Mitochondrial and Inflammatory Biomarkers in Patients with Parkinson's Disease and REM Sleep Behaviour Disorder

Fatima Afaar

A thesis submitted to fulfil the requirements for the degree of
Doctor of Philosophy

Central Clinical School
Faculty of Medicine and Health
The University of Sydney
Sydney, Australia

August 2024

Statement of originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual property of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Fatima Afaar

28 / 08 / 2024

Dedication

I dedicate all the efforts of this thesis to my beloved mother and father. This milestone belongs to you both, as every page of this thesis carries your fingerprints. Your heartfelt sacrifices have been my foundation, and your unwavering support has been my pillars of strength. Thank you for being my greatest blessing.

Acknowledgment

Their call therein will be, "Exalted are You, O Allah," and their greeting therein will be, "Peace." And the last of their call will be, "Praise to Allah, Lord of the worlds.
- The Qur'an 10:10

'Verily, knowledge is the life of the heart, the light of the eyes from blindness and the strength of the body from weakness.' – Imam Ali (a.s)

This journey was not easy, nor was it required to be. However, those who said they were up for it reached it and regardless of the difficulty endured it. My deepest gratitude goes to all those who supported this journey, from the words of encouragement to the kind gestures. Thank you all so sincerely.

I would like to express my deepest gratitude to my primary supervisor Dr. Nicolas Dzamko. I am grateful to have had the opportunity to be part of his team and learn from his invaluable expertise and knowledge. The advice, support and feedback provided was always much appreciated. I would also like to thank my co-supervisor, Prof. Glenda Halliday. As a female in science, it was truly inspirational and motivating to be part of the Halliday team led by an internationally renowned female scientist.

I would also like to express a heartfelt thank you to my co-supervisor, Dr. Priscilla Youssef, whose support, kindness, and work ethic was very much appreciated during my PhD journey. Thank you for all the lasting memories, I hope I have made a memorable PhD student.

Thank you to the entire research team at the Dzamko lab and the Forefront team at the Brain and Mind Centre for the cherished time spent together in academic and social settings. In particular, I would like to thank Marianne Hallupp for her lovely encouragement over the past few years, Siying Zhong for managing lab orders so kindly and to Dr. Jasmin Galper for always being prompt to discuss analysis-related questions.

I would also like to acknowledge and thank all the patients, their families/caregivers and all those who participated in the research studies of this thesis.

Most significantly I would like to thank my family, whom without their support this thesis would have not been possible.

To my mother, my first teacher. Your incredible faith in me was the light that illuminated every step of this challenging journey. You poured yourself so endlessly into making my dreams possible. No words of the English or Rome or even the delicate poetry of the Arabs could phrase my appreciation, admiration, and eternal gratefulness I have for you. To my father, thank you for modelling and instilling within me principles of strength, courage, and warmth that have allowed me to venture and take on challenges to grow and develop every day.
My parents, all the achievements and milestones my siblings and I have achieved we owe to your prayers - that have guided us throughout the way.

My brother Aziz, the first surgeon of our family. Thank you for always reminding me of how capable I am, especially during times of difficulty. Your words were always of deep impact and reassurance. My brother Mustapha, my rock. Thank you for always being so initiative to help and a source of light and comfort. Your constant care and compassion always made the biggest difference.

My sister Zaynab, my source of happiness. The support that you have showered me with during this journey is beyond measure. Thank you so deeply for being my anchor during the stormy seas.

Abstract

Parkinson's disease (PD) pathologically defined by the accumulation of alpha-synuclein (α-syn) aggregates and selective loss of dopaminergic neurons is the most common neurodegenerative movement disorder and one of the world's fastest-growing neurological disease. The clinical course for many patients with PD is long and slow, often occurring over decades. There are currently no treatments that can slow or stop PD, resulting in a progressively incapacitating disorder that culminates in considerable disability. A lack of simple blood-based biomarkers to diagnose and track the progression of PD is a main contributor to the current lack of treatments. Mitochondrial dysfunction, increased mitochondrial reactive oxygen species (ROS) and inflammation have presented as having significant roles in the progression of PD.

The objective of this thesis was to determine if peripheral mitochondrial and inflammatory readouts may comprise as diagnostic and prognostic biomarkers of PD. Mitochondrial measures were conducted on peripheral blood mononuclear cells (PBMCs) and monocytes while inflammatory measures were performed on plasma and serum samples of PD, idiopathic REM sleep behavior disorder (iRBD), and healthy control samples. Initially a flow cytometry assay was optimised and validated using healthy donor PBMCs. The optimised mitochondrial flow assay was utilised to measure differences in mitochondrial parameters between patient and healthy control samples at baseline and at stimulation. The study constituted of 105 subjects in total: 35 healthy controls, 35 iRBD patient and 35 PD patient PBMC samples. The data revealed significant differences in the levels of mitochondrial ROS ($p<0.05$) and mitochondrial content ($p<0.05$) in PD samples compared to controls at baseline. Positive association was also observed between iRBD monocyte mitochondrial ROS and the Mini Mental State Examination ($p<0.05$) for cognitive impairment and monocyte mitochondrial

content at baseline and the Montreal Cognitive Assessment (p<0.05) for mild cognitive dysfunction.

In line with the interest to determine if inflammatory measures can elucidate as potential diagnostic or prognostic biomarkers of PD, serum pro-inflammatory cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A and TNF-α were measured using a sensitive multiplex assay. The study constituted of 134 subjects in total: 35 healthy controls, 65 iRBD patient and 34 PD patient serum samples. The data revealed significant decrease of serum pro-inflammatory cytokine IL-2 in the iRBD compared to controls (p<0.01) and the PD group (p<0.05). Positive associations were also observed between PD serum pro-inflammatory cytokine IL-17A and the Movement Disorder Society - Unified Parkinson's Disease Rating Scale (MDS-UPDRS) III score (p<0.05) for assessment of motor symptom severity in PD and cytokine IL-6 and the Hoehn and Yahr scale (p<0.05) for symptom progression.

The final study aimed to determine if mitochondrial ROS and mitochondrial content can be measured in isolated monocytes of PD and healthy control samples and to determine if inhibition of the major PD risk protein, leucine rich repeat kinase 2 (LRRK2) and activation of the Glucocerebrosidase (GCase) enzyme could ameliorate mitochondrial dysfunction in PD patient monocytes. The study comprised a total of 49 subjects; 25 healthy controls and 24 PD patients that were age and sex matched. No difference in mitochondrial or ROS levels was observed between control and PD patient monocytes, and thus the effectiveness of PD drug to ameliorate mitochondrial dysfunction could not be concluded. A positive correlation was present between PD patients ROS levels and the MDS-UPDRS III score (p<0.05). PD patient monocytes mitochondrial function significantly correlated with α-syn (p<0.005), GFAP (p<0.05) and NfL (p<0.05) plasma levels.

Overall, the findings of this thesis suggest that PBMC mitochondrial readouts may elucidate as diagnostic biomarkers of PD and that PD monocyte mitochondrial dysfunction is associated with increased disease progression. Further longitudinal validation is required to assess the efficacy of peripheral mitochondrial measures as PD biomarkers in clinical practice.

Table of Contents

List of Figures

List of Tables

Abbreviations

α-syn	Alpha-synuclein
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
B-FGF	Basic fibroblast growth factor
BMI	Body mass index
CCL15	C-C Motif Chemokine Ligand 15
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CX3CL1	CX3 chemokine ligand 1
CX3CL1	Chemokine Fractalkine
CXCL12	C-X-C motif ligand 12 protein
DLB	Dementia with Lewy Bodies
DMSO	Dimethyl sulfoxide
EES	Epworth Sleepiness Scale
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
FACS	Fluorescence-activated cell sorting
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GBA1	Glucosylceramidase Beta 1
GCase	Glucocerebrosidase
GWAS	Genome wide association studies

H&Y	Hoehn and Yahr
HADS	Hospital anxiety and depression scale
HDL-C	High-density lipoprotein cholesterol
HI FBS	Heat Inactivated Fetal Bovine Serum
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1β	Interleukin-1 beta
iPSC	Induced pluripotent stem cells
iRBD	Idiopathic REM sleep Behaviour Disorder
KO	Knock out
LBP	Lipopolysaccharide binding protein
LBs	Lewy Bodies
LDL-C	Low density lipoprotein cholesterol
LEDD	levodopa equivalent daily dose
LNs	Lewy Neurites
LRRK2	Leucine-rich repeat kinase 2
MCP-1	Monocyte chemoattractant protein-1
MDS-PD	Movement Disorder Society for PD
MDS-UPDRS III	Movement Disorder Society- Unified Parkinson's Disease Rating Scale III
MFI	Median Fluorescence Intensisty
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
MMP-2	Metalloproteinase 2
MMSE	Mini Mental State Examination

MoCA	Montreal Cognitive Assessment
MOMP	Mitochondrial outer membrane permeabilization
MPTP	Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple System Atrophy
MSD	Meso Scale Discovery
NfL	Neurofilament light chain
NLRP3	NOD-like receptor thermal protein domain associated protein 3
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-Buffered Saline
PD	Parkinson's Disease
PINK1	PTEN induced putative kinase 1
PRKN	Parkin
PTX3	Pentraxin 3
RANTES	Regulated upon activation, normal T cell expressed and secreted
RBDSQ	REM Sleep Behaviour Disorder Screening Questionnaire
REM	Rapid eye movement
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SEM	Standard error mean
SN*pc*	Substantia Nigra pars compacta
SOD	Superoxide dismutase
SSC	Side scatter
sVCAM -1	Soluble vascular cell adhesion molecule-1
TNF-α	Tumour Necrosis Factor - alpha

UCH-L1	Ubiquitin C-terminal hydrolase L1
VPS35	Vacuolar protein sorting 35

CHAPTER ONE

General Introduction and Literature Review

1.1.1 Parkinson's Disease

First described as the 'Shaking Palsy' in 1817, by James Parkinson and later characterised and renamed to Parkinson's disease (PD) by Jean-Martin Charcot, PD is a disease of more than two centuries with deep history and impact.

1.1.2 Epidemiology of PD

Classified as the fastest growing neurological disorder globally, the incidence of PD is at a continuous rise (Dorsey et al., 2018). In Australia alone, 32 individuals are diagnosed with PD daily, giving a current estimate of 100,000 Australians living with PD (Bivol et al., 2022). Internationally, in less than 3 decades the prevalence rate of PD has doubled, with a current global estimate of 8.5 million individuals (WHO, 2022). The prevalence of PD significantly increases with age and is estimated to affect 1% of the population above the age of 60 (Tysnes & Storstein, 2017). The scorching numbers and significant disability associated with the disease have identified PD as the "Parkinson's Pandemic' (Dorsey et al., 2018).

