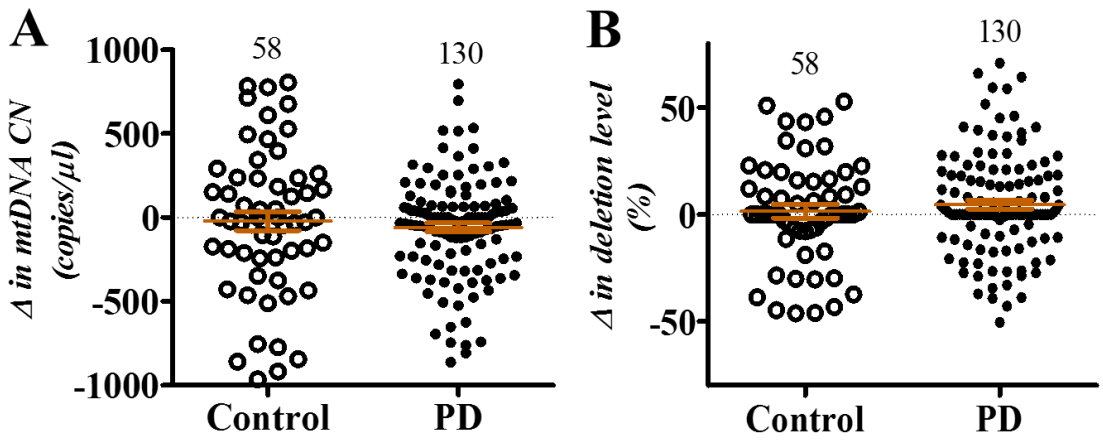

Figure 5.4. Change (Δ) in ccf-mtDNA copy number (CN) (copies/µl) (A) and deletion level (%) (B) for samples matched at baseline and 36 months. Dot plots show mean change with SEM (orange bars). N numbers are above stacks.

5.3.4 Phenotypic associations to ccf-mtDNA copy number level.

The PPMI project allowed access to a comprehensive online database containing copious phenotypic information (described in Section 5.2.3), including age of symptom onset, cognitive profiling scores (Montreal Cognitive Assessment (MoCA)), Geriatric depression scale (GDS) and numerous motor phenotype evaluations (i.e. MDS-UPDRS-III, PASE and SCOPA-AUT).

i) *Ccf-mtDNA copy number of Early onset vs Late onset PD.*

Based on age of symptom onset, PD was stratified into early onset PD (EOPD if <50 years of age, mean 44 years of age, ~14% of the cohort) and late onset PD (LOPD if >50 years of age, mean 63 years of age, ~86% of the cohort), to assess whether age of onset affected ccf-mtDNA copy number. At BL and V08 there was no significant difference in ccf-mtDNA copy number between the two disease groups and controls (Kruskal Wallis p>0.05, Figure 5.5). Furthermore, age of disease onset did not correlate with ccf-mtDNA copy number at BL or V08 (Pearson's p>0.05).

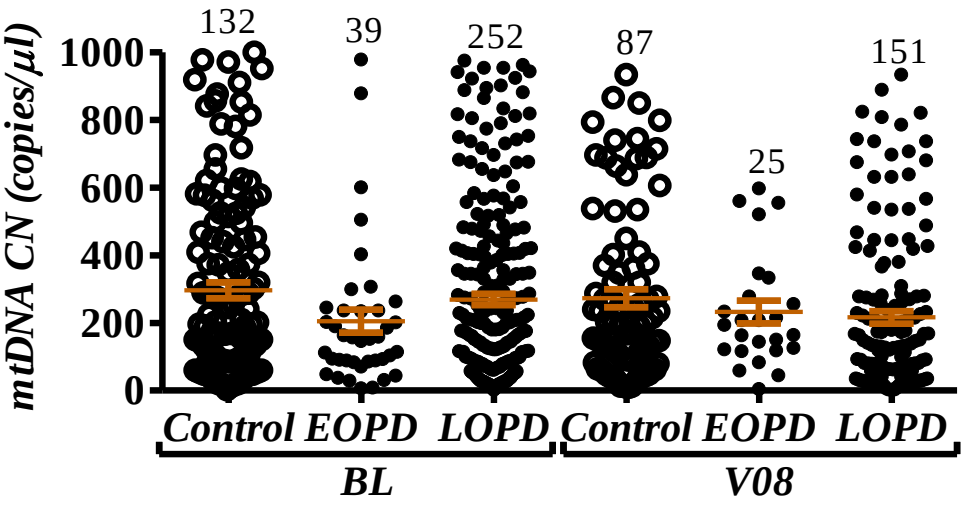

Figure 5.5. Ccf-mtDNA copy number of controls and PD patients separated into early onset (EOPD) and late onset (LOPD) Parkinson's disease, at baseline (BL) and 36 months (V08). Orange bars indicate mean copy number+SEM. N numbers are above stacks.

ii) *Analysis of mean phenotypic scores between PD cases and controls.*

Mean phenotypic scores, including GDS, SCOPA, MDS-UPDRS I-IV, PASE and MoCA were compared between PD cases and controls to assess symptom severity (Table 5.4). At BL, motor phenotype scores from SCOPA and MDS-UPDRS indicated significantly higher motor and autonomic dysfunction in PD cases compared to controls (Table 5.4, Pearson's $p<0.01$), whereas GDS scores were not significantly different between PD cases and controls at BL (Pearson's $p>0.05$). By the 36 month follow up (V08), GDS, SCOPA and MDS-UPDRS scores had increased from BL scores for PD patients but not controls, thereby maintaining a significant difference in phenotypic scores between PD patients and controls (Table 5.4), however PASE activity was no different between PD cases and controls (Mann Whitney U $p>0.05$). MoCA scores were available at 36 months but were not significantly different between cases and controls.

Phenotypic Test	Baseline			36 months		
	Controls (132)	PD (291)	Case vs Control (p)	Controls (87)	PD (176)	Case vs Control (p)
GDS	5.1±1.1	5.2±1.4	$3.76x10^{-1}$	5.1±1.0	5.5±1.3	$2.40x10^{-2}$ *
SCOPA	8.9±7.6	13.3±9.1	<0.01 **	10.0±7.6	16.7±9.5	<0.01 **
UPDRS I	1.5±0.9	2.2±1.4	<0.01 **	1.5±0.9	3.1±2.3	<0.01 **
UPDRS II	1.6±1.2	6.8±4.2	<0.01 **	1.5±0.8	10.1±5.9	<0.01 **
UPDRS III	1.1±2.0	21.3±9.2	<0.01 **	1.7±2.7	28.1±12.5	<0.01 **
UPDRS IV	-	-	-	-	1.0±2.0	-
UPDRS Total	4.1±2.9	30.3±11.9	<0.01 **	4.7±3.4	42.2±17.2	<0.01 **
PASE (household activity)	-	-	-	10.8±1.4	10.6±1.7	$4.92x10^{-1}$
PASE (leisure activity)	-	-	-	3.5±1.4	3.7±1.4	$3.74x10^{-1}$
MOCA score	-	-	-	20.5±11.0	22.2±9.3	$8.27x10^{-1}$

*Table 5.4. Summary of mean scores and standard deviation (±) from phenotypic tests carried out in the PPMI study. N numbers are in brackets. Mann Whitney U p values are present for case vs control comparison. * indicates significance at p<0.05, ** indicates significance at p<0.01. GDS: global depression score, SCOPA: Self-reported autonomic symptoms in PD, MDS UPDRS: Movement disorders unified Parkinson's Disease rating scale, PASE: Physical activity scale in the elderly: HH, house hold activity, L, leisure activity, MOCA; Montreal Cognitive assessment.*

iii) Comparison of ccf-mtDNA copy number to phenotypic assessments of PD.

To assess whether disease severity, as measured by different phenotypic scores, influenced ccf-mtDNA copy number correlation analysis was performed between phenotypic score and ccf-mtDNA copy number.

Given the link between depression and PD (Reijnders *et al.*, 2008) and mtDNA copy number (Cai *et al.*, 2015), the relationship between Geriatric Depression scale (GDS) score and ccf-mtDNA copy number was assessed. Similar to previous results (Pyle *et al.*, 2016), there was no association between GDS and ccf-mtDNA copy number in cases, controls, or the whole cohort combined at BL or 36 month (V08) follow up (Pearson's p>0.05, Table 5.5).

Cognitive impairment, as measured by MoCA, was not assessed at BL, but was assessed at 36 months (V08). This was found to be significantly negatively correlated to ccf-mtDNA copy number in controls (Pearson's r^2=-0.54, p=1.70x10^{-2}) but not PD patients, or the cohort as a whole (Table 5.4). Moreover, MDS-UPDRS II and MDS-UPDRS total scores were also correlated to V08 ccf-mtDNA copy number in controls only.

Motor phenotype severity as measured by SCOPA-AUT (scales for outcomes in PD-Autonomic), MDS-UPDRS I-IV and total (Movement disorder society-Unified PD rating scale) and PASE (physical activity scale for the elderly) assessments did not associate with ccf-mtDNA copy number (Mann Whitney p>0.05, Table 5.4) at BL nor V08 for PD cases.

Ccf-mtDNA copy number versus:	***Baseline***			***36 months***		
	Whole Cohort (423)	***Control (132)***	***PD (291)***	***Whole Cohort (263)***	***Control (87)***	***PD (176)***
GDS	4.68x10^{-1}	8.30x10^{-2}	8.33x10^{-1}	5.51x10^{-1}	2.29x10^{-1}	2.69x10^{-1}
MoCA	n/a			6.77x10^{-1}	1.70x10^{-2} *	1.59x10^{-1}
SCOPA-AUT	9.43x10^{-1}	3.71x10^{-1}	4.20x10^{-1}	3.31x10^{-1}	7.88x10^{-1}	7.67x10^{-1}
MDS UPDRS I	9.94x10^{-1}	4.09x10^{-1}	4.54x10^{-1}	8.80x10^{-1}	2.01x10^{-1}	9.70x10^{-2}
MDS UPDRS II	9.40x10^{-2}	8.70x10^{-2}	3.99x10^{-1}	3.70x10^{-2} *	6.10x10^{-2}	1.38x10^{-1}
MDS UPDRS III	4.71x10^{-1}	9.20x10^{-1}	6.94x10^{-1}	1.68x10^{-1}	7.20x10^{-2}	7.65x10^{-1}
MDS UPDRS IV	4.71x10^{-1}	-	-	2.28x10^{-1}	-	2.30x10^{-1}
MDS UPDRS Total	2.92x10^{-1}	3.37x10^{-1}	9.23x10^{-1}	6.60x10^{-1}	2.50x10^{-2} *	2.78x10^{-1}
PASE (HH)	n/a			5.01x10^{-1}	3.04x10^{-1}	7.78x10^{-1}
PASE (L)	n/a			4.35x10^{-1}	7.15x10^{-1}	6.12x10^{-1}

*Table 5.5. Correlational analysis of various phenotype measurements against ccf-mtDNA copy number in the entire cohort, and stratified by PD case or control at baseline and V08. Values are Pearson's p. N numbers are in brackets. n/a present where scores were not available. * indicates significance at p<0.05. GDS: global depression score, SCOPA: Self reported autonomic symptoms in PD , MDS UPDRS III: Movement disorders unified Parkinson's Disease rating scale III, PASE: Physical activity scale in the elderly: HH, house hold activity, L, leisure activity, MOCA; Montreal Cognitive assessment.*

5.3.5 CSF Proteomic relationships to ccf-mtDNA copy number.

A number of neurodegeneration-specific proteins (alpha-synuclein, amyloid beta, total tau and phosphorylated tau) were measured in the lumbar CSF (lCSF) of PD patients and controls enrolled in the PPMI-PD cohort and these were made available via the PPMI database. All four proteins were found to be significantly reduced in the lCSF of PD cases compared to controls at 36 months (Table 5.6), and alpha synuclein levels were also significantly reduced in PD cases at BL. However, correlation analysis failed to find any significant associations between lCSF protein abundance and ccf-mtDNA copy number at BL or 36 months (Table 5.7).

Protein of interest	Baseline			36 months		
	Controls (132)	PD cases (291)	Case vs Control (p)	Controls (87)	PD cases (176)	Case vs Control (p)
Alpha Synuclein	1878.3±942.0	1655.9±771.8	$4.20x10^{-2}$ *	1653.5±623.9	1429.1±577.5	$7.00x10^{-3}$ **
Amyloid Beta	379.5±118.1	367.7±101.0	$4.43x10^{-1}$	1010.4±444.3	848.8±403.5	$1.10x10^{-2}$ *
Total Tau	52.1±27.3	44.5±18.0	$1.50x10^{-1}$	193.2±64.9	170.5±58.1	$7.00x10^{-3}$ **
Phosphorylated Tau	17.6±11.2	15.8±10.3	$1.70x10^{-1}$	17.6±6.2	14.9±5.2	$1.00x10^{-3}$ **

*Table 5.6. Mean CSF protein levels for controls and PD cases at baseline and 36 months. Protein levels are mean and standard deviation (±). N numbers are in brackets. Case vs control are Mann Whitney U p values. * indicates significance as p<0.05, ** p<0.001.*

Protein of interest	Baseline			36 months		
	Whole Cohort	Control	PD	Whole Cohort	Control	PD
Alpha synuclein	$1.66x10^{-1}$	$6.54x10^{-1}$	$2.19x10^{-1}$	$1.90x10^{-1}$	$2.26x10^{-1}$	$3.00x10^{-1}$
Amyloid Beta	$7.36x10^{-1}$	$7.00x10^{-1}$	$2.37x10^{-1}$	$2.80x10^{-1}$	$7.97x10^{-1}$	$1.15x10^{-1}$
Total Tau	$7.95x10^{-1}$	$9.29x10^{-1}$	$8.66x10^{-1}$	$3.98x10^{-1}$	$5.93x10^{-1}$	$3.28x10^{-1}$
Phosphorylated Tau	$1.76x10^{-1}$	$4.00x10^{-1}$	$2.37x10^{-1}$	$3.55x10^{-1}$	$6.21x10^{-1}$	$2.28x10^{-1}$

Table 5.7. Pearson's correlation between CSF NDD-associated protein levels and ccf-mtDNA copy number, for the entire cohort and stratified by PD cases and controls at baseline and 36 months. Values are Mann Whitney U p, p>0.05.

5.3.6 Transmission Electron Microscopy of the lCSF.

Transmission electron microscopy was used to examine the lCSF and evaluate the presence of any cellular or organellar ultrastructures. Two samples (one PD and one control) were assessed in a preliminary test. No cellular or organellar structures were discovered in these samples, but, similar to vCSF (Section 3.4.5) an abundance of unknown proteinaceous material was present in both the PD patient (Figure 5.6, A) and control (Figure 5.6, B) lCSF samples.