1.1.3 Clinical features of PD

The clinical features of PD are categorised into classical motor manifestations and non-motor symptoms. The hallmark motor features of PD are tremor, rigidity and bradykinesia, and although PD is classified as a motor movement disorder, non-motor symptoms are common and manifest prior to the presentation of motor symptoms by decades. Non-motor symptoms

can be predicative or early indicators of disease and involve an array of disturbances impacting autonomic function, cognitive impairment, sleep disturbances and neuropsychiatric symptoms. Neuropsychiatric non-motor features of PD include rapid eye movement (REM) sleep behaviour disorder (RBD), apathy, and dementia, with dementia presenting in ~83% of PD patients following 20 years of diagnosis (Cohen et al., 2022; Hely et al., 2008; Weintraub et al., 2020). Motor and non-motor symptom severity increases with disease progression; collectively these symptoms result in a slow progressively disabling disease (Figure 1.1). (Chaudhuri et al., 2006; Chaudhuri et al., 2005).

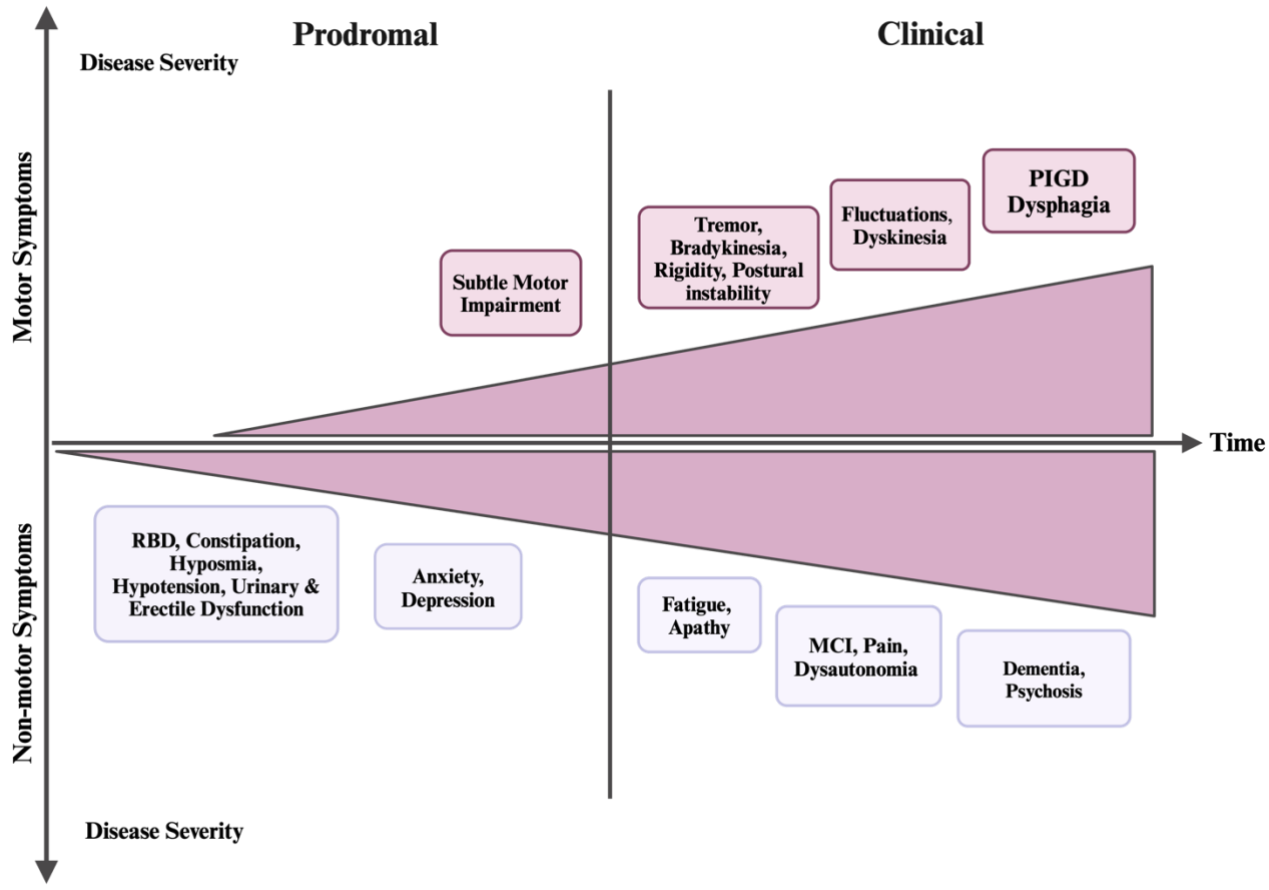


Figure 1.1 Symptom presentation and disease progression. Non-motor and motor symptoms of prodromal and clinical disease phase associated to disease progression. Figure adapted from (Jankovic & Tan, 2020). Created with BioRender.com

1.1.4 PD brain pathology

There are two distinct neuropathological features that characterise PD, namely the presence of alpha-synuclein (α-syn) containing Lewy pathologies and the loss of nigrostriatal dopaminergic neurons in the Substantia Nigra pars compacta (SN*p*c) region of the midbrain.

1.1.5 Lewy pathologies

Lewy pathologies are the key histopathological hallmark of PD and comprise of Lewy bodies (LBs) and Lewy neurites (LNs). LNs are dystrophic neurites that are mostly axonal and contain granular material and accompany LBs (Kouli et al., 2018). LBs are primarily composed of intracytoplasmic inclusions that have granular and fibrillar core with a pale-staining halo. α-syn protein forms the primary component of LBs and is abnormally aggregated, phosphorylated and misfolded in PD brains (Gómez-Benito et al., 2020). More than one LB can be present inside a neuron and they range in size, varying between 5-30 μM in diameter with two different forms being classical brainstem and cortical LBs. The core morphological differences between the two is that the classical brainstem LBs are greater in size, have a more distinct outline, and include a halo that mainly comprises α-syn (Goedert et al., 2012). The cortical LBs are smaller in size, have less distinct outline and do not include a halo (Kouli et al., 2018).

1.1.6 Braak Staging

The Braak staging hypothesis describes the sequential distribution of α-syn pathology that is found in PD brain (Figure 1.2). The theory proposes that PD initiates from two origins, either from neurons in the nasal cavity of the olfactory tract, or neurons in the gut of the vagal nerve. Both origins move into the medulla oblongata, with α-syn spreading within the Central Nervous System (CNS) and eventually leading into the neocortex through six progressive stages (Braak et al., 2003).

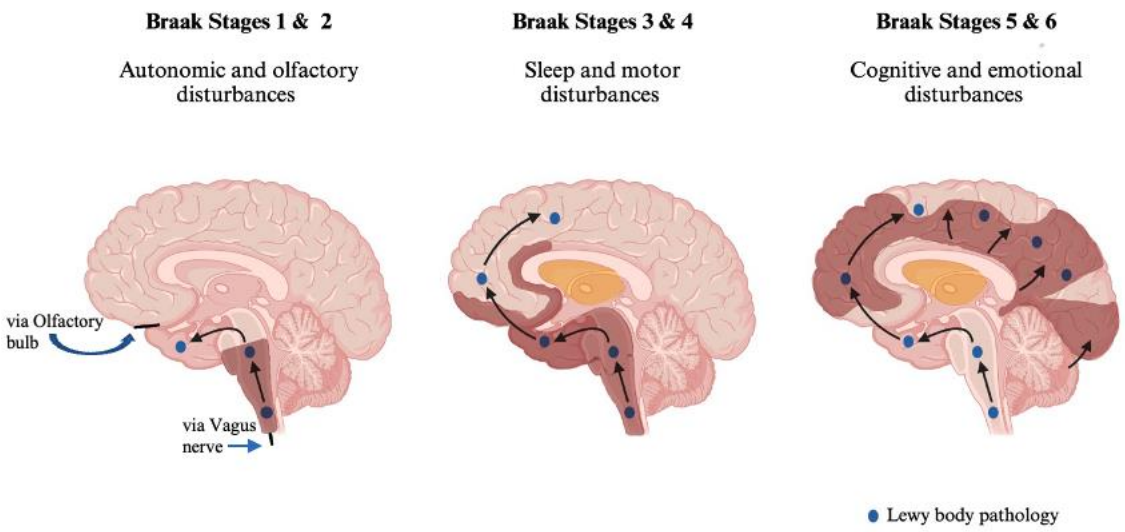


Figure 1.2 Lewy pathology and symptom progression according to Braak staging. In the initial stages Lewy pathology reaches the medulla via the Vagus nerve and the olfactory nucleus via olfactory bulb with presentation of autonomic and olfactory disturbances. Lewy pathology then spreads to midbrain resulting in sleep and motor disturbances during stages 3 and 4. In the final Braak stages 5 and 6 Lewy pathology reaches neocortex giving rise to emotional and cognitive disturbances. Figure adapted from (Halliday et al., 2011). Created with BioRender.com

1.1.7 Selective loss of dopaminergic neurons

PD is a progressive neurodegenerative disorder with symptom severity increasing proportionally to disease progression. This is particularly related to the loss of dopaminergic neurons and decreased dopamine content, which are a central pathological hallmarks of PD (Saikia et al., 2018; Zhou et al., 2023). 50-60% of dopaminergic neurons are degenerated at disease diagnosis, particularly at the appearance of motor symptoms. Furthermore, an estimate of 4-5% of further dopaminergic loss in the SN*p*c occurs per decade (Kouli et al., 2018; Saikia et al., 2018). The expression and control of movement is coordinated by communication of the neurons of the substantia nigra and neurons of the basal ganglia through dopamine neurotransmitters. Degeneration of the substantia nigra neurons results in decreased dopamine

neurotransmission in the corpus striatum. This biochemical imbalance gives rise to the classical motor symptoms of PD (Triarhou, 2013).

1.1.8 Diagnosis of PD and monitoring of symptoms

The diagnosis of clinically established PD is based upon medical history, and the presence of cardinal motor symptoms, requiring the presence of bradykinesias in combination with resting tremor or rigidity or both (Greenland & Barker, 2018). The exclusion of other cerebellar abnormalities, and a positive response to Levodopa treatment is also part of the diagnostic criteria.

To assist in the assessment of symptom severity of non-motor and motor symptoms different clinical screening tools are used. In particular, the Movement Disorder Society - Unified Parkinson's Disease Rating Scale (MDS-UPDRS) developed by the Movement Disorder Society for PD (MDS-PD) is a well-recognised symptom severity assessment composed of 50 question assessment of motor and non-motor symptoms (Balestrino & Schapira, 2019). Table 1 provides a summary of clinical assessments that are used for tracking the progression of PD symptoms. Despite the wealth of clinical tools and diagnostic criteria, tests so far remain subjective, and a definitive diagnosis of PD requires a post-mortem autopsy of the brain. Furthermore, the shared clinical features between PD and other neurodegenerative movement disorders such as dementia with Lewy bodies, Multiple System Atrophy (MSA), progressive supranuclear palsy and Corticobasal syndrome, particularly within the early stages of PD, results in an estimated 20% misdiagnosis of PD (Rizzo et al, 2016). It has therefore been proposed that in order to overcome the challenges of diagnosis and disease tracking of PD based on subjective clinical tests, objective biomarkers that directly measure disease pathophysiology are required (Wang et al., 2019).

Screening tool	Assessment	Indication	Score interpretation	Application in previous studies
MDS-UPDRS	Motor and non-motor symptom severity scale.	Motor and non-motor symptom severity.	= / <32 considered mild = / >59 severe	Holden et al. (2017)
Mini-Mental State Examination (MMSE)	Cognitive screening test assesses attention, memory, orientation.	Cognitive impairment	Total score of 25 or above classified as normal	Mamikonyan et al. (2009)
Montreal Cognitive Assessment (MoCA)	Cognitive function including attention and concentration, executive functions, visuoperception.	Mild cognitive dysfunction	Total score of 25 or above classified as normal.	Vaśquez et al. (2019)
Hoehn and Yahr (H& Y) Scale	Symptom progression relative to disease progression.	Functional disability severity scale	Stages: 0-6 0= no disability 6= Severe disability (constant assistance required)	Kataoka and Sugie (2021)
Farnsworth-Munsell Test	Colour accuracy through colour arrangement in hue.	Colour blindness	= > 4 possible colour deficiency	Postuma et al. (2011) Bertrand et al. (2012)
Epworth Sleepiness Scale (EES)	Sleep disturbances	Assessment of excessive sleepiness	= / > 8 considered as potential excessive sleep	Kumar et al. (2003)
Sniffin Sticks Test	Hyposmia. Threshold, discrimination and identification of olfactory function tests	Assessment of odour discrimination and identification		Baert et al. (2022)
Anxiety HADS	Anxiety and depression subscale	Hospital patients that may require further psychiatric attention / evaluation	>8 patient presents symptoms of anxiety and depression	Marinus et al. (2002)
RBDSQ	Sleep disturbances related to RBD	Clinical scale for RBD symptoms		Bušková et al. (2019)

Table 1.1 PD and iRBD screening tools for indication of motor and non-motor symptom severity.

1.2.1 The urge for PD biomarkers

Objective biomarkers that distinguish PD from other motor or neurodegenerative disorders are needed for earlier and more accurate diagnosis, while biomarkers that track disease pathology may allow for more targeted and specific therapeutic approach and or disease modifying therapies. Therefore, different types of biomarkers are necessary for PD.

1.2.2 Definition of biomarker

The World Health Organization defines the term biomarker as "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" (WHO,1993). Therefore, an ideal biomarker is not only providing indication to susceptibility of disease, but rather inclusive of effect of treatment, intervention or exposure of chemicals and nutrients through biological measurements that can be in the form of substance, structure or process to the human body. Depending on the context of application and the purpose different biomarkers are applied to yield specific information. For example, diagnostic biomarker may inform on the presence or not of PD, whilst a prognostic biomarker may inform on the level of disease severity or a response to therapy (Figure 1.3).

1.2.3 Diagnostic Biomarkers in PD

Most attempts to identify a diagnostic biomarker for PD have focussed on measuring the α-syn protein. Currently, seed amplification assays that measure α-syn aggregation in the cerebrospinal fluid (CSF) have shown specificity and sensitivity above 90% for the detection of α-syn aggregation in the CSF of PD patients (Kang et al., 2019; Shahnawaz et al., 2017). Though this technique offers relatively high specificity and sensitivity, the invasiveness of the

technique is not ideal for routine clinical practice (Mollenhauer, 2023). In clinical and research application peripheral diagnostic biomarkers are a more practical procedure. As blood specimens can be obtained from peripheral sources without the need for invasive procedures, therefore readily accessible requiring minimal time, near-immediate patient down time and cost-effective (Patel et al., 2011). However, the development of early and accurate peripheral diagnostic biomarkers that detect PD remains a significant gap. Therefore, the aim of the research herein is the development of peripheral diagnostic biomarkers.

1.2.4 Prognostic Biomarkers

PD is a heterogenous condition with a range of clinical presentations of motor and non-motor symptoms across different stages of disease. For such reasons, prognostic biomarkers in PD are useful in providing prediction to progression and responsiveness to disease modifying treatments. Prognostic biomarkers also provide the potential to stratify prognostic phenotypes which can be predictive of providing targeted therapeutic intervention unique to patient's case. Neurofilament light chain (NfL) is a protein of the central nervous system that is elevated in PD (Yamashita et al., 2023). Currently NfL is the best prognostic biomarker for PD, however, lacks specificity as NfL is associated to cognitive decline and not to motor symptoms (Narayanan et al., 2021). Validation of prognostic biomarkers requires specificity and sensitivity, whilst providing association to disease trend and treatment outcome. Development of peripheral prognostic biomarkers that are sensitive and specific to motor and non-motor symptoms of PD is of significant importance for the management and treatment of PD.

1.2.5 Pharmacodynamic Biomarkers

Pharmacodynamic biomarkers provide biochemical or physiological measures as a result of the engagement of specific drugs or drug doses. (Kelly & West, 2020). Thus, insightful for drug development of disease modifying treatments or therapies that diminish or decrease the

progression of PD pathology. In PD, pharmacodynamic biomarkers have emerged for alteration of gene pathways that have significant roles in the pathogenesis of familial and sporadic PD. As the *Glucosylceramidase Beta 1* (*GBA1)* and *leucine-rich repeat kinase 2* (*LRRK2*) gene mutations are the most common form of gene variants in PD, previous studies have assessed the potential of pharmacodynamic biomarkers in measuring the effect of small drug modulators to improve PD pathology (Kelly & West, 2020; Merchant et al., 2023). Specifically, the phosphorylated form of LRRK2, pS935 in PD peripheral blood mononuclear cells (PBMCs) has identified as potential disease modifying pharmacodynamic biomarker (Padmanabhan et al., 2020; Vissers et al., 2023) and is currently in clinical trials.

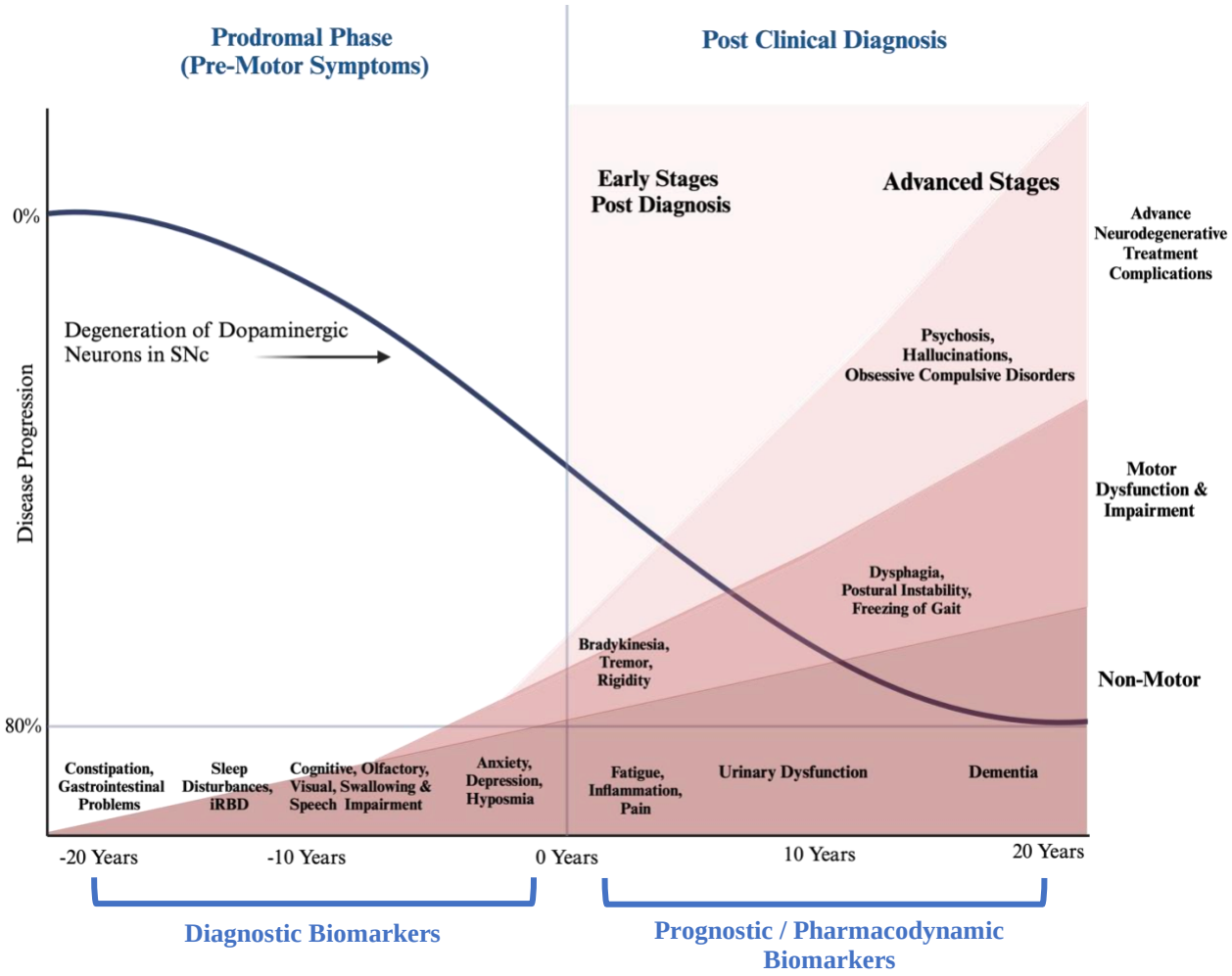


Figure 1.3 Progression of non-motor and motor PD symptoms during prodromal and clinical disease phase with fitting biomarkers for different disease stages. Idealistically, utilisation of diagnostic biomarkers during prodromal disease phase for early disease diagnosis and therapeutic interventions. Prognostic and pharmacodynamic biomarkers will allow for the monitoring of disease progression and for development and stratification of disease modifying therapies targeted for different PD forms. Figure adapted from (Ravenhill et al., 2023). Created with Biorender.com

1.3.1 Prodromal groups for the study of early PD

Validating earlier diagnostic biomarkers of PD requires the study of subject cohorts before they are clinically diagnosed. Advances in understanding the genetics of PD, as well as the identification of clinically at-risk groups, has helped to build these needed cohorts.