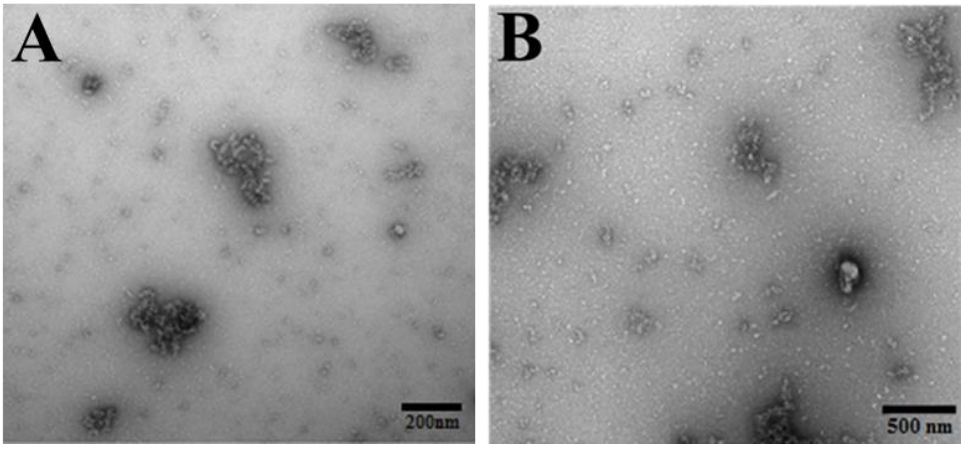

Figure 5.6. TEM of (A) PD and (B) control lCSF, showing the presence of proteinaceous materials but no cellular or mitochondrial structures.

5.3.7 Sample heterogeneity or external variables may be confounding replication.

The lack of replication of previous results, in addition to the detailed individual data provided by the PPMI database, prompted further investigation to determine reasons behind this disparity. Moreover, the great deal of inter-individual variation of ccf-mtDNA copy number within the cohort, (coefficient of variance at baseline was 90% in PD, and 98% in controls, and at 36 months (V08) was 91% in PD and 99% in controls) indicated large sample heterogeneity or potential confounding factors which may be influencing ccf-mtDNA copy number.

5.3.8 PD medication affects ccf-mtDNA copy number.

In the initial preliminary study in the ICICLE-PD cohort (Pyle *et al.*, 2015b) the majority of PD cases (90%) were receiving treatment at BL and 18 months (100% on L-DOPA). In contrast, no PPMI PD cases were receiving treatment for PD at BL. However, at 36 months 90% of PPMI PD cases were receiving one or more PD treatment. Therefore, the V08 (36 months) cohort was stratified into controls (87), treatment naïve (17) and treated PD patients (159) to establish if pharmacological treatment was affecting ccf-mtDNA copy number.

Ccf-mtDNA copy number was found to be significantly reduced in the lCSF of treated PD patients compared to controls (Figure 5.7, A), where mean ccf-mtDNA copy number was 208±216 copies/µl in 159 treated PD patients compared to 273±250 copies/µl in 87 controls (Mann Whitney U $p=2.66x10^{-2}$). The same trend was present between treated PD patients compared to treatment naïve patients (208±216 copies/µl in 159 compared to 313±263 copies/µl in 17), though this was not significant (Mann Whitney U $p>0.05$). Treatment naïve PD patients and controls showed no significant difference in ccf-mtDNA copy number (Mann Whitney U $p>0.05$) (Figure 5.7, A).

Comparison of matched samples ccf-mtDNA copy number over time showed a reduction for PD patients who received treatment at V08 (-66±297 copies/µl), whereas untreated PD patients showed a minor elevation in ccf-mtDNA copy number over time (+13±209 copies/µl), however, this was not significantly different (Mann Whitney U $p>0.05$) (Figure 5.7, B).

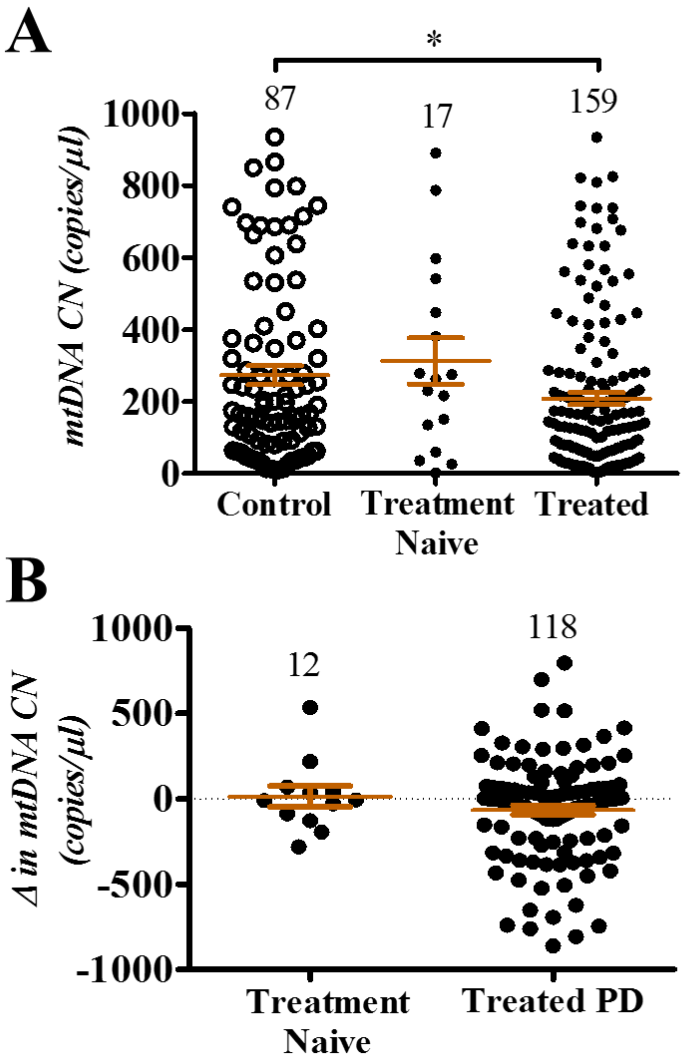

*Figure 5.7. Illustrating the effect of PD treatment on ccf-mtDNA copy number. (A) 36 month follow up cohort stratified into controls, treatment naïve and treated PD patients. (B) Matched samples with measurements at BL and V08 stratified by treatment status (naïve or treated). Orange bars represent mean+SEM. N numbers are present above stacks. * indicates significance at p<0.05, calculated by Mann Whitney U.*

Furthermore, the PD patients receiving treatment could be further subdivided into three main PD drug classes; Levocarb (Levodopa/carbidopa combination: LDOPA), dopamine agonists (DOPAG) and monoamine oxidase B inhibitors (MAOBIs) and a further group for patients on a cocktail of pharmaceuticals (i.e. two or more of the previous three drug classes, from now on referred to as combination treatment) (Figure 5.8, A).

Stratification by drug class revealed that the significant reduction of ccf-mtDNA copy number was specific to only the PD patients receiving Levocarb treatment (mean ccf-

mtDNA copy number in 49 patients on LDOPA treatment was 188±208 copies/µl compared to 273±250 copies/µl in 87 controls, Mann Whitney U $p=2.51x10^{-2}$) or a combination of medications including Levocarb (mean ccf-mtDNA copy number in 83 PD patients on combination therapy was 200±209 copies/µl, Mann Whitney U $p=4.27x10^{-2}$). PD patients on MAOBIs showed a similar trend towards lower ccf-mtDNA copy number, however, this was not significant (mean ccf-mtDNA copy number for 15 MAOBI PD patients was 211±241 copies/µl, Mann Whitney U $p>0.05$). Dopamine agonist (DOPAG) treated patients showed a different trend to the other drug classes, displaying an overall elevation in ccf-mtDNA copy number compared to controls and treatment naïve patients, however, this did not reach significance (Figure 5.8, A).

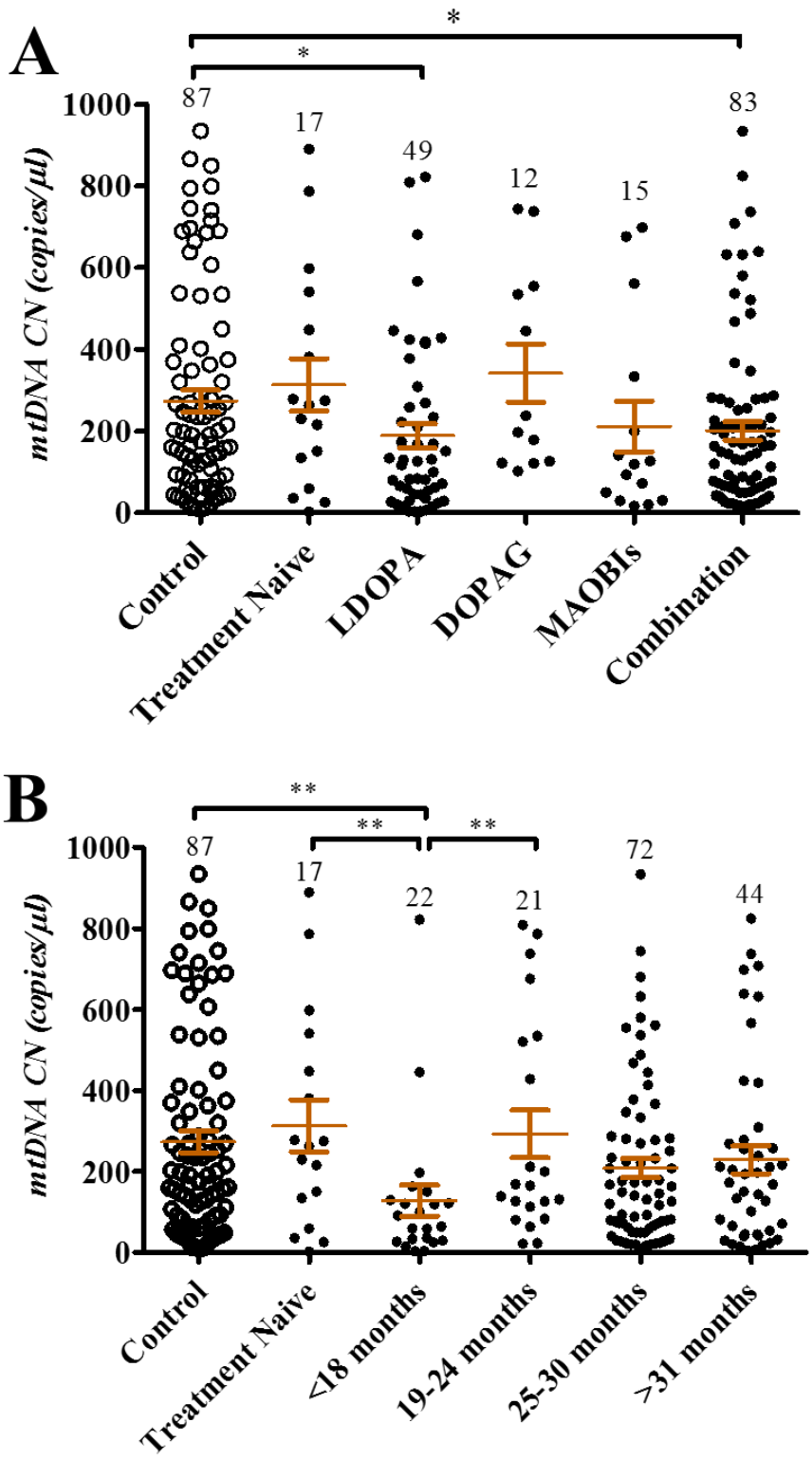

*Figure 5.8. Ccf-mtDNA copy number stratified by (A) drug class or (B) duration of time on treatment at the 36 month follow up (V08), with mean+SEM (orange bars). Treatment naïve refers to PD patients not receiving typical PD medication by V08. LDOPA; Levocarb treatment, DOPAG; dopamine agonists, MAOBIs; monoamine oxidase B inhibitors, Combination; LDOPA+DOPAG/MAOBI. Numbers above stacks are n. * indicates significance at p<0.05, ** p<0.01, calulcated by Mann Whitney U.*

Given the link between PD treatment and ccf-mtDNA copy number, subsequent analysis was performed comparing ccf-mtDNA copy number to treatment duration. Treatment duration was available for all but one of the cohort (159/160), varying from 8 to 35 months. Ccf-mtDNA copy number was found to initially decrease in PD patients with treatment duration <18 months (compared to controls and non-treated PD patients, Mann Whitney U $p=1.50x10^{-3}$ and $p=7.80x10^{-3}$, respectively) and then significantly increased with treatment duration after this point (Figure 5.8, B; mean ccf-mtDNA for 22 PD patients <18 months treatment was 128±182 copies/µl versus 293±269 copies/µl for 21 PD patients 19-24 months treatment, Mann Whitney U $p=7.80x10^{-3}$). This effect did not appear to be treatment specific, as each of the four treatment groups showed reduced ccf-mtDNA copy number level at 18 months, subsequently rising back to control and non-treated PD patient levels as treatment continued (Figure 5.9, A-D).

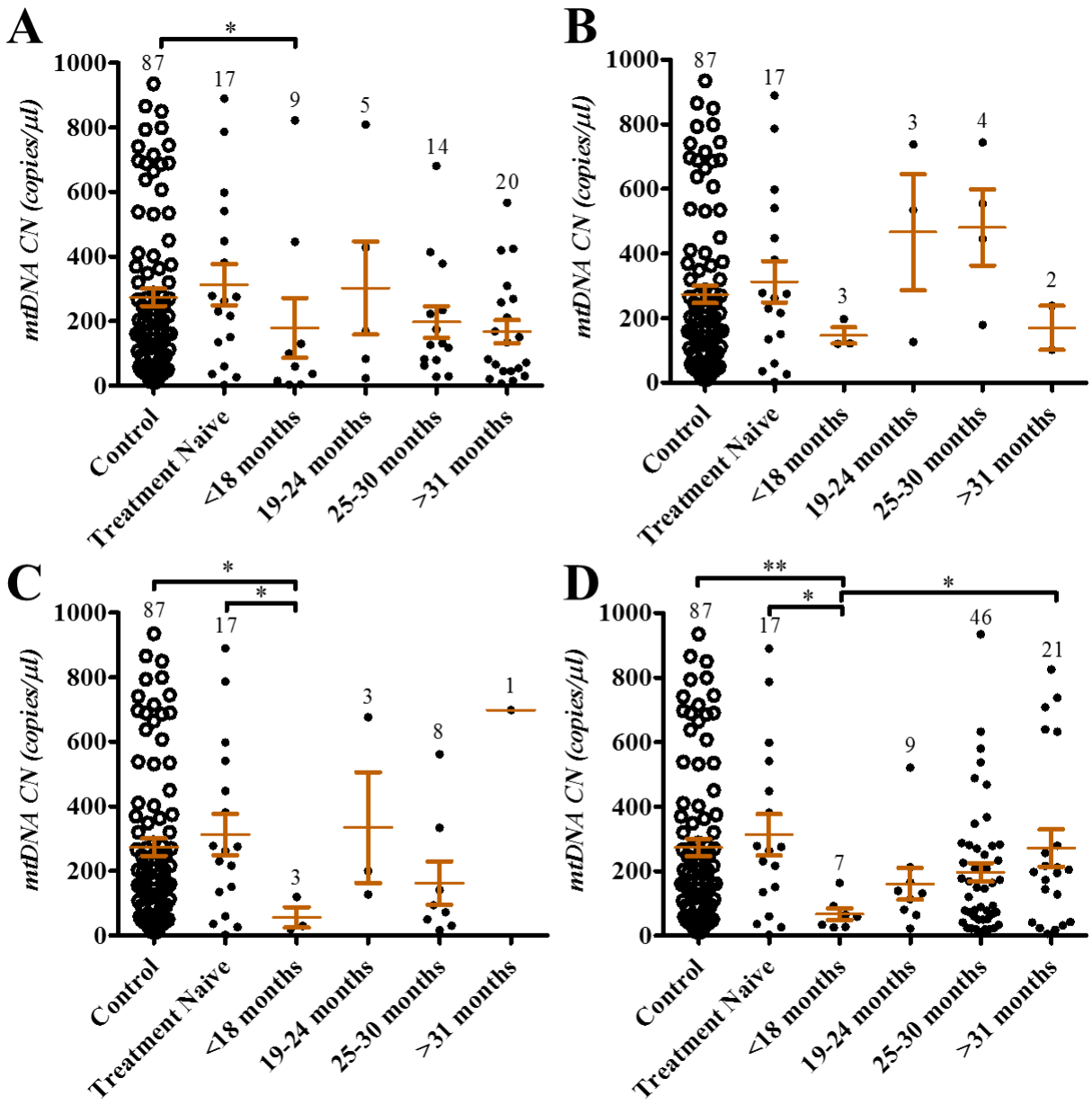

*Figure 5.9. Ccf-mtDNA copy number levels grouped by time on PD treatment. Each drug category is displayed separately, (A) LDOPA; levocarb, (B) DOPAG; dopamine agonist, (C) MAOBIs; monoamine oxidase B inhibitors, (D) combination therapy; LDOPA+DOPAG/MOABI. Orange bars represent mean +SEM. N numbers are above stacks. * indicates significance at p<0.05, ** p<0.01.*

5.3.9 *Incidence of comorbidities and secondary phenotypes in the PPMI-PD cohort.*

The PPMI database included detailed clinical information regarding previous and current comorbidities and secondary phenotypes of PD patients and controls. Over 1000 different conditions were listed in the PPMI database, including the major secondary phenotypes associated with PD (i.e. anxiety and depression (Kano *et al.*, 2011), gastroesophageal reflux disease (GERD) (Maeda *et al.*, 2013), insomnia (Gjerstad *et al.*, 2007)), common comorbidities associated with PD or aging (i.e. diabetes (Cereda *et al.*, 2013), arthritis (Loeser, 2010)), various other comorbidities (i.e. cancer, coronary heart disease (CHD)) and numerous other conditions (i.e. allergies, vision and hearing problems, respiratory disease). Due to the vast scope of conditions reported in the PPMI-PD cohort database, limitations were set to analyse only the comorbidities that were common secondary phenotypes of PD, previously associated with ccf-mtDNA copy number or present in >10% of the cohort. This led to subsequent analysis of 10 conditions including, hypertension, anxiety/depression, hypercholesterolemia, arthritis, GERD, constipation, CHD, cancer, diabetes and insomnia (Table 5.8).

Comorbidity Type	Baseline			36 months		
	Control (% of 132)	PD (% of 291)	p	Control (% of 87)	PD (% of 176)	p
Hypertension	36	32	$3.36x10^{-1}$	28	37	$1.32x10^{-1}$
Anxiety/Depression	23	20	$5.66x10^{-1}$	31	23	$1.46x10^{-1}$
Hypercholesterolemia	19	19	$9.41x10^{-1}$	25	22	$5.72x10^{-1}$
Arthritis	17	19	$5.81x10^{-1}$	20	22	$6.25x10^{-1}$
GERD	5	8	$2.82x10^{-1}$	7	9	$6.47x10^{-1}$
Constipation	5	8	$3.95x10^{-1}$	8	10	$5.70x10^{-1}$
Cancer	3	5	$2.68x10^{-1}$	9	7	$6.11x10^{-1}$
Diabetes	4	5	$6.38x10^{-1}$	5	10	$1.54x10^{-1}$
Insomnia	2	5	$1.00x10^{-1}$	8	6	$5.87x10^{-1}$
CHD	1	2	$3.30x10^{-1}$	2	5	$3.70x10^{-1}$

Table 5.8. Table showing the prevalence of the 10 selected comorbidities in the PPMI-PD cohort at baseline and 36 months. Numbers are percentages. P values show no difference in prevalence between PD cases and controls, Pearson Chi-square test. Orange fill indicates inclusion based on prevalence in >10% of the population, green fill indicates association with PD and yellow fill indicates previous association with ccf-mtDNA copy number in the literature. GERD; gastroesophageal reflux disorder, CHD; coronary heart disease.

Furthermore, the prevalence of one or more of these 10 comorbidities was extremely high in the PPMI-PD cohort; >65% had one or more at BL and >70% had one or more at 36 months (V08) (Table 5.9).

No. Comorbidities	% of cohort at baseline	% of cohort at 36 months
0	34	29
1	33	30
2	18	19
3	10	13
4	4	7
5	1	2
6	0	1

Table 5.9. Summary of the percentage of the cohort with comorbidities (%) at baseline and 36 months. Number of comorbidities ranges from 0-6.

5.3.10 Ccf-mtDNA copy number measurement may be confounded by comorbidities.

At BL, secondary phenotypes of anxiety/depression and insomnia in PD patients were associated with significantly higher ccf-mtDNA copy number than PD patients with no comorbidities (Figure 5.10, A, mean ccf-mtDNA copy number was 235±259 copies/µl in 96 PD patients with no comorbidities compared to 333±288 copies/µl in 59 PD patients with anxiety/depression (Mann Whitney U $p=1.29x10^{-2}$) and 490±357 copies/µl in 14 PD patients with insomnia (Mann Whitney U $p=6.20x10^{-3}$)). The same effect of anxiety/depression on ccf-mtDNA copy number was also present in controls, however, not significantly (Figure 5.11, A). Interestingly, at the 36 month follow up (V08), these trends were no longer seen in the PD patients (Figure 5.10, B) and remain non-significant for the control population (Figure 5.11, B).

PD patients with CHD or diabetes showed non-significant elevations in ccf-mtDNA copy number at BL compared to PD patients with no comorbidities (where BL mean ccf-mtDNA copy number was 433±303 copies/µl in 6 PD patients with CHD and 295±272 copies/µl in 14 PD patients with diabetes, compared to 235 copies/µl in 96 PD patients with no comorbidities, Mann Whitney U $p>0.05$, Figure 5.10, A). This effect was still present at V08 (36 months) in PD patients, however, to a much lesser degree (where V08 mean ccf-mtDNA copy number was 243±268 copies/µl in 8 PD patients with CHD and 280±255 copies/µl in 17 PD patients with diabetes, compared

to 187±200 copies/µl in 48 PD patients with no comorbidities, Mann Whitney U $p>0.05$, Figure 5.10, B).

Cancer as a comorbidity to PD, was associated with a non-significant increase in ccf-mtDNA copy number at baseline (where mean ccf-mtDNA copy number was 327±250 copies/µl in 16 PD patients with cancer compared to 235±259 copies/µl in 96 PD patients with no comorbidities, Mann Whitney U $p>0.05$, Figure 5.10, A), but a reduction in ccf-mtDNA copy number at V08 (where mean ccf-mtDNA copy number was 115±92 copies/µl in 13 PD patients with cancer compared with 187±200 copies/µl in PD patients with no comorbidities, Mann Whitney U $p>0.05$, Figure 5.8, B). Whilst the opposite trend was present in controls, showing a non-significant reduction at BL (where mean ccf-mtDNA copy number was 151±117 copies/µl in controls with cancer compared to 260±238 copies/µl in controls with no comorbidities, Mann Whitney U $p>0.05$, Figure 5.11, A) and a slight elevation at V08 (where mean ccf-mtDNA was 272±247 copies/µl for controls with cancer compared to 247±232 copies/µl for controls with no comorbidities, Mann Whitney U $p>0.05$, Figure 5.11, B).

All other comorbidities investigated (hypertension, hypercholesterolemia, arthritis, GERD and constipation) were found to have no consequence on ccf-mtDNA copy number levels in PD cases or controls. In addition, linear regression of ccf-mtDNA copy number against status with comorbidities as a covariate was not significant ($p>0.05$ at BL and V08), suggesting that overall number of comorbidities did not influence ccf-mtDNA copy number.

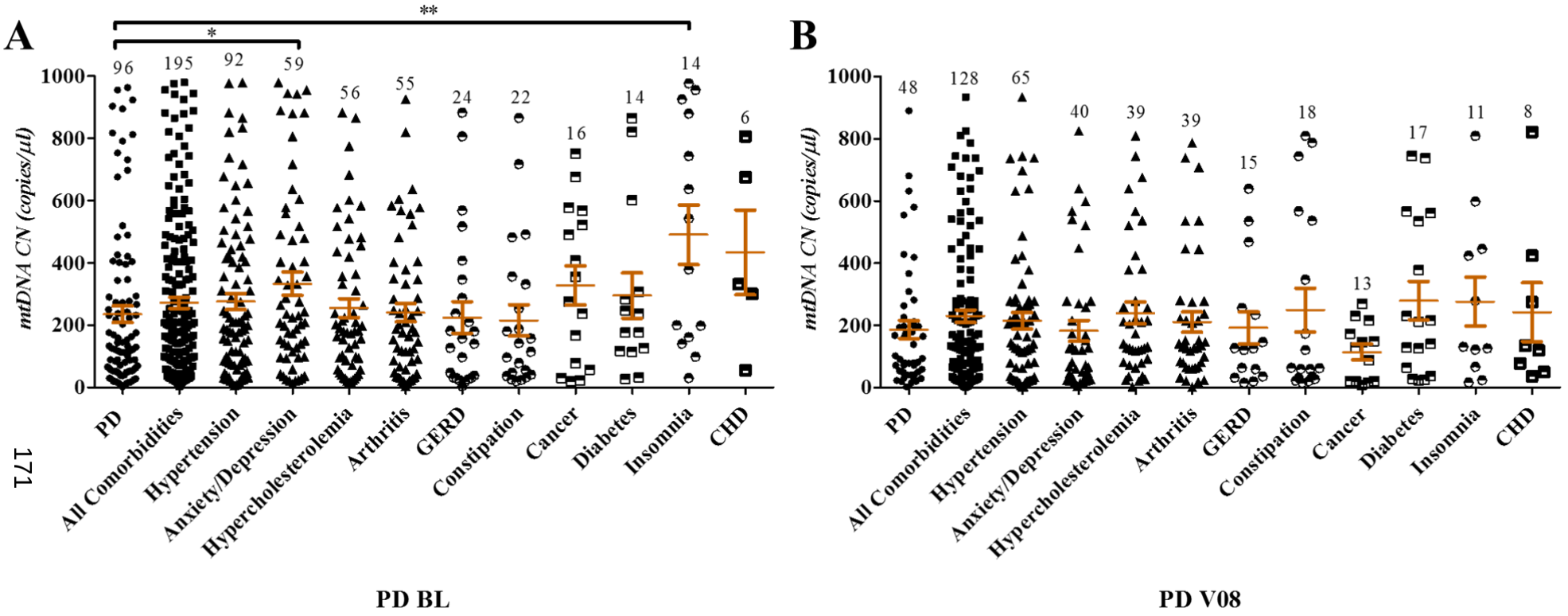

*Figure 5.10. Dot plots of ccf-mtDNA copy number for PD cases without comorbidities (PD) compared to PD patients with a comorbidity (all comorbidities) and stratified for each comorbidity at baseline (A) and 36 months (B). Orange bars represent mean +SEM. N numbers are above stacks. * indicates significant difference at p<0.05, ** p<0.01.*

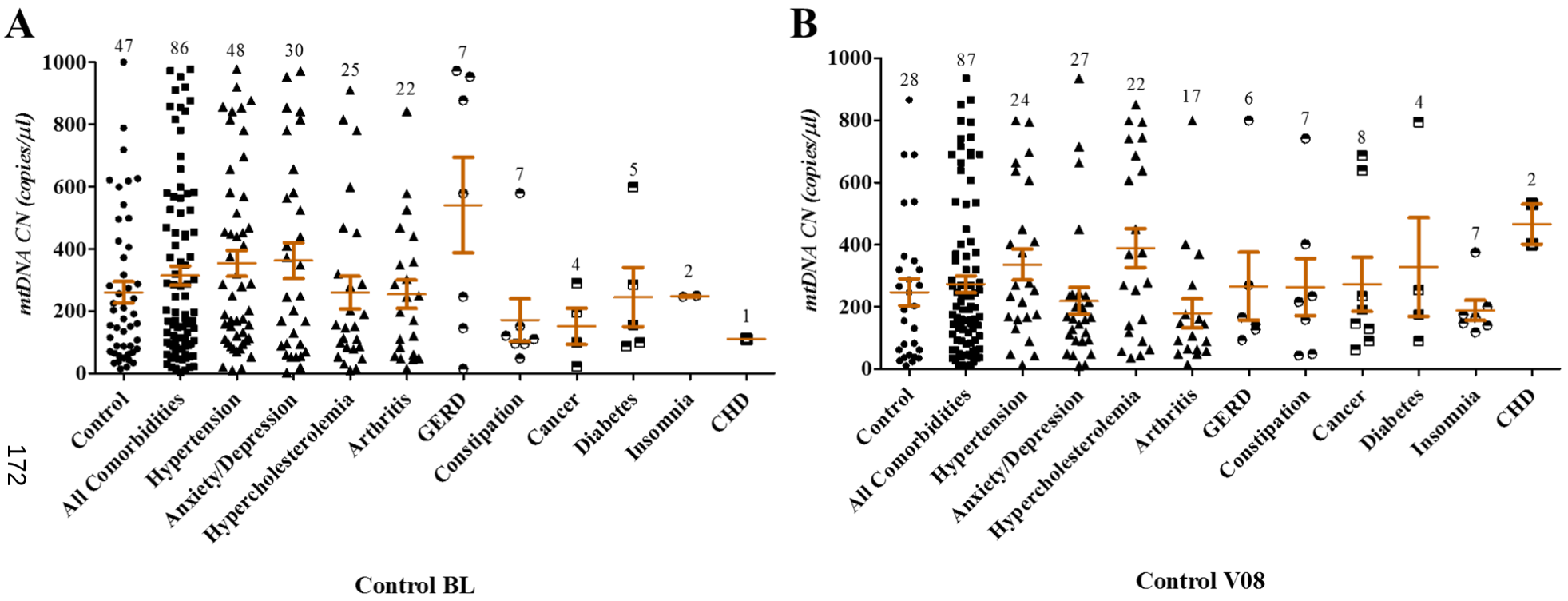

Figure 5.11. Dot plots of ccf-mtDNA copy number for controls without comorbidities (Control) compared to controls with a comorbidity (all comorbidities) and stratified for each comorbidity at baseline (A) and 36 months (B). Orange bars represent mean +SEM. N numbers are above stacks.