1.3.2 The genetics of PD

PD is currently classified into forms, sporadic and familial. Sporadic PD is the most common form of PD constituting of 85-90% of all PD cases (Tran et al., 2020). The median age of sporadic PD onset is 60 years with age being the primary risk factor, therefore typically presenting as late onset (Ryan et al., 2015). The aetiology of sporadic PD is unknown however genetic polymorphism and environmental factors have multifactorial contribution to the onset of PD (Schmidt et al., 2023). In particular, genome wide association studies (GWAS) have identified at least 90 common gene variants that increase the risk of developing PD, at least slightly (Nalls et al., 2019). Another gene that constitutes a major genetic risk factor for PD is *GBA1*, which encodes the enzyme glucocerebrosidase (GCase). Approximately 300 *GBA1* gene variants have been identified that have severe, mild and risk severity on PD pathology (Smith & Schapira, 2022). The prevalence of the *GBA1* mutation in PD patients is estimated to be around ~ 5%, while the penetrance is ~30% PD patients below the age of 80 (Stoker et al., 2018). In contrast to sporadic PD, familial PD risk genes are substantially higher, but with

increased penetrance for developing disease. Familial PD results from the inheritance of specific gene mutations and constitutes the minority of PD cases with estimated ranging between 5-15% of all PD (Balestrino & Schapira, 2019; Kouli et al., 2018,). Genes that are associated with the development of familial PD are labelled with locus name "PARK", and currently 23 PARK genes have been confirmed causal for PD (Table 1.2). PARK gene mutations are of two categories: either being autosomal dominant or autosomal recessive inheritance. Inheritance of autosomal dominant PD requires a single copy of gene mutation and includes the risk genes alpha-synuclein (*SCNA*) and *LRRK2*. Autosomal recessive PD is the inheritance of a gene variant from both parents that do not present symptoms of PD, with examples being homozygous mutations in *Parkin* (*PRKN*) and *PTEN induced putative kinase* 1 (*PINK1*) (Day & Mullin, 2021; Kouli et al., 2018; Singleton et al., 2013; Tran et al., 2020). The discovery of autosomal dominant and recessive gene mutations in familial PD has significantly progressed the understanding of the aetiology and molecular mechanisms of sporadic PD (Chai & Lim, 2013). Moreover, genetic at-risk cohorts can be identified for the development of PD biomarkers and therapeutic intervention. Non-manifesting genetic mutation carriers define the genetically at-risk PD populations. Mutation of the *LRRK2*, *SNCA* and *VPS35* genes are linked to autosomal dominant PD inheritance, whereas mutation of the *PARKIN, PINK1 and DJ-1* genes are associated to autosomal recessive PD inheritance. Specific ancestral populations such as the African Arab Berber population and the Ashkenazi Jewish population have increased incidence of the *LRRK2 G2019S* mutation, approximately being 40% and 20% respectively (Schulte & Gasser, 2011). Mutation of the *GBA1* gene also comprises a significant risk factor of PD, in which 5-15% of sporadic PD patients carry *GBA1* mutations. The clinical manifestation of GBA-associated PD is very similar to sporadic PD. The primary difference being the early onset of GBA-PD, preceding sporadic PD by 5 years (Smith & Shcapira, 2022) and increased risk of greater cognitive dysfunction (Zheng & Fan,

2022). *GBA1* mutations are also prominent in the Ashkenazi Jewish population (Cheon et al., 2012). Asymptomatic mutation carriers of these risk genes have the potential to identify biomarkers in the pre-clinical phase of PD. For example, assessment of peripheral mitochondrial function in non-manifesting LRRK2 mutation carriers revealed increased mitochondrial DNA damage and inflammation, demonstrating early peripheral disease processes prior to the clinical diagnosis of PD (Dzamko et al., 2016; Qi et al., 2023).

Locus name	**Gene**	**Chromosome**	**Inheritance**	**Onset**	**Reference**
PARK1	*SNCA*	4q21-q22	Autosomal dominant	Classic/early onset PD	Klein & Westenberger (2012)
PARK2	*PRKN*	6q25.2–q27	Autosomal recessive	Early-onset PD	Kitada et al. (1998)
PARK3	-	2p13	Autosomal dominant	Classic PD onset	Klein & Westenberger (2012)
PARK4	*SNCA*	4q21–q23	Risk factor	Early-onset PD	Klein & Westenberger (2012)
PARK5	*Ubiquitin C-terminal hydrolase L1*	4p13	Autosomal dominant	Classic PD onset	Nishikawa et al. (2003)
PARK6	*PINK1*	1p36.12	Autosomal recessive	Early-onset PD	Valente et al. (2004)
PARK7	*DJ-1*	1p36.23	Autosomal recessive	Classic PD onset	Bonifati et al. (2002)
PARK8	*LRRK2*	12q12	Autosomal dominant / risk factor	Classic PD onset	Zimprich et al. (2004)

PARK9	ATP13A2	1p36	Autosomal recessive	Early-onset, atypical with dementia	Ramirez et al. (2006)
PARK10	Ubiquitin specific peptidase 24	1p32	Risk factor	Classic PD onset	Farrer et al. (2006)
PARK11	*GYF protein 2*	2q36-7	Autosomal dominant	Late-onset PD	Klein & Westenberger (2012)
PARK 12	-	Xq21-q25	Risk factor	Classic PD onset	Klein & Westenberger (2012)
PARK13	HtrA serine peptidase 2	2p13.1	Autosomal dominant / risk factor	Classic PD onset	Klein and Westenberger (2012)
PARK14	*PLA2G6*	22q13	Autosomal recessive	Early-onset PD	Paizan-Ruiz et al. (2008)
PARK15	*F-box protein 7*	22q12.3	Autosomal recessive	Early-onset PD	Fonzo et al. (2008)
PARK16	*RAB7L1*	1q32	Risk factor	Classic PD onset	Tan et al. (2010)
PARK17	*VPS35*	16q11.2	Autosomal dominant	Classic PD onset	Wider et al. (2008)
PARK18	*EIF4G1*	3q27.1	Autosomal dominant	Classic PD onset	Chartier-Harlin et al. (2011)
PARK19	*DNAJC6*	1p31.3	Autosomal recessive	Early-onset PD	Olgiati et al. (2016)
PARK20	*SYNJ1*	21q22.11	Autosomal recessive	Early-onset PD	Krebs et al. (2013)
PARK21	*DNAJC13*	3q22.1	Autosomal dominant	Classic PD onset	Vilariño-Güell et al. (2014)
PARK22	*CHCHD2*	7p11.2	Autosomal dominant	Classic/early PD onset	Funayama et al. (2015)

PARK23	*VPS13C*	15q22.2	Autosomal recessive	Early-onset PD	Lesage et al. (2016)

Table 1.2 Summary table of 23 PARK genes and associated chromosome, inheritance and onset.

1.3.3 iRBD

In addition to genetically at-risk cohort for the identification and potential development of early diagnostic biomarkers and/or therapeutic intervention for PD, idiopathic rapid eye movement (REM) sleep behaviour disorder (iRBD) patients also present as an advantageous cohort.

1.3.4 Clinical features of iRBD

iRBD is characterised by rapid eye movement, loss of muscular atonia and abnormal and disruptive sleep enactment during sleep (Aurora et al., 2010). Sleep enactment of iRBD patients can be potentially dangerous, in which 33-66% of iRBD patients experience sleep-related injury to self or bedroom partner (Aurora et al., 2010). iRBD is a middle to older age adult parasomnia disorder with the prevalence estimated to be between 1.06 to 1.23 % in middle to older adults (Sumi et al., 2022).

1.3.5 Diagnosis of iRBD

The diagnosis of iRBD requires the presence of dream enactment behaviour that manifests motor events during sleep that is not influenced by neurological disorders (Bramich et al., 2022). Screening questionaries such as the REM Sleep Behaviour Disorder Screening Questionnaire (RBDSQ) can aid the assessment of iRBD (Bramich et al., 2022). Importantly, 80% of iRBD patients phenoconvert to alpha-synucleinopathies within 10 years, primarily being MSA, Dementia with Lewy bodies and PD (Mangia et al., 2017; Sumi et al., 2022). Therefore, through iRBD is an ideal prodromal PD cohort the pheonconversion of iRBD to

different neurodegenerative diseases introduces difficulty in studying iRBD as a primary precursor to PD. As the onset of PD is estimated to be 10-20 years prior to the onset of significant motor symptoms, the majority of subjects with iRBD are likely to be within the prodromal phase of PD (Postuma et al., 2019; Schenck et al., 2013).

1.3.6 iRBD as prodromal PD

iRBD patients often manifest PD symptoms including cognitive and motor deficits, autonomic disturbances such as constipation and hyposmia and sleep disturbances (Diaconu et al., 2021; Slow et al., 2014). The shared clinical markers of prodromal PD and iRBD present up to decades prior to the clinical diagnosis of PD (Moscovich et al., 2020), with autonomic disturbances manifesting 11-20 years prior to the clinical diagnosis of PD (Postuma et al., 2013). The shared symptom manifestations and significant phenoconversion rate of iRBD to alpha-synculeinopathies is due to the mutual pathology of abnormal deposition of α-syn protein (Howell & Schenck, 2015). Furthermore, Schaffrath et al. (2023) measured α-syn in stool of PD, iRBD and healthy controls and found a significantly increased concentration of α-syn aggregates in iRBD patients compared to controls (Schaffrath et al., 2023). This finding is indicative of α-syn pathology in prodromal disease phase of PD. Further, combined assessment of grey matter volume, polysomnography data and motor and non-motor features of 66 iRBD patients, found clinical subtypes of PD present during the prodromal disease phase of PD (Seger et al., 2023). The mutual pathology of PD and iRBD, shared symptom presentation and powerful conversion rate of iRBD to PD identify the iRBD cohort as the best clinically defined at risk group to develop PD. Therefore, iRBD patients are an ideal cohort to define prodromal PD for the fundamental importance of understanding disease causes, better and more accurate diagnosis of PD.