5.3.11 Genotyping and quantitative trait analysis.

i) *Association of previously reported PD-risk variants to ccf-mtDNA copy number.*

Several genetic variants are associated with PD (Section 1.5.3, 1.5.4, Klein and Westenberger (2012) and Nalls *et al.* (2014)). It is possible, given that these variants can modulate disease severity and progression, and in some instances are in genes with mitochondrial function (i.e. *PINK1, Parkin* (Nuytemans *et al.*, 2010), that they may be associated with a release of ccf-mtDNA in PD.

Initially, QTL analysis was used to investigate associations between 32 previously reported PD-risk variants (identified in a recent large scale meta-analysis (Nalls *et al.*, 2014), shown in Table 5.10) and ccf-mtDNA copy number. Of the 32 variants investigated, only 22 (69%) could be located on the NeuroX array and 11 (34%) on the Immunochip array, with an overlap of 9 variants on both arrays (eight variants were missing completely, and thus are not present in Table 5.10).

Several variants were found to be associated with the onset of PD (highlighted with an asterisks, Table 5.10) including rs356182 (*SNCA*), rs76904798 (*LRRK2*), rs34311866 (*TMEM175*), rs591323 (*FGF20*), and rs10797576 (*SIPA1L2*), with similar p values being generated by both arrays (when available). However, there was no correlation between any of these previously reported PD SNPs and ccf-mtDNA copy number level in PD cases or controls at BL or V08 (36 months) (Table 5.10).

dbSNP	CHR	Gene	Immunochip Array				NeuroX Array			
			PD cases v Control		SNP vs ccf-mtDNA copy number		PD cases v Control		SNP vs ccf-mtDNA copy number	
			p	OR (95% CI)	Baseline (p)	36 months (p)	p	OR (95% CI)	Baseline (p)	36 months (p)
rs10797576	1	*SIPA1L2*	n.a.				$4.8x10^{-2}$ *	1.47	$6.3x10^{-1}$	$8.8x10^{-1}$
rs823118	1	*RAB7L1*	$6.30x10^{-1}$	0.9	$8.8x10^{-1}$	$7.4x10^{-1}$	$7.5x10^{-1}$	0.96	$7.5x10^{-1}$	$9.4x10^{-1}$
rs6430538	2	*ACMSD/TMEM163*	n.a.				$3.10x10^{-1}$	0.88	$8.0x10^{-1}$	$3.2x10^{-1}$
rs12637471	3	*MCCC1*	$9.00x10^{-2}$	0.7	$3.27x10^{-1}$	$4.14x10^{-1}$	$3.80x10^{-1}$	0.87	$4.34x10^{-1}$	$8.49x10^{-1}$
rs34016896	3	*NMD3*	$3.50x10^{-1}$	1.15	$3.78x10^{-1}$	$2.46x10^{-1}$	$4.70x10^{-1}$	1.11	$4.50x10^{-1}$	$3.46x10^{-1}$
rs11724635	4	*BST1*	$6.00x10^{-2}$	0.77	$9.59x10^{-1}$	$5.04x10^{-1}$	$3.60x10^{-2}$	0.76	$5.47x10^{-1}$	$6.46x10^{-1}$
rs34311866	4	*TMEM175*	$4.50x10^{-2}$ *	1.4	$7.31x10^{-1}$	$5.67x10^{-1}$	$2.00x10^{-2}$ *	1.5	$7.16x10^{-1}$	$8.03x10^{-1}$
rs356182	4	*SNCA*	$3.00x10^{-3}$ **	1.55	$1.20x10^{-1}$	$9.57x10^{-1}$	$1.00x10^{-3}$ **	1.57	$1.43x10^{-1}$	$8.80x10^{-1}$
rs6812193	4	*FAM47E/SCARB2*	$9.30x10^{-1}$	0.99	$2.11x10^{-1}$	$6.51x10^{-1}$	$7.80x10^{-1}$	1.04	$6.73x10^{-1}$	$4.64x10^{-1}$
rs199347	7	*GPNMB*	n.a.				$4.10x10^{-1}$	0.9	$9.29x10^{-1}$	$8.10x10^{-1}$
rs591323	8	*FGF20*	$3.10x10^{-2}$ *	0.71	$2.68x10^{-1}$	$1.10x10^{-1}$	$1.10x10^{-1}$	0.79	$3.42x10^{-1}$	$2.18x10^{-1}$
rs60298754	8	*MMP16*	$1.10x10^{-1}$	0.79	$3.42x10^{-1}$	$2.18x10^{-1}$	n.a.			
rs7077361	10	*ITGA8*	n.a.				$6.10x10^{-1}$	0.9	$1.42x10^{-1}$	$8.33x10^{-1}$
rs329648	11	*MIR4697*	n.a.				$9.40x10^{-1}$	1.01	$8.23x10^{-1}$	$8.51x10^{-1}$
rs3793947	11	*DLG2*	n.a.				$1.30x10^{-1}$	0.82	$7.67x10^{-1}$	$3.20x10^{-1}$
rs11060180	12	*CCDC62*	$9.40x10^{-1}$	0.99	$7.40x10^{-2}$	$5.02x10^{-1}$	$9.30x10^{-1}$	1.01	$5.50x10^{-2}$	$8.49x10^{-1}$
rs76904798	12	*LRRK2*	$3.60x10^{-2}$ *	1.54	$5.23x10^{-1}$	$5.60x10^{-2}$	n.a.			
rs11158026	14	*GCH1*	n.a.				$3.60x10^{-2}$	1.54	$5.71x10^{-1}$	$2.33x10^{-1}$
rs2414739	15	*VPS13C*	n.a.				$5.80x10^{-2}$	0.76	$6.50x10^{-1}$	$1.93x10^{-1}$
rs14235	16	*BCKDK/STX1B*	n.a.				$2.20x10^{-1}$	1.18	$9.55x10^{-1}$	$6.84x10^{-1}$
rs11868035	17	*SREBF/RAI1*	n.a.				$6.40x10^{-1}$	1.07	$6.90x10^{-2}$	$9.10x10^{-2}$
rs17649553	17	*MAPT*	n.a.				$9.00x10^{-1}$	1.02	$7.70x10^{-1}$	$6.91x10^{-1}$
rs12456492	18	*RIT2*	n.a.				$7.70x10^{-1}$	1.04	$4.60x10^{-1}$	$2.34x10^{-1}$
rs2823357	21	*USP25*	n.a.				$2.00x10^{-1}$	0.84	$3.42x10^{-1}$	$2.83x10^{-1}$

*Table 5.10. QTL of previously associated PD-risk SNPs using NEUROX and IMMUNOchip array systems. PD case v control column shows p values for association between SNP and PD and odds ratio (OR) indicates effect direction. SNP vs ccf-mtDNA copy number column shows p values at BL and 36 months (V08). CHR is chromosome, BP is base pair position, n.a. indicates absence of SNP from array. * indicates significance at p<0.05, ** indicates significance at p<0.01.*

Analysis was further extended to unbiased QTL, utilising all NeuroX and Immunochip array variants to try and identify associations between SNP genotype and phenotype (ccf-mtDNA copy number level).

Power calculation

Assuming a mean difference in ccf-mtDNA of >20% between individuals (based on published data (Pyle *et al.*, 2015b) and the data presented in Chapter 3) and a SNP minor allele frequency of >0.10, this QTL has ~20% power to detect a significant association, with an effect size of >1.8, between ccf-mtDNA copy number and variants at baseline and 36 months under an additive model (power calculation conducted using Quanto (Gauderman, 2002)). Therefore, given the discovery nature of this experiment and the limited samples available for analysis, significance was set at $p<0.05$, under the understanding that any significant result would need to be replicated in an independent cohort.

ii) *Novel SNP associations to PD status and ccf-mtDNA copy number*

Initial analysis was performed assessing relationships between SNP genotype and PD status (Figure 5.12, A Immunochip and Figure 5.13, A NeuroX), identifying 9 significant SNPs ($p<0.05$). Two SNPs (rs2290402 and rs1397596) were associated with a 2-fold increase in PD risk, whereas the other 7 SNPs were found to protect against PD (Table 5.11). In addition, 4 of the 9 SNPs identified to be associated with PD status, were also associated with ccf-mtDNA copy number at $p<0.05$ significance level (Table 5.11).

Variant ID	CHR	Gene	Immunochip Array				NeuroX Array			
			PD cases v Control		SNP vs ccf-mtDNA copy number		PD cases v Control		SNP vs ccf-mtDNA copy number	
			p	OR (95%CI)	Baseline p	36 months p	p	OR (95%CI)	Baseline p	36 months p
1kg_2_207267535	2	*DYTN*	$6.66x10^{-7}$	0.03	$5.52x10^{-1}$	$2.29x10^{-3}$	n.a.			
rs2290402	4	*GAK/TMEM175/DGKQ*	$5.90x10^{-6}$	2.70	$5.47x10^{-1}$	$4.33x10^{-1}$	$1.10x10^{-6}$	2.63	$5.85x10^{-1}$	$4.29x10^{-1}$
rs458006	5	*NDUFS6/IRX2*	$1.23x10^{-5}$	0.46	$9.52x10^{-1}$	$1.39x10^{-1}$	n.a.			
rs2200204	11	*DLG2*	$4.09x10^{-6}$	0.39	$1.43x10^{-1}$	$2.23x10^{-1}$	n.a.			
rs1397596	11	*OR10A6/OR10A3*	$1.14x10^{-5}$	2.49	$7.55x10^{-1}$	$1.71x10^{-1}$	n.a.			
exm259930	2	*CPO*	n.a.				$2.57x10^{-6}$	0.04	$5.55x10^{-1}$	$1.98x10^{-3}$
exm2269661_ver2	3	*Unknown*	n.a.				$3.88x10^{-6}$	0.38	$4.31x10^{-1}$	$1.07x10^{-1}$
exm319695_ver2	3	*Unknown*	n.a.				$4.57x10^{-6}$	0.07	$6.45x10^{-1}$	$7.26x10^{-4}$
exm2271626	11	*TENM4*	n.a.				$5.08x10^{-6}$	0.16	$1.15x10^{-1}$	$2.06x10^{-4}$

Table 5.11. Top 'hits' for association to Parkinson's disease using the Immunochip and Neurox array, including variant ID, chromosome locus and gene name. p values are present for case versus control and for SNP versus ccf-mtDNA copy number. Odds ratio (OR) shows direction of association. n.a. present when SNP was not present on array. Red p values are significant.

iii) *Novel SNP association to ccf-mtDNA copy number*

QTL analysis was then performed twice for each time point (BL and 36 months); once as a linear regression of ccf-mtDNA copy number against SNPs, and a second time with status as a covariate. Results from these analyses were identical suggesting that PD status did not affect association between SNP genotype and ccf-mtDNA copy number.

A number of variants were found to be associated with ccf-mtDNA copy number (Table 5.11), highlighted on Manhattan plots; three significant SNPs were reported on the Immunochip array at BL (Figure 5.12, B) and 36 months (V08, Figure 5.12, C), four were reported on the NeuroX array at baseline (Figure 5.13, B) and two at V08 (Figure 5.13, C).

Immunochip Array

Of the six significant SNPs identified by the Immunochip array, none were found to be associated with PD (Table 5.12, *PD cases vs Controls, p*). Two SNPs (rs662776, Chr8 (Figure 5.12, B) and rs2963114, Chr5 (Figure 5.12, C)) were in uncharacterised regions with unknown functions. One was found to be associated with calcium signalling (Chr12, Figure 5.12, C), whilst the other three significant SNPs (rs7555149, chr1, imm_20_44200345, Chr20 (Figure 5.12, B) and rs4915296, Chr1 (Figure 5.12, C)) were found to be in genes associated with the immune response and inflammation.

Immunochip and NeuroX Array

One of the SNPs discovered on the Immunochip (rs4915296, Chr1, Figure 5.12, C) was also identified as significantly associated with ccf-mtDNA copy number by the NeuroX array (exm2263867, Chr1, Figure 5.13, C). Additionally, the SNP was also found to be significantly associated with PD on the NeuroX array.

NeuroX Array

Of the six significant SNPs identified by the NeuroX array (Table 5.12), two were uncharacterised (exm1578720, Chr21 (Figure 5.13, C) and exm995587, Chr12 (Figure 5.13, B)), whilst the other three were within genes that are not associated with mtDNA, mitochondrial function, or neurodegenerative disease.

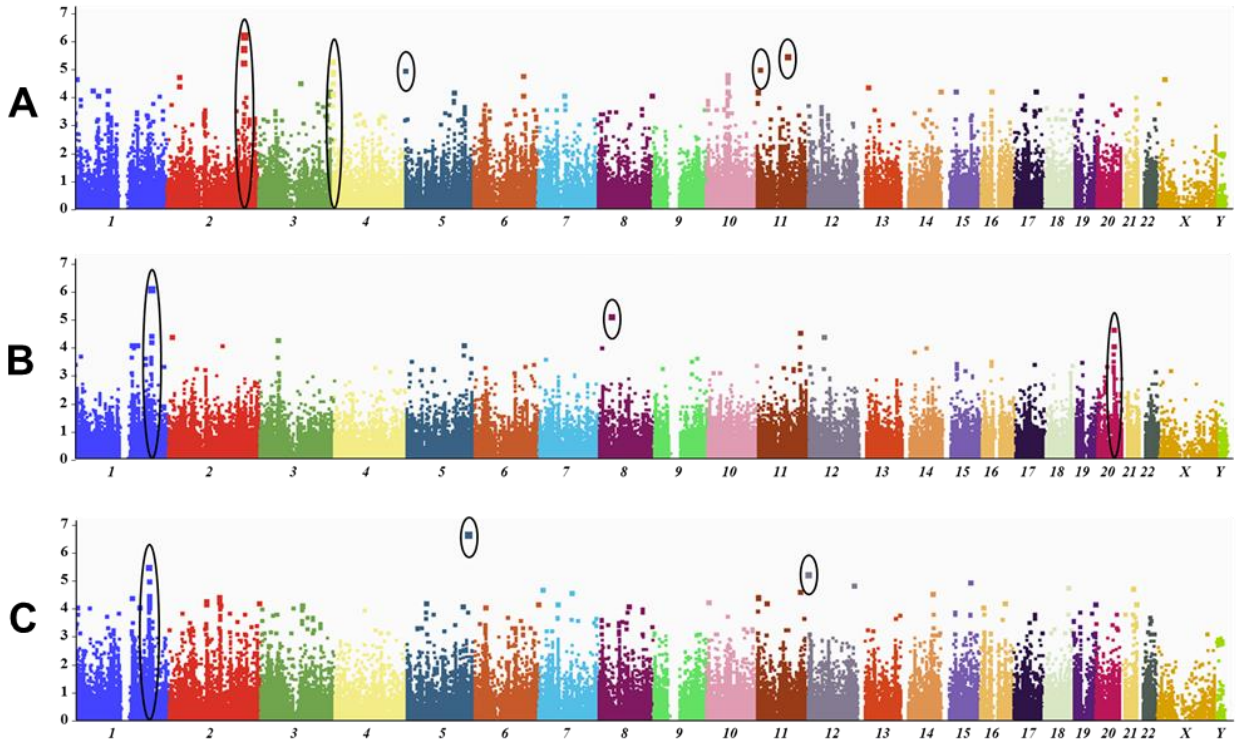

Figure 5.12. Manhattan plot of SNPs called on the Immunochip array showing p values for association of nuclear encoded SNPs to ccf-mtDNA copy number. A) Case vs control SNP association. B) Baseline QTL C) 36 month QTL. Black circles highlight significant SNPs. Chromosomes are different colours for clarity.

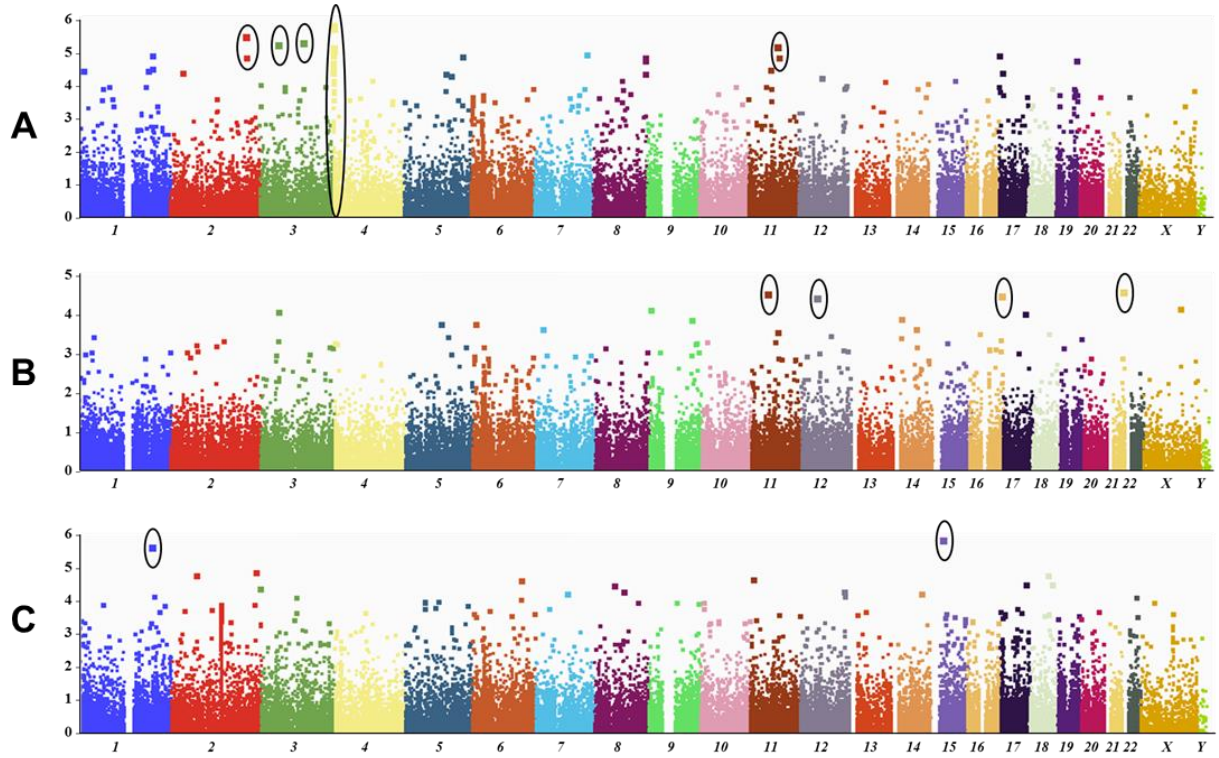

Figure 5.13. Manhattan plot of SNPs called on the NeuroX array showing p values for association of nuclear encoded SNPs to ccf-mtDNA copy number. A) Case vs control SNP association. B) Baseline QTL C) 36 month QTL. Black circles highlight significant SNPs. Chromosomes are different colours for clarity.

Variant ID	CHR	Gene	N Significant SNPs	Immunochip Array				NeuroX Array			
				PD cases v Control		SNP vs ccf-mtDNA copy number		PD cases v Control		SNP vs ccf-mtDNA copy number	
			(~50Kbp Flank)	p	OR (95% CI)	Baseline p	36 months p	p	OR (95% CI)	Baseline p	36 months p
rs7555149	1	*RASSF5/ EIF2D*	26	$3.27x10^{-1}$	1.17	$9.2x10^{-7}$	$9.2x10^{-2}$	n.a.			
rs4915296	1	*NEK7/ATP V1G3*	34/1	$8.87x10^{-1}$	0.96	$2.0x10^{-1}$	$4.0x10^{-6}$	$8.13x10^{-3}$	0.28	$1.99x10^{-1}$	$1.67x10^{-6}$
rs2963114	5	*LOC1053 77703*	1	$5.51x10^{-1}$	1.41	$9.4x10^{-1}$	$2.6x10^{-7}$	n.a.			
rs662776	8	*Intergenic*	1	$1.72x10^{-1}$	0.69	$9.1x10^{-6}$	$5.9x10^{-2}$	n.a.			
exm901502	11	*C11orf96*	3	n.a.				$5.32x10^{-1}$	1.65	$2.69x10^{-5}$	$9.74x10^{-1}$
imm_12_2240040	12	*CACNA1C*	46	$7.12x10^{-2}$	na	$1.2x10^{-1}$	$7.3x10^{-6}$	n.a.			
exm995587	12	*Intergenic*	5	n.a.				$4.52x10^{-1}$	0.72	$3.47x10^{-5}$	$2.33x10^{-1}$
exm1145538_ver3	15	*DPH6-AS1*	7	n.a.				$3.32x10^{-1}$	0.56	$2.71x10^{-1}$	$1.00x10^{-6}$
exm1266488	16	*PIEZ01*	1	n.a.				$7.58x10^{-1}$	0.84	$3.01x10^{-5}$	$3.46x10^{-1}$
imm_20_44200345	20	*CD40/CD H22*	44	$4.21x10^{-1}$	1.12	$2.6x10^{-5}$	$9.0x10^{-1}$	n.a.			
exm1578720	21	*Intergenic*	2	n.a.				$5.32x10^{-1}$	0.62	$2.41x10^{-5}$	$5.18x10^{-1}$

Table 5.12. Top hits for novel SNP associations to ccf-mtDNA copy number at baseline and 36 months, including variant ID, chromosome locus, gene ID and number of SNPs within a 50Kbp flank representing strong linkage disequilbrium. P values are present for case versus control analysis and for ccf-mtDNA association. Odds ratio (OR) determines the direction of association. Red p values are significant.

5.3.12 Extrapolation to serum

The results gained from this Chapter, Chapter 3 and Pyle *et al.* (2015b) indicated a potential utility for ccf-mtDNA copy number as a biological marker of therapeutic intervention and disease. However, due to the invasive, potentially complicated nature of CSF collection, blood based biomarkers are emerging as a more attractive and practical alternative (Blennow, 2017). Therefore, CSF-matched serum samples were requested from the original ICICLE-PD cohort to assess if ccf-mtDNA copy number level in the serum reflected trends observed with the CSF. The total serum cohort included 213 samples consisting of 73 controls and 140 PD patients (described in Section 2.1.1 *Serum samples*). Gender and age of PD cases and controls were matched (Table 5.13) and neither gender, age nor disease duration correlated to serum ccf-mtDNA copy number (Table 5.14, $p>0.05$).

Serum was observed to have significantly lower ccf-mtDNA copy number than lumbar CSF (Figure 5.14, A) (mean ccf-mtDNA copy number in 49 matched serum and lCSF samples were 26 copies/µl and 132 copies/µl, respectively, Mann Whitney U $p<0.0001$) and there was no correlation present between matched serum and lCSF ccf-mtDNA copy number (Pearson's correlation, $p>0.05$).

Furthermore, comparison of serum ccf-mtDNA copy number between PD cases and controls revealed a significant reduction in ccf-mtDNA copy number in PD cases (Figure 5.14, B) (mean serum ccf-mtDNA in 73 controls was 63 copies/µl compared to 33 copies/µl in 140 PD cases, Mann Whitney U $p=4.84x10^{-2}$). Similar to previous work (Section 5.3.2, Chapter 3 and 4) a minority of samples had serum ccf-mtDNA with deleted portions (>10%, 17/73 controls and 3/140 PD cases), however, there was no significant difference in deletion level between PD cases and controls (Mann Whitney U $p>0.05$).

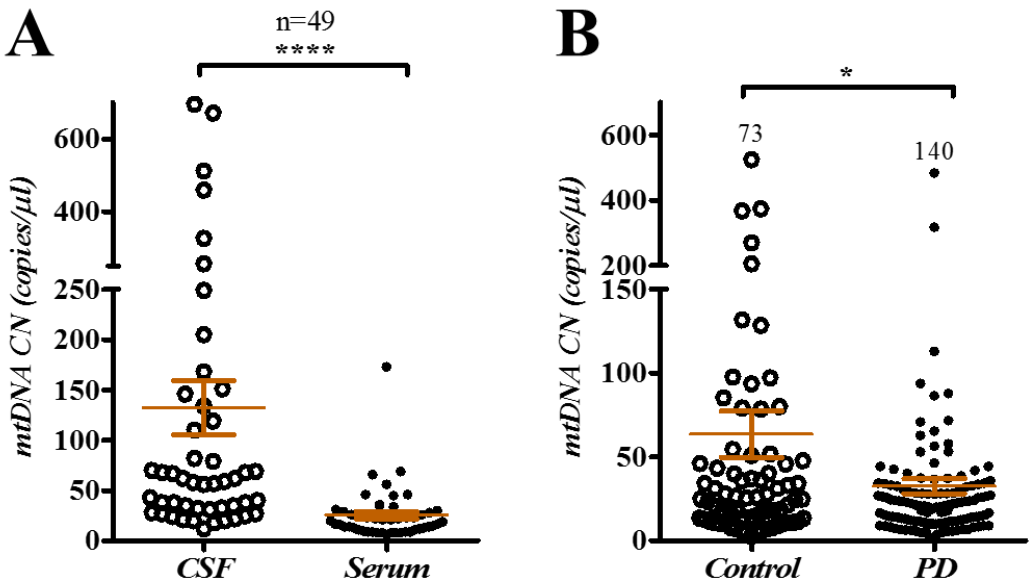

*Figure 5.14. Comparison of serum ccf-mtDNA copy number. (A) shows difference between serum and CSF ccf-mtDNA copy number levels in 49 matched participants. (B) shows comparison between PD cases and controls. Orange bars indicate mean +SEM. * indicates significance at p<0.05, ****p<0.0001.*

MoCA and MMSE scores (indicating cognitive function) were significantly lower for PD cases compared to controls (Table 5.13), however scores did not correlate to ccf-mtDNA copy number (Table 5.14). MDS-UPDRS scores and serum alpha synuclein levels were only available for PD cases and did not correlate to ccf-mtDNA copy number (Table 5.14). Levodopa equivalent dose (LEDD) was not significantly correlated to ccf-mtDNA copy number (Pearson's, $p>0.05$), and there was no difference in ccf-mtDNA copy number between treated (n=18) and non-treated PD (n=114) (Mann Whitney U $p>0.05$). Serum brain derived neurotrophic factor (BDNF) levels were elevated in PD cases compared to controls, but not significantly, and were found to significantly correlate to ccf-mtDNA copy number (r^2=-0.34, Pearson's $p<0.001$).