1.3.7 Prospective PD cohorts

PD presents as a complex multifactorial heterogenous disorder, with genetic and environmental influences contributing to disease pathogenesis. The identification of different early-stage disease presentations can contribute to an increased understanding of disease pathobiology, leading to more targeted treatment intervention (Rees et al., 2024). Apart from iRBD patients and genetically at-risk PD cohorts, individuals that present prodromal PD features may also constitute prodromal PD cohorts. Prospective cohorts can be identified through the presence of risk markers or prodromal markers. Risk markers include family history of PD, male sex, pesticide or solvent exposure, caffeine consumption, smoking status and type II diabetes status. Prodromal markers include the presence of parkinsonism, urinary dysfunction, constipation, hyposmia, depression or anxiety and cognitive impairment (Mahlknecht et al., 2022).

1.4.1 Mitochondrial and inflammatory dysfunction in PD

As outlined above, the study of genetic forms of PD has greatly enhanced the mechanistic study of PD, with mitochondrial dysfunction and inflammation routinely presenting as two key mechanisms that underly the pathology of PD (Henrich et al., 2023; Peggion et al., 2024). An understanding of the interconnected intricate relationship between inflammation and mitochondrial dysfunction in PD, if one mechanism precedes or initiates the other remains elusive (Peggion et al., 2024). Moreover, since these biological processes are closely linked to PD pathology, measures of these pathways may provide needed objective PD biomarkers.

1.4.2 Mitochondria

Mitochondria are intercellular membrane bound organelles found in the cytoplasm of eukaryotic cells that range between 0.5 – 1 uM in size. The primary role of mitochondria is the generation of energy in the form of Adenosine triphosphate (ATP), which is required for many

biochemical and cellular processes. ATP is produced through oxidative phosphorylation process, that involves the chemiosmosis of electrons Nicotinamide-adenine dinucleotide (NADH) and Flavin adenine dinucleotide (FADH2) to oxygen across mitochondrial complexes I, II, III and IV of the mitochondrial electron transport chain (ETC) as illustrated in Figure 1.4 (Deshpande & Mohiuddin, 2022). Apart from the primary function of the generation of metabolic energy through oxidative phosphorylation and the ATP synthase, mitochondrial organelles perform other multifunctional roles critical to maintaining normal cellular function (Bractic & Larsson, 2013). Mitochondrial calcium homeostasis, which involves the regulation of calcium signalling and buffering is essential for normal neuronal functioning. Calcium signalling is critical for synaptic release of neurotransmitters, neuronal plasticity and axonal support. Whilst calcium buffering is crucial to sustained release of dopamine essential for normal functioning of the striatum and to cell health, as upregulated calcium levels lead to apoptosis and cell death (Nicoletti et al., 2021). Apoptosis programmed cell destruction, eliminates aged, damaged or mutated cells is an essential function for maintaining proliferation and preventing disease. This cell autonomous process is initiated through a mitochondrial event termed mitochondrial outer membrane permeabilization (MOMP). The activation of MOMP leads to the selective release of proteins of the intermembrane space that lead caspase activity which causes cell death (Tait & Green, 2013). Furthermore, mitochondrial organelles have significant roles in cell signalling as initiators and transducers. Cell signalling is regulated through two forms, through physical platforms for protein-protein interaction and through the regulation of intracellular signalling of ROS and calcium molecules (Tait & Green, 2012). Addition to the above mitochondria regulate fission and fusion processes that are essential in maintaining cell cycle regulation and cellular functions (Spurlock et al., 2021). Fission the division of larger mitochondria and fusion the combination of smaller mitochondria maintain mitochondrial size and shape for optimal mitochondrial function.

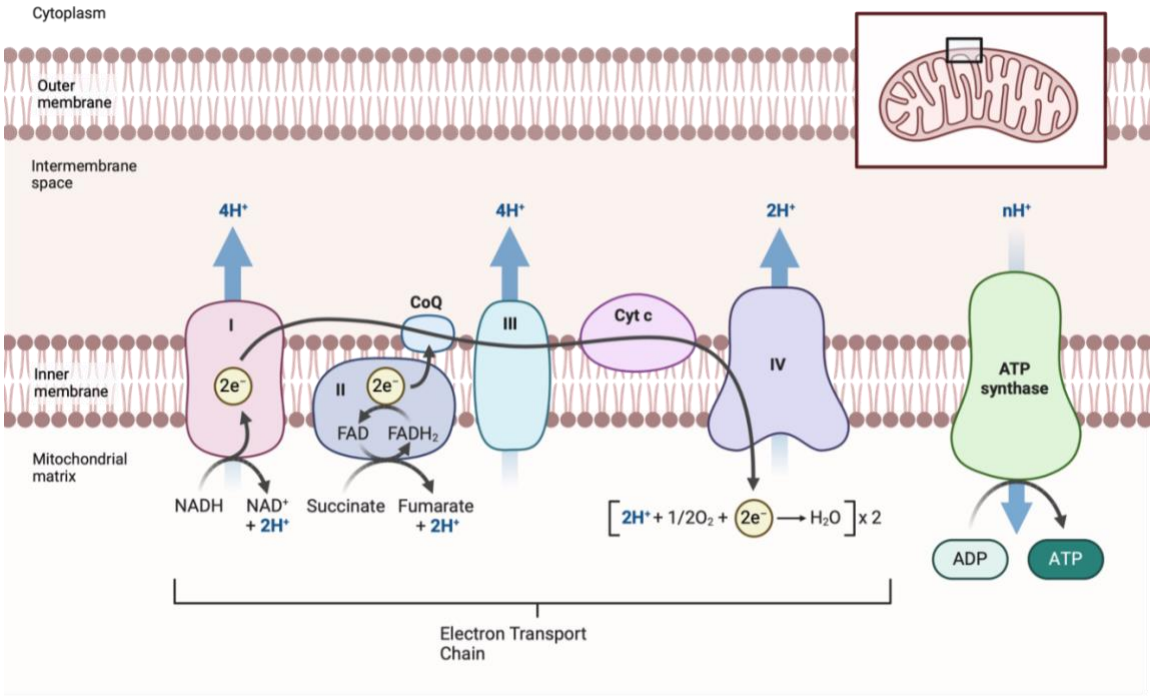


Figure 1.4 Inner mitochondrial membrane space of the ETC. Constituting of mitochondrial complexes I, II, III and IV for the generation of ATP synthase. Figure adapted from (Wu et al., 2022). Created with Biorender.com

1.4.3 Significance of mitochondrial health

The primary function of mitochondrial organelles is the production of ATP to sustain cellular survival. Though the human brain weighs 2% of the total body weight it is the most energy-intensive organ, requiring 20% of body oxygen while at rest for energy production (Maldonado & Alsayouri, 2023). Therefore, mitochondrial function is essential for neuronal homeostasis. Unfortunately, defective mitochondrial function can result in elevated levels of mitochondrial ROS, leading to oxidative stress and potential degeneration of neuronal cells (Klemmensen et al., 2024; Olagunju et al., 2023).

1.4.4 Defective Mitochondria and Mitochondrial ROS generation

The oxidative phosphorylation process that generates ATP, occurs in the intermembrane space of the mitochondria and requires complexes I and III to convert oxygen to superoxide anions (O_2^-). Further mitochondrial complex III generates superoxide anions. Impaired function of complexes I and III due to age related deterioration or exposure to chemical or environmental toxins impairs the function of these complexes. Impaired function results in diminished expression of mitochondrial complex I and increases oxidation production. Increased oxidative stress, damages complex I and subsequently causes defective transport of electron resulting in increased electron leakage to oxygen and production of superoxide anions (O_2^-). Superoxide dismutase (SOD) is an enzymatic antioxidant, that has an essential role in catalysing the dismutation of superoxide ions into hydrogen peroxide (H_2O_2) to eliminate the potential of damaging oxygen molecules. The Fenton reaction reduces hydrogen peroxide (H_2O_2) to hydroxyl radical ($^{\bullet}OH$) which causes significant mitochondrial oxidative stress. The generation of mitochondrial ROS occurs during the Fenton reaction as mitochondrial iron defects the efficiency biosynthesis. Further imbalance in iron homeostasis due to increased mitochondrial iron results in increased production of free radicals which generates oxidative stress (Lee et al., 2023; Long et al., 2023). Furthermore, mitochondrial dysfunction leads to impaired structure and function through impaired mitophagy, abnormal fission and fusion and defective biogenesis and cristae morphology (Figure 1.5). In the pathology of sporadic and familial PD, impaired mitochondrial function has a central role to disease pathogenesis (Bose & Beal, 2016). Post-mortem studies of PD brains have identified the contribution of oxidative stress to PD pathogenesis, particularly impacting mitochondrial function and antioxidant system including SOD (Jenner & Olanow, 1996). Furthermore, more recently mitochondrial abnormalities were observed in fibroblasts, erythroblasts and in dopamine neurons differentiated from induced pluripotent stem cells of PD patients (Barnhoorn et al., 2024). The observed findings from

central and peripheral levels, indicate the key role of mitochondrial dysfunction in the pathophysiology of PD patients.