Variable	Controls (73)	PD cases (140)	Case vs Control (p)
Gender (M/F)	41/31	92/46	-
Age (years)	67.7±8	66.4±10	$4.58x10^{-1}$
Disease duration (months)	-	6±74	-
MoCA	27±3	25±4	$1.00x10^{-4}$ ***
MMSE	29±1	28±1	$3.00x10^{-3}$ **
MDS-UPDRS	-	26±11	-
Alpha Synuclein (pg/mL)	-	3222±4904	-
BDNF (pg/mL)	915±840	2461±1177	$4.43x10^{-01}$

*Table 5.13. Table of ICICLE serum cohort demographics (gender, age, disease duration), phenotypic scores (Montreal cognitive assessment (MoCA), Mini-mental state exam (MMSE) and Movement disorders- Unified PD rating scale (MDS-UPDRS)) and protein levels (alpha synuclein and brain derived neurotrophic factor (BNDF)). Values are mean±standard deviation, except gender (male n/female n) and case versus control (Mann Whitney U, p values; ** indicates significance at p<0.01, *** p<0.001).*

Variable vs ccf-mtDNA copy number	Whole Cohort	Control	PD
Male vs Female	$29.49x10^{-1}$	$2.04x10^{-1}$	$7.40x10^{-2}$
Age	$3.91x10^{-1}$	$2.82x10^{-1}$	$8.73x10^{-1}$
Disease duration	-	-	$3.49x10^{-1}$
MoCA	$8.33x10^{-1}$	$6.04x10^{-1}$	$7.33x10^{-1}$
MMSE	$3.07x10^{-1}$	$4.55x10^{-1}$	$9.71x10^{-1}$
MDS-UPDRS	-	-	$5.02x10^{-1}$
Alpha Synuclein	-	-	$8.27x10^{-1}$
BDNF	$2.00x10^{-3}$ **	$2.79x10^{-1}$	$3.00x10^{-3}$ **
LEDD	-	-	$5.64x10^{-1}$

*Table 5.14. Pearson's correlation of ICICLE serum cohort demographics (gender, age, disease duration), phenotypic scores (Montreal cognitive assessment (MoCA), Mini-mental state exam (MMSE) and Movement disorders- Unified PD rating scale (MDS-UPDRS)), protein levels (alpha synuclein and brain derived neurotrophic factor (BNDF)) and daily levodopa equivalent dose (LEDD) to serum ccf-mtDNA copy number. Values are p (Mann Whitney U, p values; ** indicates significance at p<0.01).*

5.4 Discussion

Previous work in Chapter 3 and in preliminary data (ICICLE-PD, Pyle *et al.* (2015b)) indicated that CSF ccf-mtDNA copy number is significantly reduced in PD cases compared to controls and this had >80% predictive validity as a biomarker for PD. This chapter aimed to validate the biomarker potential of CSF ccf-mtDNA copy number by performing a replication study utilising a large, longitudinal cohort of well-characterised PD patients and controls (PPMI-PD cohort). At first glance, this study failed to significantly replicate previous findings, observing that although ccf-mtDNA copy number was lower in PD patients compared to controls, this was not robust enough to significantly distinguish PD from controls.

However, analysis of this larger, deeply phenotyped cohort revealed several confounders, including PD-specific treatment and comorbidity, which may impact ccf-mtDNA copy number levels and could therefore explain the disparity in findings reported here and in previous work (Chapter 3, and Pyle *et al.* (2015b).

5.4.1 Ccf-mtDNA copy number is not associated with sporadic PD but is associated with pharmacological treatment of PD.

The major finding from this large scale replication study failed to reproduce initial findings and disproves the original hypothesis that CSF ccf-mtDNA copy number is a valid biomarker for sporadic PD. Alternatively, investigation of this large, well-characterised, longitudinal cohort revealed that PD treatment may in fact be the driving force behind the changes in CSF ccf-mtDNA copy number, in addition to PD status. As discussed in Section 5.3.8, in contrast to the ICICLE-PD cohort, all of the PPMI-PD cohort were treatment naïve at baseline, with >90% beginning treatment sometime between baseline and the 36 month follow up (V08). Stratification of the 36 month cohort into treated PD patients, untreated PD patients and controls, indicated that reduced ccf-mtDNA copy number was correlated with the use of PD medication and to some extent the treatment duration, with ccf-mtDNA levels dropping within the first 18 months and subsequently rising as treatment continues. Not only does this replicate findings reported in preliminary work (Pyle *et al.*, 2015b) (where >90% of PD patients were receiving treatment at baseline and 18 month follow up), but it also presents the novel idea that PD pharmacological intervention could potentially modulate ccf-mtDNA copy number, a hypothesis which has been suggested

previously in studies of HIV (Payne *et al.*, 2011) and depression (Lindqvist *et al.*, 2018).

The depletion in ccf-mtDNA copy number was most prominent in PD patients receiving Levocarb (LDOPA) and combination treatment, but was also present in patients receiving MAOBIs.

LDOPA and MAOBIs could possibly effect ccf-mtDNA copy number levels by their direct, but opposing, actions on mitochondrial function. LDOPA has been shown to increase the production of ROS, thereby leading to the promotion of mitochondrial damage (Soliman *et al.*, 2002; Dorszewska *et al.*, 2014) which could potentially reduce mtDNA copy number and subsequently ccf-mtDNA export. Whereas MAOBIs are reported to reduce ROS production and enhance mitochondrial function (Youdim *et al.*, 2006), thereby potentially reducing ccf-mtDNA copy number through retention of mtDNA by selective survival processes or anti-apoptotic means.

Alternatively, the effect of PD medication on ccf-mtDNA copy number may be attributable to their impact on dopamine bioavailability. LDOPA and MAOBIs exert their therapeutic action by crossing the blood brain barrier (BBB) and increasing the bioavailability and prolonging the activity of dopamine in the striatum (Connolly and Lang, 2014): LDOPA is converted to dopamine by dopa decarboxylase (Blaschko, 1942), whilst MAOBIs block MAOB preventing the breakdown of dopamine (Fernandez and Chen, 2007) (Figure 5.15). Differently, dopamine agonists do not alter the levels of dopamine in the striatum, instead they simply cross the BBB and mimic dopamine action by binding to and activating dopamine receptors (Connolly and Lang, 2014)(Figure 5.15).

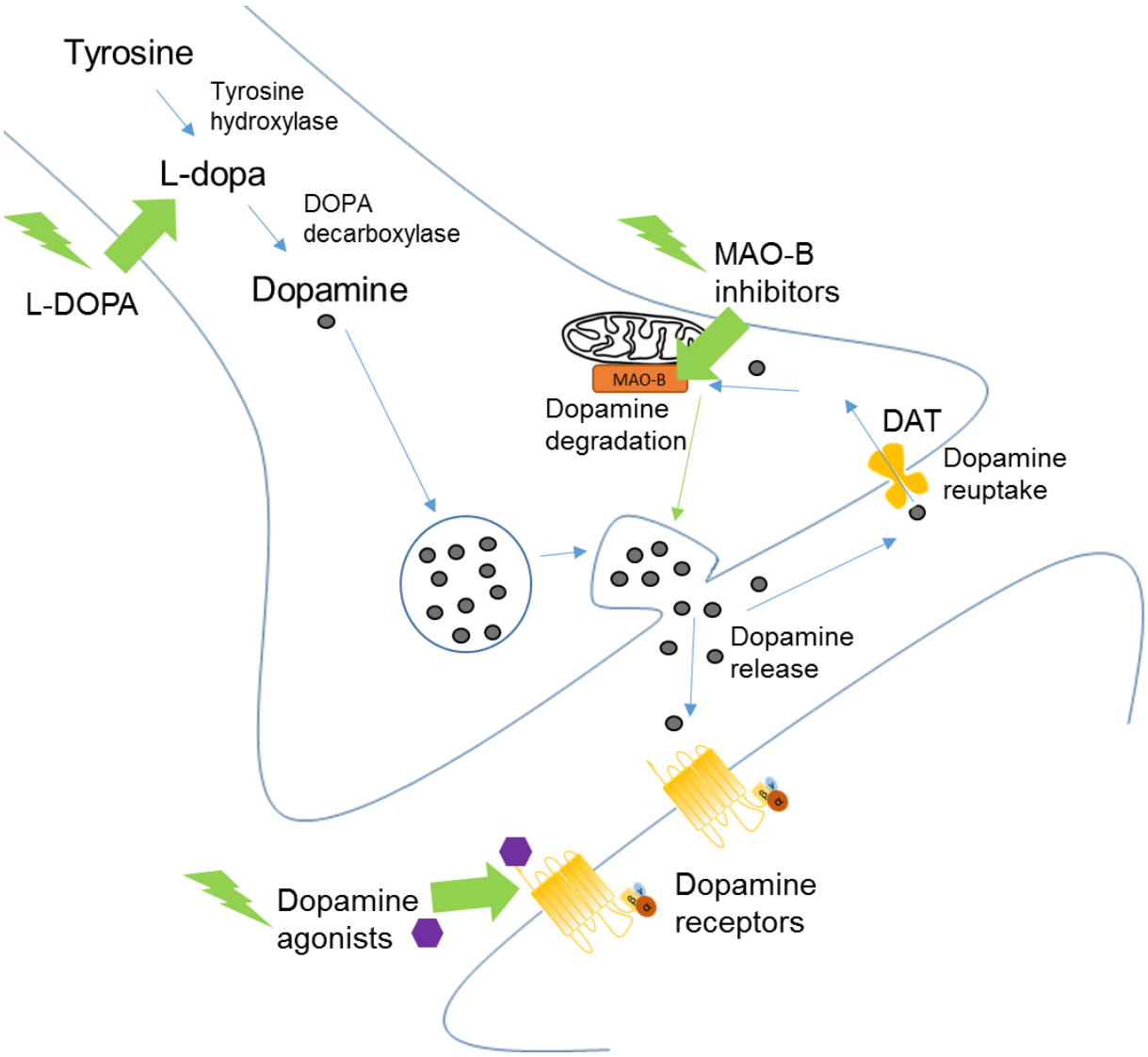

Figure 5.15. Schematic of PD treatment action in the neuron. All three medications are administered orally and circulate in the blood stream before crossing the blood brain barrier to exert their effects. Exogenous L-DOPA (levodopa) is converted to dopamine in the presynaptic neuron by dopa decarboxylase to replenish dopamine stores in presynaptic vesicles. Monoamine oxidase B inhibitors bind to MAOB and prevent the breakdown of dopamine, thereby prolonging its action in the synaptic cleft. Dopamine agonists directly target dopamine receptors in the post-synaptic neuron to mimic endogenous dopamine and reverse the dopaminergic deficit.

As dopamine agonists did not have the same effect as LDOPA, MAOBIs and combination treatment on ccf-mtDNA copy number, it could be postulated that the modulation of ccf-mtDNA copy number is specific to drugs that alter dopamine levels within the brain, possibly owing to the neurotoxic auto-oxidative nature of exogenous cytosolic dopamine (Munoz *et al.*, 2012; Hauser and Hastings, 2013). Numerous in vitro and in vivo studies have shown exogenous dopamine to induce apoptosis (Luo

et al., 1998; Jones *et al.*, 2000) and contribute to neurodegeneration (Chen *et al.*, 2008) through its actions of mitochondrial dysfunction (Bondi *et al.*, 2016), oxidative stress (ROS production) (Asanuma *et al.*, 2003; Chen *et al.*, 2008) and mtDNA copy number depletion (Naydenov *et al.*, 2010). Additionally, conditions such as Schizophrenia (Kageyama *et al.*, 2018; Shivakumar *et al.*, 2018) and drug addiction (Feng *et al.*, 2013) which are associated with excessive dopamine levels within the brain also show reduced neuronal mtDNA copy number.

This evidence therefore suggests that PD drug treatments that increase dopamine levels within the brain may modulate ccf-mtDNA copy number by altering the amount of mtDNA available for release from the cellular mtDNA pool. This is further supported by work reporting depletion of mtDNA copy number in peripheral blood lymphocytes and Substantia Nigra tissue of end-stage PD patients (Pyle *et al.*, 2015a), potentially attributed to chronic PD treatment.

In addition, results regarding the duration of treatment indicate a transient effect of PD medication on ccf-mtDNA copy number, where PD patients on treatment for less than 18 months show a significant depletion in ccf-mtDNA copy number compared to controls (Figure 5.16, A). Patients receiving treatment for longer than 18 months appear to separate into two groups (albeit, not clearly defined); one where ccf-mtDNA copy number increases (shown in blue in Figure 5.16) and the other where ccf-mtDNA copy number remains depleted (shown by purple in Figure 5.16). This may be explained by the slowly progressing nature of PD due to the lack of disease modifying treatments and the decreasing tolerability of LDOPA and other PD medications overtime, resulting in dyskinesia's, detrimental side effects and progressing symptoms (Fahn, 2006). This could subsequently cause exacerbation of non-dopaminergic neuronal death (Stansley and Yamamoto, 2014), possibly leading to increased ccf-mtDNA copy number in line with the cell-death theory of ccf-mtDNA export.

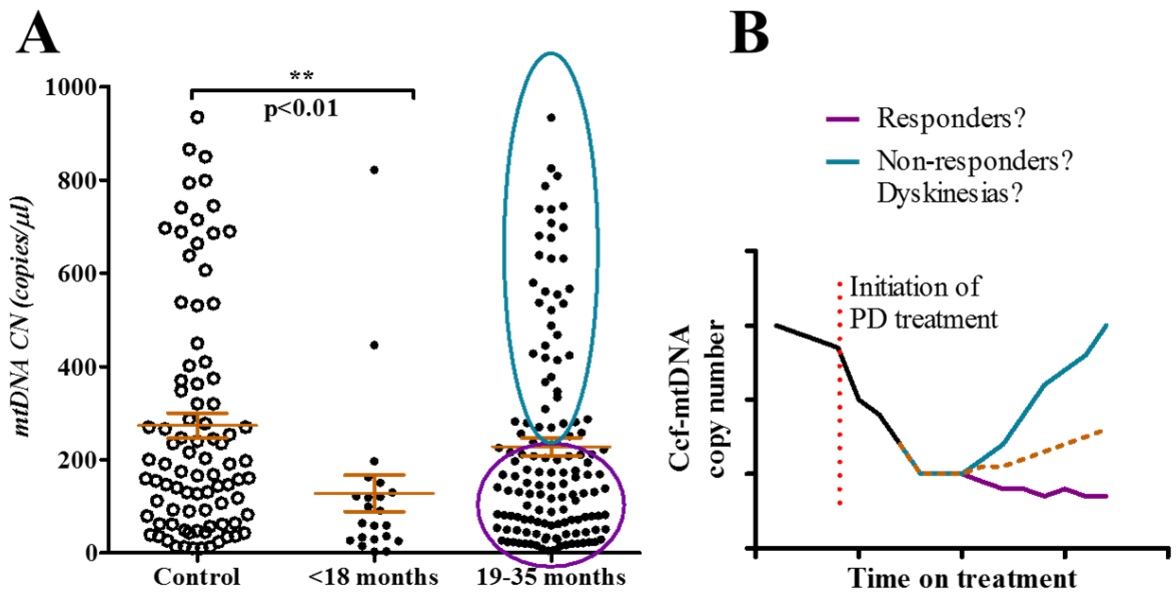

Figure 5.16. Summary of the effect of duration of treatment on ccf-mtDNA copy number. A) shows data from Section 5.3.8 adapted to show two separate groups for PD patients on treatment for 19 months and longer; those with increased ccf-mtDNA copy number (blue circle) and those who remain depleted (purple circle). B) shows a model graph of ccf-mtDNA copy number against treatment duration, indicating potential mechanisms behind the why ccf-mtDNA increasing for some PD patients but remains low for others.

5.4.2 Correlation of phenotypic scores to ccf-mtDNA copy number.

Phenotypic tests were carried out by the PPMI study and scores were available in the PPMI database. Analysis of depression (GDS), motor dysfunction (MDS-UPDRS), autonomic dysfunction (SCOPA-AUT), daily activity (PASE) and cognitive impairment (MoCA) revealed typical trends for PD, with PD patients showing significantly higher depression scores (Reijnders *et al.*, 2008), motor dysfunction (Holden *et al.*, 2018) and autonomic dysfunction (Damian *et al.*, 2012). PASE scores are generally lower in PD patients compared to controls (Mantri *et al.*, 2018), however, this was not observed in the PPMI-PD cohort. Furthermore, MoCA scores indicated mild cognitive impairment in both PD cases and controls with no significant difference between mean scores.

Despite significant differences in important phenotypic severity scores between PD cases and controls, there were no significant associations between these scores and ccf-mtDNA copy number in PD cases, similar to previous literature (Pyle *et al.*,

2015b). Interestingly, there were two significant associations observed in controls, between MoCA and MDS-UPDRS total and ccf-mtDNA copy number.

In Chapter 3, both controls and NDD cases showed an elevation in ccf-mtDNA copy number in cognitively impaired individuals (MMSE ≤23) compared to cognitively normal (MMSE ≥24), whereas this trend is only present for controls in the PPMI-PD cohort. The discrepancy between these findings may relate to the difference in prevalence of cognitive impairment in the NDD group (NDD-vCSF cohort) and the PD group (PPMI-PD cohort), as >80% of the NDD group were cognitively impaired, compared to only 22% of the PD group. Controls in both NDD-vCSF and PPMI-PD cohorts had an incidence of ~20% cognitive impairment. This could present the notion that in early stage NDD (i.e. the PPMI-PD cohort) pharmaceutical intervention or presence of disease may lower ccf-mtDNA copy number, however, as the disease progresses and cognitive impairment worsens ccf-mtDNA copy number rises and may overcome the depletive effect of medications or disease on ccf-mtDNA copy number.

The correlations between MDS-UPDRS (II and total score) and ccf-mtDNA copy number in controls are largely uninformative as unsurprisingly, scores given by control participants are extremely low and do not reach the threshold to indicate any motor disability (control mean MDS-UPDRS total 5/199) (Goetz *et al.*, 2008).

In addition, it is important to note that although PPMI PD patients showed differences in phenotypic severity compared to controls, the scores gained for the PD patients indicated particularly mild, early stage PD patients with minor motor or cognitive dysfunction. Therefore, this could make it difficult to generalise findings to the wider PD population.

5.4.3 CSF protein levels are associated with PD, but not ccf-mtDNA copy number.

In keeping with previous reports, CSF α-synuclein (Tokuda *et al.*, 2006; Mollenhauer *et al.*, 2008; Hong *et al.*, 2010; Mollenhauer *et al.*, 2011; Tateno *et al.*, 2012), amyloid beta (Alves *et al.*, 2010; Montine *et al.*, 2010; Siderowf *et al.*, 2010), total tau and phosphorylated tau (Mollenhauer *et al.*, 2011) were observed to be reduced in the CSF of PD cases compared to controls. In addition, these proteins were not found to correlate to ccf-mtDNA copy number levels in the entire cohort, or when stratified by

PD cases and controls, supporting previous work indicating no correlation between ccf-mtDNA and amyloid beta, total tau, phosphorylated tau (Pyle *et al.*, 2015b; Cervera-Carles *et al.*, 2017), and α-synuclein ((Pyle *et al.*, 2015b) and Chapter 3).

In addition, TEM was performed on two CSF samples, similarly to Chapter 3, and indicated the absence of any organellar or mitochondrial structures, supporting the idea that ccf-mtDNA circulates in the CSF outside of the mitochondrion.

5.4.4 Secondary phenotypes and comorbidities underlie sample heterogeneity and create huge variance in ccf-mtDNA copy number levels.

As described in Section 5.3.9 the PPMI database contained well-characterised clinical history for all participants, including comorbidities and secondary phenotypes. With over 500 different comorbidities in the database, limitations were applied to investigate only the most common (i.e. >10% of the population; hypertension, hypercholesterolemia and arthritis), those associated with PD (i.e. GERD (Maeda *et al.*, 2013), constipation (Pedrosa Carrasco *et al.*, 2018), anxiety/depression (Kano *et al.*, 2011), insomnia (Gjerstad *et al.*, 2007)) and those with previous association with ccf-mtDNA copy number (i.e. cancer (Yu, 2012), CHD (Liu *et al.*, 2016), diabetes (Liu *et al.*, 2015)). As aging is associated with multimorbidity (Barnett *et al.*, 2012), it is not surprising that 65-70% of the PPMI-PD cohort had one or more secondary phenotype or comorbidity and although few significant differences were reported between groups, interindividual variability in ccf-mtDNA copy number was shown to be large (see Section 5.4.6).

Importantly, the trends observed at baseline, were supported by those reported in previous literature, showing a general increase in ccf-mtDNA copy number to be associated with anxiety/depression (Lindqvist *et al.*, 2018), CHD and diabetes (Liu *et al.*, 2015; Liu *et al.*, 2016) and cancer (Mehra *et al.*, 2007; Ellinger *et al.*, 2008; Zachariah *et al.*, 2008; Ellinger *et al.*, 2009; Kohler *et al.*, 2009; Ellinger *et al.*, 2012; Mahmoud *et al.*, 2015; Lu *et al.*, 2016). At 36 months later, these differences were no longer present and in some cases (i.e. anxiety/depression and cancer), were reversed. This is likely due to the commencement of PD treatment as this is observed to lower ccf-mtDNA copy number and therefore could potentially overcome or diminish the effect of elevated copy number associated with these conditions.

5.4.5 QTL analysis discovered novel associations between genetic variants and ccf-mtDNA, but not within known PD risk genes.

QTL analysis supports the notion that ccf-mtDNA copy number is not associated with PD, as somewhat predictably, none of the previously reported PD-related genes were found to be associated with ccf-mtDNA copy number. A small number of variants (nine) were found to be associated with ccf-mtDNA copy number in the whole cohort, and of the six characterised SNPs, three were found to be located in genes association with inflammation and the immune response; RASSF5 (rs7555149),(Volodko et al., 2014) CD40 (imm_20_44200345)(Rizvi et al., 2008) and NEK7 (rs4915296/exm2263867)(He et al., 2016). mtDNA and ccf-mtDNA has previously been described as a damage-associated molecular pattern (DAMP)(Gögenur et al., 2017), shown to be important in activating the immune response and promoting inflammation (Collins et al., 2004; Zhang et al., 2010; Nakayama and Otsu, 2018). Thus, the identification of significant genetic associations between known inflammatory genes and ccf-mtDNA copy number provide further support for this. In addition, two significant SNPs were in genes previously associated with cognitive function and psychiatric illness; CACNA1C, a voltage gated calcium ion channel, mutations in which are associated with schizophrenia (Bigos *et al.*, 2010) and DPH6-AS1, an RNA gene associated with cognitive decline (Li *et al.*, 2015). However, no SNPs were found to be associated with specific neurodegenerative diseases or mitochondrial genes.

Due to the *a priori* nature of this QTL analysis the generated p values are uncorrected, therefore, replication is needed in a large cohort to enable greater statistical power.

5.4.6 Ccf-mtDNA copy number is variable

Another important finding from this study was the large dispersion in ccf-mtDNA copy number within individuals of the same diagnostic group. A number of factors such as treatment type, treatment duration, comorbidities and phenotypic severity influenced ccf-mtDNA copy number and created large variance and overlap between ccf-mtDNA copy number of PD cases compared to controls. The inter-assay coefficients of variance were low, indicating that the high levels of inter-individual variability were

not an artefact of the qPCR assay. Rather, it is more likely that this variation is owing to the use of a large cohort, limited to an aged population and studying a disease with large biological and phenotypic heterogeneity (Lewis *et al.*, 2005). This likely explains the disparity between results reported here and in previous work (ICICLE-PD and Chapter 3) as previous cohorts lacked the size and deep clinical characterisation needed to observe this effect. Furthermore, a recent validation study by Cervera-Carles *et al.* (2017) supports this observation, as they report large variability in ccf-mtDNA copy number in a large replication cohort of AD patients compared to initial findings in a smaller cohort of AD (Podlesniy *et al.*, 2013).

Therefore, although ccf-mtDNA copy number is an attractive biomarker for disease, due to its ease of sampling and measurement, results from this validation study indicate limited utility of ccf-mtDNA copy number due to a lack of specificity and susceptibility to modulation by a number of confounding factors.

5.4.7 Extrapolation to Serum

Serum ccf-mtDNA is observed to be associated with various diseases (Zhong *et al.*, 2000; Kohler *et al.*, 2009; Yu, 2012; Yu *et al.*, 2012; Liu *et al.*, 2015; Mahmoud *et al.*, 2015; Lindqvist *et al.*, 2018) (Section 1.2.4) but has not been investigated in NDDs due to its peripheral localisation away from the CNS. However, due to the invasive and potentially complicated nature of CSF biopsy (Blennow, 2017), research is focusing on the identification of blood based biomarkers as more cost-effective, non-invasive alternatives. Additionally, CSF is filtered and reabsorbed into the venous blood in the subarachnoid space (Brinker *et al.*, 2014), therefore, serum may provide a peripheral measure reflecting CNS and CSF changes. Therefore, due to the interesting associations observed between CSF ccf-mtDNA copy number and PD in the NDD-vCSF, ICICLE-PD and PPMI-PD cohorts, CSF-matched serum samples were obtained from the ICICLE-PD cohort to assess ccf-mtDNA copy number.

Ccf-mtDNA copy number could be detected in the serum of PD cases and controls, however, this was at significantly lower levels than in the CSF and did not correlate to CSF ccf-mtDNA copy number. This low level serum ccf-mtDNA copy number has been reported in numerous studies with typical ranges between 1-100 copies/µl (Zhong *et al.*, 2000; Kohler *et al.*, 2009; Yu *et al.*, 2012; Liu *et al.*, 2015; Mahmoud *et al.*, 2015; Lindqvist *et al.*, 2018) compared to 1-1000copies/µl in the lCSF (Podlesniy *et al.*, 2013; Pyle *et al.*, 2015b; Perez-Santiago *et al.*, 2016; Podlesniy *et al.*, 2016b;

Cervera-Carles *et al.*, 2017; Pérez-Santiago *et al.*, 2017; Varhaug *et al.*, 2017; Leurs *et al.*, 2018) and is in-keeping with NDD-associated protein abundance (i.e. tau 2-300pg/mL in the CSF (Sjogren *et al.*, 2001) but <5pg/mL in the serum (Zetterberg *et al.*, 2013)). This suggests that serum composition does not directly reflect that of the CSF, potentially owing to the dilution of molecules as they move through circulation, or the filtration of molecules out of the CSF or blood.

Interestingly, although serum ccf-mtDNA copy number did not associate with that of matched CSF, it was observed to exhibit the same trend (Pyle *et al.*, 2015b), showing significantly reduced ccf-mtDNA copy number in PD cases compared to controls. This therefore raises the possibility that serum ccf-mtDNA copy number may be a potential blood-based biomarker for PD. However, this was limited by the variation within disease groups (coefficient of variance was 186% for controls and 164% for PD cases) and subsequently serum ccf-mtDNA did not have the sensitivity or specificity to distinguish PD patients from controls (with ROC curve analysis generating an area under the curve of 0.58). Furthermore, unlike CSF ccf-mtDNA copy number, PD treatment was not observed to affect serum ccf-mtDNA copy number and ccf-mtDNA copy number did not correlate to levodopa equivalent dose. However, all participants were receiving levodopa carbidopa combination (Levocarb), thereby blocking the metabolism of L-DOPA into dopamine in the periphery (Nutt *et al.*, 1985), hence an effect in the blood may not be expected.

Finally, similar to CSF ccf-mtDNA copy number (Pyle *et al.*, 2015b; Cervera-Carles *et al.*, 2017), no phenotypic scores correlated to serum ccf-mtDNA copy number. However, unexpectedly serum BDNF levels were significantly negatively correlated to ccf-mtDNA copy number in PD cases. This could provide support for the idea that ccf-mtDNA results from cell death, as elevated BDNF levels are known to reduce apoptosis and inflammation and promote mitochondrial function *in vitro* (Xu *et al.*, 2017) and *in vivo* (as reviewed in Markham *et al.* (2014)).

5.4.8 Further work

The work carried out in this Chapter has revealed a novel association for ccf-mtDNA copy number with PD medication, alongside the potential association to PD status (Chapter 3 and Pyle *et al.* (2015b)). To further assess this relationship, *in vitro* methods could be employed, using cell culture to observe the direct effect of PD medication on neuronal populations. Previous work has indicated that induction of

cellular stress leads to the expulsion of mitochondria and mtDNA (Melentijevic *et al.*, 2017) into culture medium. Therefore, in line with this, future work could involve dosage of neuronal culture with various PD medication and analysis of the culture medium to assess mitochondrial and mtDNA content.

In addition, the PPMI study has collected CSF and serum samples at 6 months intervals from baseline to 60 months. Therefore, it may be useful to request samples at more intervals to give a more complete image of how ccf-mtDNA copy number changes over time. This would also enable accurate investigation into the effect of treatment on ccf-mtDNA copy number, as CSF taken at the nearest time point following initiation of PD treatment could be analysed and ccf-mtDNA copy number could be followed more specifically. Furthermore, as the ICICLE-PD serum cohort indicated a significant depletion of serum ccf-mtDNA copy number in PD cases compared to controls, it would be interesting to assess this within the PPMI cohort, as a blood based biomarker would have far greater clinical practicality than CSF.

Furthermore, it may be interesting to compare ccf-mtDNA within different PD phenotypes. The early-stage of the PPMI-PD samples somewhat restricted this analysis, as the majority of patients present with only bradykinesia and tremor, but few other symptoms. However, as sample collection is on-going, the latter stages may have more exaggerated phenotypes (i.e. more severe MDS-UPDRS or MoCA scores) and thus could reveal more pronounced phenotypic differences in ccf-mtDNA copy number. Additionally, prodromal features of PD may also link to ccf-mtDNA copy number, therefore, a cohort such as the Parkinson's Repository of Biosamples and Network Datasets could be employed to assess this.

Another important avenue to investigate is the potential for ccf-mtDNA as a biological outcome measure for treatment. Currently, the most common outcome measure for PD medication is the MDS-UPDRS (Ramaker *et al.*, 2002; Holden *et al.*, 2018). Published data from the PPMI study indicates that MDS-UPDRS score is associated with dopaminergic therapy, where PD patients receiving treatment showed a smaller change in their MDS-UPDRS scores over time compared to untreated PD patients (Simuni *et al.*, 2018). Therefore, it would be interesting to correlate ccf-mtDNA copy number to MDS-UPDRS score over time, with the use of more samples at a range of time points. To assess this fully, patient information regarding tolerability, side effects, dyskinesia, 'on-off' symptoms and overall response to treatment would also be useful for further comparisons to ccf-mtDNA copy number.