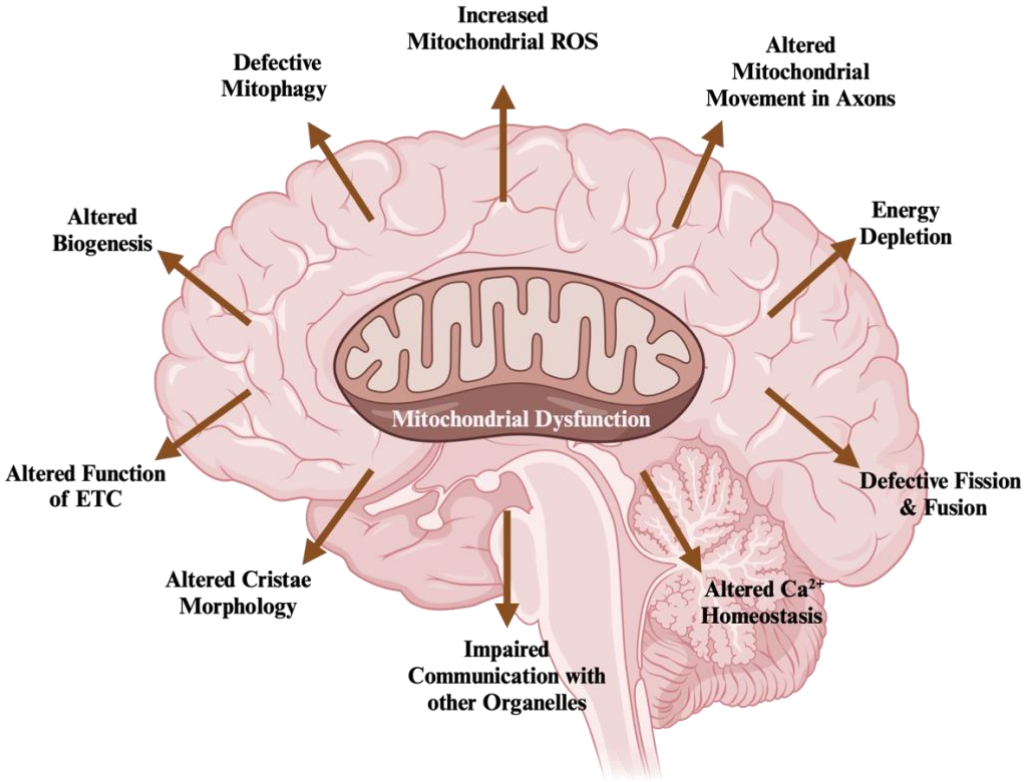


Figure 1.5 Impacted pathways due to mitochondrial dysfunction. Impaired mitochondrial function impacts the ETC therefore depleting energy, affects mitochondrial dynamics through altered fission and fusion, impairs integrity and structure due to defective biogenesis and cristae morphology. Also results in elevated ROS, impaired mitophagy function, calcium homeostasis and axonal transport ultimately impacting communication with other organelles. Figure adapted from (Peggion et al., 2024). Created with Biorender.com

1.4.5 Impaired mitophagy

Mitophagy is mitochondrial specific macro-autophagy that is responsible for the clearance of defective mitochondrial turnover to prevent the accumulation of damaged mitochondria that produce excessive ROS (Doxaki & Palikaras, 2021; Song et al., 2020; Springer & Macleod,

2016). Impaired mitochondrial quality control due to diminished mitophagy mechanism has been associated to familial and sporadic PD. PINK1 and Parkin are proteins that are involved in maintaining mitochondrial quality control through monitoring mitochondrial integrity and signalling clearance of malfunctional mitochondria (Tanaka, 2020). Furthermore, post-mortem, cellular and experimental studies on toxin-induced animal studies have suggested the role of mitophagy in the pathogenesis of neuropathological features present in PD (Gao et al., 2017). Due to the role of mitophagy in regulating mitochondrial homeostasis and the involvement of mitophagy in familial and sporadic PD (McWilliams & Muqit, 2017), improving mitophagy has been hypothesised as a potential disease modifying therapeutic strategy for PD (Masaldan et al., 2022).

1.4.6 Mitochondrial Dysfunction in PD and iRBD disease pathogenesis

The involvement of mitochondrial dysfunction to the pathogenesis of PD, was first observed in 1983, through recreational drug users. Patients developed acute and irreversible parkinsonism directly following intravenous injection of synthetic heroin that contained 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The toxic oxidation of MPTP to MPP^+, reached dopamine nerve terminals via dopamine transporters (Subramaniam & Chesselet, 2013). The patients presented with typical motor symptoms including tremor, non-motor symptoms, degeneration of dopaminergic neurons and immediate response to Levodopa treatment (Langston, 2017). The clinical focus of the 7 unique cases and the effect of the neurotoxin MPTP on mitochondria consequently causing the degeneration of dopaminergic neurons paved a paradigm shift in PD research, later establishing mitochondrial dysfunction as a pivotal pathological hallmark of disease (Langston, 2017). A proteomic study of post-mortem PD brains in early to mid-stage of disease, categorised between Braak stages 3 – 4, demonstrated that mitochondrial dysfunction pathology precedes neuronal loss and α-syn

depositions (Toomey et al., 2022). Further, in early stages of PD, impaired mitochondrial pathways are present in pathologically unaffected brain regions, primarily being the frontal cortex, and is trajectory to worsen with disease progression. This finding is indicative that mitochondrial dysfunction has a key role in early PD pathogenesis (Toomey et al., 2022), as impaired mitochondria significantly impacts cellular homeostasis and function. The findings from Payne et al. (2023) suggest that impaired mitochondrial function in the central and periphery of PD patients is linked to impaired mitophagy and disturbed bioenergetics of dopamine striatal nerve terminals. The multimodal parallel study revealed elevated levels of mitochondrial content and impaired mitophagy in PD patient fibroblasts parallel to increased intracellular ATP levels of the striatal dopaminergic nerve terminals that exceeded that observed in the midbrain of PD patients (Payne et al., 2023). Central and peripheral mitochondrial impairment observed in PD patients was suggested to be linked to impaired mitophagy and mitochondrial and metabolic deficiencies (Payne et al., 2023). Furthermore, more recently the assessment of the peripheral bioenergetic profiles of PD patients has gained noteworthy emphasis. Study conducted by Schirinzi et al. (2022) that assessed the PBMC mitochondrial respiration of 16 PD and 14 age and sex matched healthy controls found association between PD patients elevated bioenergetic profiles and clinical measures. The measured mitochondrial function parameters presented increased maximal and spare respiratory capacitates and although not significant increased ATP production in PD PBMCs. Elevated maximal and spare compensatory capacities correlated to MDS-UPDRS III scores and disease duration. Schirinzi et al. (2022) suggested increased respiratory mechanisms is a compensatory adaption to clinical progression of PD. Significantly elevated ATP levels and altered gene expression of mitochondrial oxidative phosphorylation genes and mitochondrial quality control genes was observed in PD PBMCs (Gezen – Ak et al., 2020). The study constituted of 107 PD patients that were divided into subgroups according to age, PD onset and

familial background and 49 age matched healthy controls (Gezen – Ak et al., 2020). Gezen – Ak et al. (2020) proposed that altered mitochondrial gene expression is potentially associated to sporadic PD and that elevated ATP levels is a compensatory response mechanism. Consistent with previous studies, research conducted by Navarro et al. (2021) found alterations in mitochondrial gene expression in peripheral monocytes of sporadic PD patients. In addition to altered expression of mitochondrial DNA from previous studies, recent findings by Qi et al. (2023) presented increased levels of damaged mitochondrial DNA in PD patient PBMCs. These findings indicate that mitochondrial dysfunction may be associated to the pathophysiology of PD and therefore comprise as a biomarker.

1.4.7 Mitochondrial dysfunction and α-syn accumulation

Mitochondrial dysfunction and α-syn aggregation are central neuropathological features that lead to the degeneration of dopaminergic neurons. The interaction between α-syn and impaired mitochondrial function has been established in experimental studies and PD patient studies (Calabresi et al., 2023; Thorne & Tumbarello, 2022). Whether α-syn deposition occur prior to mitochondrial dysfunction or if mitochondrial dysfunction causes α-syn accumulation remains debatable (Zaltieri et al., 2015). Over expression of α-syn in hypothalamic neuronal cell lines (GT1-7) led to compromised mitochondrial function and generation of ROS (Hsu et al., 2000). Altered mitochondrial function, preceding the α-syn pathology in PD has been observed in several animal model studies. Of those study conducted by Cannon et al. (2009) that demonstrated administration of rotenone to male Lewis Rats to model PD, found mitochondrial impairment and oxidative damage to precede α-syn accumulation and aggregation.

1.4.8 Mitochondrial dysfunction and dopaminergic cell death

Neurons at resting or at stimulation require mitochondrial - derived energy to maintain neuronal homeostasis and synaptic activity. Impaired mitochondrial function depletes mitochondrial oxidative phosphorylation generation of ATP which negatively effects the regulation of ROS and calcium buffering essential for neuronal and synaptic homeostasis (Faria-Pereira & Morais, 2022). Animal model study of MPTP- treated mice found that enhanced mitochondrial function improved motor dysfunction. Treatment of MPTP-treated mice with ethyl pyruvate and SA4503 enhanced ATP production in the substantia nigra pars compacta and enhanced calcium influx, resulting in improved cognitive and motor symptoms severity (Haga et al., 2019).

1.4.9 Mitochondrial dysfunction as a peripheral PD biomarker

As outlined above, mitochondria are intrinsically linked to numerous aspects of PD pathology and the study of mitochondrial function may provide insight into disease mechanisms and objective biomarkers. Efforts to develop peripheral mitochondrial biomarkers are scant with limited studies assessing the utility of mitochondrial parameters as diagnostic and prognostic biomarkers. Previous studies that have measured peripheral mitochondrial dysfunction in PD patients have done so either through skin fibroblasts (Antony et al., 2020; Milanese et al., 2019; Ongari et al., 2024; Yakhine-Diop et al., 2019) or PBMCs (Qadri et al., 2018; Smith et al., 2018). PD patient fibroblasts have been suggested to be a potential biospecimen source to study mitochondrial bioenergetic impairment for potential biomarker development due to their biochemical and metabolic relationship with neurons (Olesen et al., 2022). However, the invasive procedure required for obtaining patient fibroblasts impacts their practical clinical utility as an ideal peripheral biomarker source for diagnostic and prognostic screening. In which for the application of clinical screening PBMCs are likely a more advantageous peripheral biospecimen source. PBMCs are reflective of deeper brain pathology (Alexovic et

al., 2023) and neuronal and immune alterations, (Lauritsen & Romero-Ramos, 2023). Furthermore, as mitochondrial ROS levels are a key hallmark of PD, the direct measurement of PBMC ROS levels is a highly useful indication to mitochondrial dysfunction in PD patients. Although elevated levels of mitochondrial ROS have been observed in PD patient PBMCs (Qadri et al., 2018; Smith et al., 2018), further validation is required to determine the feasibility of mitochondrial dysfunction as diagnostic and prognostic PD biomarkers. Specifically, the measurement of mitochondrial ROS and mitochondrial content levels in PD and iRBD patients PBMCs in larger cohort groups, whilst assessing their association to clinical measures to determine the feasibility and practicality of mitochondrial measures as PD diagnostic and prognostic biomarkers is required. Additionally, a review of previous studies that have assessed the utility of mitochondrial based biomarkers in detecting mitochondrial dysfunction in mitochondrial diseases and disorders suggested that different biochemical markers should measure specific mitochondrial components. As a single biomarker that provides 100% sensitivity and specificity for mitochondrial diseases is not yet accessible (Hubens et al., 2022).