5.5 Conclusion

Replication of the ICICLE-PD study in a large-scale longitudinal validation cohort has revealed that ccf-mtDNA copy number is not a valid biomarker for PD status. However, this study does indicate a novel effect of PD medication in the significant depletion of CSF ccf-mtDNA copy number. Further investigation of this finding is warranted to establish whether this could serve as an important biological outcome measure for PD treatment. Furthermore, alongside medication effects on ccf-mtDNA copy number, this work has revealed numerous other factors, including phenotypic heterogeneity and comorbidities, that can influence ccf-mtDNA copy number leading to large dispersion and interindividual variation. This further limits the utility of ccf-mtDNA copy number as a biological marker as it is lacks robust specificity and is susceptible to influence from various factors.

Chapter 6. General Discussion

6.1 General Discussion

The discovery of sensitive and specific, novel fluid-based biomarkers for age-related neurodegenerative disease (NDD) are crucial for better diagnosis and subsequent disease treatment and management. Currently, clinical diagnosis has good accuracy, but heavily relies on presentation of cardinal symptoms of disease and still requires confirmation neuropathologically (Rachakonda *et al.*, 2004). Reliance on post-symptomatic diagnosis for NDD generally indicates a large degree of neuronal death has already occurred, meaning treatment can only manage symptoms rather than slow or reverse the disease course. Therefore, investigation into prodromal biomarkers of NDDs are crucial, particularly in our increasingly aged population, to enable earlier diagnosis and in the development of disease-modifying therapies.

Is ccf-mtDNA copy number a biomarker for neurodegenerative disease?

Preliminary work in Parkinson's disease (PD) (Pyle *et al.*, 2015b), Alzheimer's disease (AD) (Podlesniy *et al.*, 2013), relapsing remitting multiple sclerosis (RRMS) (Varhaug *et al.*, 2017) and progressive MS (PMS) (Leurs *et al.*, 2018), raised the possibility that ccf-mtDNA copy number may serve as a biomarker for neurodegenerative and neuroinflammatory conditions. This thesis aimed to evaluate this hypothesis through an in-depth analysis of ccf-mtDNA copy number in a broad range of neurodegenerative conditions (Chapter 3), a cohort of PMS patients (Chapter 4) and a large independent validation cohort of PD patients (Chapter 5).

Analysis of ccf-mtDNA copy number in the post-mortem ventricular CSF (vCSF) revealed significant disease-specific associations between PD (Chapter 3) and PMS (Chapter 4) patients and low ccf-mtDNA copy number, but failed to support an association to broad NDD. This disagrees with my original hypothesis, and suggests that reduced ccf-mtDNA copy number is a phenomenon that may be restricted to specific NDDs; likely those that have established mitochondrial and inflammatory components (i.e. PD and PMS (Mahad *et al.,* 2008; Winklhofer and Haass, 2010; Ferrari and Tarelli, 2011; Sliter *et al.,* 2018)).

With regard to PMS, the findings reported here (Chapter 4) are particular important as they focus on the prominent neurodegenerative processes underlying PMS, suggesting that low ccf-mtDNA copy number could be a biomarker of neurodegeneration in this condition. In addition, as previous literature suggests that

RRMS patients and early (ante-mortem) PMS patients have elevated ccf-mtDNA copy number (Varhaug *et al.*, 2017; Leurs *et al.*, 2018), this could potentially serve as a prognostic marker for MS, with the ability to distinguish individuals with a more neurodegenerative profile from those with an inflammatory course of disease. Therefore, ccf-mtDNA could be useful in predicting the conversion from a clinically isolated syndrome (CIS) to RRMS, and then to PMS. Currently, there are no biological markers in clinical utility that can distinguish these conditions, therefore, this could have major implications in the future management and treatment of MS. To investigate this fully, future work is warranted in a longitudinal population of MS patients, potentially following individuals over time from initial CIS or MS diagnosis through to end-stage PMS with sampling of lumbar CSF at regular intervals to assess changes in ccf-mtDNA copy number.

The observation of low vCSF ccf-mtDNA copy number in PD patients (Chapter 3), supports previous work by Pyle *et al.* (2015) and independently validates the biomarker potential of ccf-mtDNA copy number in PD. However, due to small sample size, this conclusion still lacked population validity and was limited by post-mortem effects.VCSF is the closest body fluid to the lesion site in NDD and PMS and therefore is more sensitive than lumbar CSF (lCSF) and serum to detect biological markers (Mollenhauer, 2014; Jeromin and Bowser, 2017). However, the post-mortem nature of the vCSF limits this sensitivity in biomarker discovery as these samples are representative of end-stage disease rather than early onset or prodromal stages. Therefore, vCSF samples are highly useful in ‘proof of principle’ studies and are instrumental in disease mechanism analysis, however, subsequent work would be required in ante-mortem samples to validate findings in a clinical setting.

In hindsight it could have been useful to perform a power calculation prior to all experimental laboratory work to estimate the minimum required number of samples needed to predict a biomarker ability of ccf-mtDNA in NDD (as was performed for the PPMI-PD cohort). However, human CSF samples are difficult to obtain in large numbers and due to the time restrains of a PhD project on-going recruitment to increase the cohort size was unpredictable (due to post-mortem nature) and generally not fruitful. Therefore, experiments and analysis were carried out and conclusions were drawn acknowledging this limitation.

The work performed in Chapter 5, aimed to address these limitations, utilising a large, longitudinal cohort of well characterised PD patients and matched controls to validate the results gained in Chapter 3 and Pyle *et al.* (2015). At first glance, results from this large-scale study failed to significantly replicate previous work, showing that although ccf-mtDNA copy number was depleted in PD cases compared to controls, this did not reach statistical significance in this large population. However, subsequent work in Chapter 5 revealed a great deal of dispersion in ccf-mtDNA copy number in both PD patients and controls. This variation, which is in-line with other large scale CSF biomarker replication studies (Ewers *et al.,* 2015; Cervera-Carles *et al.,* 2017) was likely a result of the use of a large, aged, biological and phenotypically heterogeneous cohort.

Due to the well-characterised nature of the PPMI-PD cohort, subsequent investigations were carried out to explore the effects of subject heterogeneity, assessing the influence of secondary phenotypes, comorbidities and medications on ccf-mtDNA copy number. This analysis revealed significant associations to a number of secondary phenotypes (i.e. anxiety and depression and cognitive impairment (also seen in Chapter 3)) and comorbidities (i.e. cancer) in both PD patients and controls.

Most interestingly, this analysis also discovered a significant effect of PD medication. PD patients receiving dopamine-replacement treatments (i.e. levodopa and monoamine oxidase B inhibitors) were found to exhibit significantly lower ccf-mtDNA copy number than non-treated PD patients and controls. This presented the novel hypothesis that PD medication may directly affect the mitochondria and regulation of ccf-mtDNA copy number, a notion that has previously been reported for nucleoside reverse transcriptase inhibitors in HIV (Payne *et al.,* 2011) and serotonin specific reuptake inhibitors in depression (Lindqvist *et al.,* 2018). This effect may also explain the results observed in the ICICLE-PD cohort analysis (Pyle *et al.,* 2015b), where >90% of PD patients enrolled were on dopamine replacement treatment, and in Chapters 3 and 4, where PD and PMS patients were end-stage and thus, were undoubtedly receiving disease-specific treatment for some years prior to death. Although previous research has suggested that CSF ccf-mtDNA copy number is a biomarker of disease state (Podlesniy *et al.,* 2013; Pyle *et al.,* 2015; Podlesniy *et al.,* 2016; Varhaug *et al.,* 2017; Leurs *et al.,* 2018), few have considered the effect of treatment, and therefore their findings may in fact be an artefact related to medication

as well as disease state. Studies that have accounted for potential treatment effects support the results in this thesis. For example, Leurs *et al.* (2018), showed that fingolimod (a common disease-modifying therapy for MS) caused a reduction in lCSF ccf-mtDNA copy number in RRMS patients. Thus, it would be recommended that future ccf-mtDNA studies include treatment as a confounding variable.

Moreover, further work investigating the effects of therapeutics on ccf-mtDNA copy number is warranted. For example, additional time points could be requested from the PPMI, particularly focussing on the time point at which patients began treatment, to allow more specific analysis of the effect of treatment on ccf-mtDNA copy number. Cellular and animal models could also be used to assess this effect, analysing ccf-mtDNA copy number within their extracellular fluid (i.e. culture medium or rodent CSF) pre- and post-treatment. This may be of particular importance for the development of new NDD therapeutics where ccf-mtDNA copy number could be used as an independent indicator of drug action or efficacy. Furthermore, given the limitations of the existing outcome measure for PD (the MDS-UPDRS (Holden *et al.,* 2018)), this may have even greater utility in these patients, providing an immediate, easily measurable outcome for drug treatment. Alternatively, it would also be interesting to assess the relationship of ccf-mtDNA copy number in patients with different treatment-related conditions i.e. treatment-induced dyskinesias, non-responders and various other side effects. This may reveal mechanistic or biomarker links between ccf-mtDNA copy number and patients who are more or less at risk of responding to treatment or developing side effects or dyskinesias.

Finally, although ccf-mtDNA appears to be stable and relatively straightforward to assay and analyse, future work should be sensitive to the potential impact of phenotypic and biological heterogeneity, to minimise the confounding impact of these variables. If time permitted, it would have been interesting to delve further into the complex heterogeneity of the PPMI-PD cohort, particularly investigating control participants, to disentangle and better understand relationships to ccf-mtDNA copy number without the influence of a primary disease. This work could be essential in deciphering important regulatory factors underlying ccf-mtDNA abundance.

What has 'omics' investigations revealed about ccf-mtDNA export.

Due to a gap in our understanding surrounding the mechanisms underlying ccf-mtDNA export, this thesis also aimed to identify proteomic associations to ccf-mtDNA copy number and differences in ccf-mtDNA integrity.

The mechanisms underlying ccf-mtDNA export are not fully elucidated, however, are most commonly believed to associate with neuronal death or active release (Aucamp *et al.*, 2018). Chapters 3 and 4 involved targeted protein investigation in the post-mortem vCSF, but largely failed to identify significant associations to ccf-mtDNA copy number. No protein markers of cell death (Chapter 3), neurodegeneration (Chapter 3-5), vesicular transport (Chapter 3) or neuro-inflammation (Chapter 4) correlated to ccf-mtDNA copy number in health or disease, possibly suggesting that ccf-mtDNA copy number is not directly related to cell death or proteomic measures of disease. The weak but significant correlation to SDHA in the entire NDD-vCSF cohort may postulate that ccf-mtDNA is initially exported with the entire mitochondria. However, once released from the cell the individual protein components of the mitochondria are quickly degraded, whereas the mtDNA resists this and persists in circulation. This is supported by transmission electron microscopy work in Chapter 3, which identified fragmented and partially degraded mitochondria in the post-mortem CSF. The significant correlation between CHI3L1 protein and ccf-mtDNA copy number in PMS cases may support the pro-inflammatory role for ccf-mtDNA as a damage-associated molecular pattern (DAMP) and to some degree supports the cell death theory of ccf-mtDNA export. Whereby the anti-inflammatory effects of CHI3L1 protects neuronal function in PMS (Bonneh-Barkay *et al.*, 2012; Wiley *et al.*, 2015), leading to reduced ccf-mtDNA copy number.

Although targeted protein screens have their advantages (i.e. increased assay sensitivity and specificity (Ghosh *et al.,* 2014)), they can also be somewhat limited, due to their basis on previously known protein interactions and reliance on currently available primary antibodies. This limits the scope of the protein screen, targeting just a minority of proteins, and introduces a bias associated with *a priori* assumptions, which can prevent novel avenues being explored (Chen-Plotkin, 2014). To remove this bias, further work could employ the use of mass spectrometry to carry out a large proteomic screen in the CSF (Aebersold *et al*., 2013) to generate a CSF proteomic profile which can then be used to guide investigation into novel associations to ccf-mtDNA.

In addition, investigation of the vCSF ccf-mtDNA integrity indicated very few differences between disease cases (NDD and PMS) and controls, and indicated that ccf-mtDNA are generally intact, with only a minority of subjects exhibiting low level deletions in their ccf-mtDNA and all samples showing low level heteroplasmy. This suggests that ccf-mtDNA are not preferentially selected for export from the cell based on mutational load and could therefore imply that ccf-mtDNA export simply corresponds to normal cell turnover, whereby it is pushed out of the cell at the same rate as it is being produced. Consequently, the depletion of ccf-mtDNA copy number reported in post-mortem PD and PMS cases (Chapter 3 and 4) may directly reflect a pre-existing cellular mtDNA depletion in the brain, as reported in previous literature (Mao and Reddy, 2010; Pyle *et al.*, 2015a). Furthermore, tying in with results from Chapter 5, this pre-existing cellular mtDNA depletion, may in fact be a result of medication use. As PD and PMS medication have neuronal actions, it is far more likely that they disrupt cellular mtDNA copy number, which subsequently affects ccf-mtDNA copy number. If there had been more time, it would have been interesting to measure mtDNA copy number in the CSF-matched brain tissue, analysing multiple sections including the major disease-specific regions and areas of the ventricular system (particularly the choroid plexus) to give insight into the relationship between cellular mtDNA copy number and ccf-mtDNA copy number. Moreover, *in vitro* work could also be useful in this investigation, as mtDNA copy number of neuronal culture could be directly compared to ccf-mtDNA in the culture medium. This could be performed prior to, and immediately following dosage with treatment.

Final Conclusions

The work in this thesis has provided a comprehensive review and investigation into the biomarker ability of ccf-mtDNA copy number for neurodegenerative disease (NDD). Through in-depth analysis of ccf-mtDNA copy number in a range of NDDs and a large-scale validation study in PD, it may be concluded that ccf-mtDNA copy number is not a valid biomarker for broad NDD or PD. However, although at an early stage, preliminary assessments of serum ccf-mtDNA copy number show promise (Chapter 5) and warrant further investigation.

Additionally, this work has revealed an exciting novel prospect for ccf-mtDNA copy number as a potential outcome measure for response to treatment, which could have major implications in future drug development and therapeutic management of neurodegenerative disease. Furthermore, omics investigations performed in this thesis suggests that ccf-mtDNA circulates as an intact, organelle free molecule, likely released as a by-product of normal cell turnover.

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