1.4.10 Peripheral measurement of mitochondrial function and inflammation in PD and iRBD patient samples

Monocytes are significant contributors to the peripheral innate immune system, due to their multifaceted roles in immune defence, phagocytosis, cytokine production and tissue repair. Increasing evidence from previous studies have indicated that monocyte function of the peripheral innate immune system of PD patients differs to healthy controls (Harms et al., 2021; Magistrelli et al., 2020; Massacci et al., 2024; Wijeykoon et al., 2018). Furthermore, alterations of the peripheral innate immune system in PD patients are reflective of disease progression and symptoms presentation (Nissen et al., 2022). Recent phosphoproteomic characterisation of PD patient PBMCs revealed that alterations in peripheral immune cells contribute to disease progression and are associated to disease staging (Massacci et al., 2024). To determine if

mitochondrial function alterations in patient monocytes can utilises as peripheral diagnostic and prognostic biomarkers, mitochondrial function of patient's samples were analysed through flow cytometry. Application of flow cytometry for biomarker discovery and development is highly useful (Barnard, 2012) due to the versatility of technique and detailed analysis of single cell measurement. In previous research flow cytometry has enabled for the measurement of GCase activity for the potential of assessing and restoring GCase function through molecular modifying therapies (Hughes et al., 2020). Furthermore, mitochondrial function of patient and healthy control peripheral monocytes has been measured through flow cytometry technique, in which the protocol of this thesis was adapted from (Smith et al., 2018). Measurement of mitochondrial function of peripheral monocytes was performed through staining samples with specific mitochondrial fluorescent probes to provide measures of mitochondrial ROS and mitochondrial content. Elevated levels of mitochondrial ROS and mitochondrial content are indication of mitochondrial dysfunction, as explained in above section of this literature review. To measure mitochondrial ROS and mitochondrial content cells were stained with MitoSOX Red (ThermoFishser, #M36008) and MitoTracker Deep Red (ThermoFisher #M22426). The selected mitochondrial probes allow for the live measurement of mitochondrial parameters in cells through omitted fluorescent signal, whilst allowing for high-throughput analysis of significant quantity of cells.

1.5.1 Inflammation

The involvement of inflammation in the pathogenesis of PD is increasingly recognised, with immune responses occurring in the CNS referred to as neuroinflammation, and inflammation occurring at peripheral referred to as peripheral inflammation. Inflammatory activation is prompted by the immune system as self defence mechanism in response to clear pathogenic stimuli. Peripheral innate immune cells constituting of myeloid cells including monocytes and dendritic cells are the first immune response to stress or infection. Monocytes and dendritic

cells are antigen – presenting cells that activate t-cells through the major histocompatibility complex system that in turn activate B-cells and result in humoral response being prompted including the secretion of cytokines, chemokines, and initiation of phagocytosis (Dzamko, 2023; Terkelsen et al., 2022). The contribution of cytokines implicated in the peripheral innate immune system response and monocyte alterations have been increasingly recognised in PD and iRBD.

1.5.2 Cytokine imbalance in PD and iRBD

Imbalance of cytokine levels at central and peripheral have been observed in PD and iRBD patients. Alterations in pro-inflammatory and anti-inflammatory cytokine levels, resulting in peripheral dysregulation contributes to neuroinflammation, enhances α-syn aggregation and the degeneration of dopaminergic neurons (Dzamko, 2023; Kasen et al., 2022). Peripheral cytokine alterations in PD patients are substantially focused upon, emphasising the role of the peripheral immune system and inflammatory response in the pathogenesis or potential aetiology of PD. Further, peripheral inflammation in PD is associated with degeneration of dopaminergic neurons. A Study of the association of peripheral inflammatory cytokines to PD motor symptoms revealed upregulated interleukin (IL)-4 and diminished IFNγ was associated to increased motor tremors (Diaz et al., 2022). Furthermore, metanalysis of 25 studies totalling to 2564 PD patients found elevated levels of inflammatory cytokines IL-6, IL-2, IL-10, tumour necrosis factor (TNF), C-reactive protein, and RANTES in peripheral blood of PD patients (Qin et al., 2016). Limited studies have focused upon the peripheral inflammatory cytokine profiles of iRBD patients, hence the limited number of studies within the past few years. The first study to investigate peripheral alteration in an iRBD cohort was conducted by Kim et al. (2019) that observed single significant finding. Of the 5 plasma cytokines measured being IL-1β, IL-2, IL-6, IL-10, and TNF-α, IL-10 anti-inflammatory cytokine was elevated in the iRBD patient's cohort (Kim et al., 2019). Case control study of 15 iRBD patients and 22 controls

that assessed immune activation and dopamine terminal function using PET imaging and measured PBMC monocytic immune markers using flow cytometry found immune changes in peripheral blood associated to immune and neuronal changes (Farmen et al., 2021). This finding is indicative of the crosstalk between the central and peripheral immune system and the potential of monocyte markers as diagnostic biomarkers for iRBD.

1.5.3. Peripheral inflammatory cytokines as PD biomarkers

The clinical diagnosis of PD occurs after significant neurodegeneration, currently preventing early intervention. PD has been suggested to commence as an immune event, and therefore inflammatory biomarkers that can detect and monitor alterations in inflammatory molecules at initial stages of disease pathology would be a major advantage (Liu et al., 2022). Much interest has been placed in the identification of plasma and serum inflammatory and pro-inflammatory cytokine measures as peripheral diagnostic and prognostic biomarker tools in PD. Though evident alterations in PD patients' inflammatory cytokine levels compared to controls has been identified in a vast number of studies, the utilisation of inflammatory cytokines as PD biomarkers remains insufficient. This is likely due to the inconsistency in literature findings, as trends in peripheral cytokine levels are not consistent across PD or different PD forms (Table 1.3). Additionally, inconsistency can be associated to cohort size differences and the lack of longitudinal studies (Table 1.3). Further, the lack of specificity and sensitivity in assessed inflammatory cytokine biomarkers decreases their practical utility as diagnostic and prognostic biomarkers (Qu et al., 2023). Sensitivity and specificity are also attributed to the form of technique used to measure cytokine levels. It is also significant to highlight, inflammatory profiles of PD patients change with disease progression. Inflammation is most prominent during the early stages of PD and then displays trends of maintained inflammation (Zimmermann & Brockmann, 2022). Furthermore, the overlapping inflammatory mechanisms

in disorders of the CNS, such as elevated levels of pro-inflammatory cytokine levels that can be observed in Alzheimer's disease, multiple sclerosis and PD challenges the development of disease specific biomarkers and targeted treatments (Cantero-Fortiz and Boada, 2024). Additionally, there is much to explore surrounding alterations in protein levels due to environmental and genetic factors in both healthy individuals and those with neurological disorders (Selvam and Ayyavoo, 2024). For the development of peripheral inflammatory biomarkers longitudinal studies that include multimodal cohorts and multiplex techniques that are of high sensitivity and specificity is required. Tables 1.3 and 1.4 provide summary of previous studies that have assessed the efficacy of peripheral inflammatory cytokines for PD biomarker application.

Peripheral Sample source	**Samples**		**Assay type / Marker**	**Summary**	**Reference**
	PD	**HC**			
Plasma	66	41	ELISA / PTX3	PTX3 may utilise as potential biomarker	Lee et al. (2011)
Plasma	36	36	ELISA / YKL040	YKL-40 involved in PD pathology	Zhao et al. (2022)
Blood	101	60	Nephelometric / CRP	CRP levels were significant indicators of peripheral inflammation	Solmaz et al. (2018)
Plasma	204	204	Nephelometric / CRP	Elevated CRP may be associated to PD severity	Yang et al. (2020)
Plasma	33	33	ELISA / SVCAM-1	Significant elevated levels of sVCAM1 in PD patients	Perner et al. (2019)
Serum	27	15	ELISA / NLRP3,	Elevated NLRP3	Chatterjee et al. (2020)

			IL-1β	IL-1β indication of peripheral inflammation	
Serum	55	40	ELISA / sTREM2	sTREM2 may utilise as potential biomarker	Peng et al. (2020)
Serum	58	60	- / SAA	Elevated levels of SAA in PD patients	Wu et al. (2021)
Plasma	76	76	CXCL12 CX3CL1 IL-8	Elevated levels of CXCL12, CX3CL1 and IL-8	Li et al. (2022)

Table 1.3 Summary of single cytokine inflammatory alterations observed in biofluid of PD patients for potential biomarker application.

Peripheral Sample source	Samples		Assay Type	Markers	Reference
	PD	HC			
Plasma	76	76	MSD	IL-8, CXCL12, CX3CL1, and CCL15	Li et al. (2022)
Plasma	248	149	ELISA	IL-6, IL-17A, TNF-α, LBP, age and sex	Chen et al. (2021)
Plasma	204	204	Nephelometric	CRP, HDL-C, LDL-C, SOD, cholesterol	Yang et al. (2020)
Serum	20	30	Luminex Xmap 7 markers	αlpha-synuclein, MMP-2 S100β and UCHL1	Calvani et al. (2020)
Serum	20	30	Luminex Xmap 7 markers	IL-8, IL-9, MIP-1α, MIP-1β, Citrulline, Phosphoetha-nolamine and Proline	Calvani et al. (2020)

Table 1.4 Summary of multiple cytokine inflammatory alterations observed in PD patients biofluid as potential biomarker application.

1.6.1 Thesis Aims

The identification of biomarkers for PD is of paramount interest. Due to their role in underlying PD pathology, mitochondrial and immune system readouts may comprise such biomarkers. The aim of this thesis is to determine if readouts of mitochondrial dysfunction and peripheral innate immune system inflammation can be utilised as diagnostic and / or prognostic biomarkers for PD.

Aim 1 (Chapter 3) - Optimise and validate a mitochondrial flow assay for the measurement of mitochondrial function using peripheral blood mononuclear cells.

Aim 2 (Chapter 4) – Use an optimised flow cytometry assay to measure mitochondrial function in subjects with PD, iRBD and healthy controls to determine if mitochondrial function can distinguish between groups or associates with clinical phenotypes.

Aim 3 (Chapter 5) - Measure serum pro-inflammatory cytokines in PD, iRBD and healthy controls to determine if cytokines can distinguish between groups or associate with clinical phenotypes.

Aim 4 (Chapter 6) – To determine if small molecular drug modulators of LRRK2 and GBA can ameliorate mitochondrial ROS present in PD PBMCs.

CHAPTER TWO

Materials and Methods

2.1 Participant recruitment

For optimisation of the mitochondrial flow cytometry assay, buffy coat samples were provided by the Australian Red Cross Lifeblood association with ethical approval granted by the University of Sydney human research ethics committee (#2017/847). Parkinson's disease (PD), idiopathic REM sleep behaviour disorder (iRBD) and neurologically normal control patients were recruited via the Parkinson's disease research clinic at the Brain and Mind Centre, University of Sydney. All recruited participants provided written informed consent. iRBD patients were diagnosed according to clinical history and video-polysomnography (Cesari et al., 2022) and PD patients were diagnosed according to clinically established criteria (Postuma et al., 2015). Recruited iRBD participants were excluded from studies if they did not meet the clinical history and video polysomnography of REM sleep, while PD participants were excluded if they did not meet the clinically established criteria. Collection of biosamples from recruited iRBD and PD patients was performed during ON therapy conditions. The Movement Disorders Society - Unified Parkinson's Disease Rating Scale (MDS-UPDRS) III, Montreal Cognitive Assessment (MoCA), Mini Mental State Examination (MMSE), Hoehn and Yahr staging scale (H & Y) and disease duration were recorded at the time of blood collection by a trained neurologist. To perform research studies ethical approval was obtained from the University of Sydney Human Research Ethics Committee (#2016/363) and (#2017/826).

Demographical, clinical and biomarker data of research participants is provided in subsequent chapters.

2.2 Isolation of peripheral blood mononuclear cells from whole blood

Up to 18 ml of whole blood was collected by venepuncture into sodium heparin tubes (BD). PBMCs were isolated from whole blood following the SepMate manufacturer instructions (StemCell Technologies). Whole blood was gently mixed with an equal volume of Phosphate Buffered Saline (PBS) containing 2% heat inactivated low endotoxin qualified fetal bovine serum (HI-FBS) (ThermoFisher, #10100-147) in a 50 mL tube. Diluted blood was then carefully transferred to a 50 mL SepMate tube (StemCell Technologies, #85450) containing 17 mL of density gradient medium. Samples were centrifuged at 1200 x g for 10 min at 21°C (acceleration at 9 and deceleration at 9) and the enriched PBMC layer transferred to a new 50 mL tube. PBMCs were then washed with PBS containing 2% HI-FBS and centrifuged at 300 x g for 8 min at 21°C (acceleration at 9 and deceleration at 9). Wash buffer was discarded, and cells were resuspended in 10 mL of PBS containing 2% HI-FBS, and the cell count was determined using 0.4% trypan blue stain (ThermoFisher, #T10282) and a Countess automated cell counter (ThermoFisher, #C10228). A final centrifugation at 300 x g for 8 min at 21°C (acceleration at 9 and deceleration at 9) was then performed. PBMCs were resuspended in cryopreservation media comprised of Roswell Park Memorial Institute (RPMI) 1640 Medium (ThermoFisher, #11875093), 20% HI-FBS and 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, #D2438), in cryovials at a density of 4 x 10^6 cells / mL and then placed in a MrFrosty Freezing Container (ThermoFisher, #5100-001) containing isopropanol (Sigma-Aldrich, ##190764) overnight in a -80°C freezer. PBMC samples were transferred to a vapour phase liquid nitrogen tank for long-term storage until required for experiments.

2.3 Isolation of peripheral blood mononuclear cells from buffy coat

To isolate peripheral blood mononuclear cells (PBMCs) from buffy coat, 15 mL of Ficoll Paque PREMIUM (GE Healthcare, #17-5442-02) was pipetted into a 50 mL centrifuge tube (Corning, #430829). The centrifuge tube was then held at an acute angle and 25 mL of buffy coat was carefully pipetted to the side of the centrifuge tube to prevent blood from disturbing the Ficoll Paque gradient. Once buffy coat was layered, samples were centrifuged at 400 x g for 30 min at room temperature (acceleration at 4 and deceleration at 0). A sterile glass pasteur pipette was used to gently collect the PBMC layer, which was then transferred into a new 50 mL centrifuge tube. PBMCs were then gently topped up with 40 mL of pre-warmed complete RPMI 1640 Medium (ThermoFisher, #11875093) supplemented with, 10% HI FBS (ThermoFisher, #10100-147), 1% Penicillin- Streptomycin (ThermoFisher, #15140-122) and 1% L-Glutamine (ThermoFisher, #25030081). Diluted PBMCs were then centrifuged at 300 x g for 10 min at room temperature (acceleration at 9 and deceleration at 9). Supernatant was discarded and PBMCs were gently resuspended in 30 mL of complete RPMI and cell count was determined as described in section 2.7. A final centrifugation at 300 x g for 10 min at room temperature (acceleration at 9 and deceleration at 9) was then performed, supernatant was removed and PBMCs were resuspended in chilled cryopreservation media that constituted of RPMI medium, 20% HI-FBS and 10% DMSO (Sigma-Aldrich, #D2438). PBMCs were aliquoted into cryovials at a density of 1 x 10^{7} cells / mL and then placed in a MrFrosty Freezing Container (ThermoFisher, #5100-0001) containing isopropanol (Sigma-Aldrich, #190764) overnight in a -80°C freezer. Following PBMC samples were transferred to a vapour phase liquid nitrogen tank until required for experiments.

2.4 Isolation of monocytes from peripheral blood mononuclear cells

To isolate monocytes from PBMCs, the EasySep Human Monocyte Isolation Kit (STEMCELL Technologies, #19359) was used. Cryopreserved PBMC samples were thawed and incubated with DNASE I solution (StemCell Technologies, #07900) at a concentration of 100 µg/mL for 15 min at room temperature. Cells were then filtered through a 37 µm cell strainer (StemCell Technologies, #27250), resuspended at 5 x 10^7 cells/mL in PBS medium (ThermoFisher, #14190144) containing 2% HI-FBS and 1 mM ethylenediamne tetraacetic acid (EDTA) (Ca++ and Mg++ free) and transferred to a 5 mL polystyrene round-bottom tube. 50 µL/mL of EasySep Human Monocytes isolation cocktail was added to sample, mixed well, and incubated at room temperature for 5 min. Magnetic capture beads were vortexed for 30 sec and added to cells. Samples were then mixed well and incubated for 5 min at room temperature, then topped up to 2.5 mL with PBS medium containing 2% HI-FBS and 1 mM EDTA (Ca++ and Mg++ free) and mixed gently by pipetting up and down three times. Tubes were then placed into the EasySep Magnet (StemCell Technologies, #18000) and incubated at room temperature for 2.5 min. In one motion EasySep Magnet containing sample was inverted into a new tube that collected enriched monocyte cell suspension. Cells were counted as described in section 2.7 and frozen down to 1 x 10^6 / mL.

2.5 Collection of plasma

Blood from each participant was collected in 10 mL K2-ethylenediaminetetraacetic acid (EDTA) - coated vacutainer tubes (BD Biosciences, #367525). Blood samples were gently inverted 8 times and then centrifuged 1500 x g for 15 min at 21°C (acceleration at 9 and deceleration at 5). The plasma layer was then transferred to a new 15 mL centrifuge tube,

aliquoted in 500 μL sizes, and immediately placed in a -80˚C freezer until required for experiments.

2.6 Collection of serum

Serum from each participant was collected in 8.5 mL Serum Separator Tubes (SST) II Advanced tubes (BD, Biosciences, #367958). Serum samples were incubated at room temperature for 30 min to allow blood to clot and were then gently inverted 5 times prior to centrifugation at 1500 x g for 15 min at 21˚C (acceleration at 9 and deceleration at 5). Serum was then transferred to a new 15 mL centrifuge tube, aliquoted in 500 μL sizes, and immediately placed in a 80˚C freezer until required for experiments.

2.7 Cell counting and cell viability

To determine cell counts and viability, 10 μL of cell suspension was gently mixed with 10 μL of trypan blue stain (0.4%), (ThermoFisher, #T10282) for a 1:1 ratio. 10 μL was then pipetted into countess chamber slide and inserted into Countess ™ automated cell counter (ThermoFisher, #C10228). Cell focus was adjusted through the touch screen and then data captured for cell count and viability percentage.

2.8 Flow cytometry

Cryopreserved PBMC samples were thawed in a 37°C bead bath until small ice crystals remained. Cells were then slowly pipetted into a 15 mL centrifuge tube that contained 7 mL of pre-warmed complete RPMI media. Samples were then centrifuged at room temperature at 300 x g for 10 min. Supernatant was removed and cells were resuspended in 8 mL of complete RPMI media. Cell count and viability was determined as outlined above. For experiments, cells were plated according to required cell concentration as detailed in subsequent chapters and left

to recover from thawing for up to 2 h in a 37°C tissue culture incubator. Cells were then treated as detailed in subsequent chapters. Following treatment, cells were then topped up with 150 μL of room temperature fluorescence-activated cell sorting (FACS) buffer (1x PBS, 1mM EDTA, 25mM HEPES, 1% HI- FBS) to terminate reaction samples were centrifuged at room temperature at 300 x g for 5 min. Cells were then stained in the dark with mitochondrial probes MitoSOX Red (ThermoFisher, #M36008) at 5 μM and MitoTracker Deep Red (ThermoFisher, #M22426) at 100 nm for 30 min at 37°C tissue culture incubator, shielded from light. Cells were then washed twice with FACS buffer and stained with surface stain master mix, constituting of Live/Dead viability dye (BD Biosciences, #564997) or (BD Biosciences, #565388) at 1:1000 dilution and FcR Blocking Reagent (Miltenyi Biotec, #130-059-901) at 1:20 dilution and incubated for 30 min at 4°C, shielded from light. Cells were then washed with FACS buffer and incubated with PE-Cy7 conjugated CD14 primary antibody (BD Biosciences, #557742) at 1:40 dilution for 30 min at 4°C, shielded from light. Cells were then washed twice with FACS buffer and resuspended in 300 μL of FACS buffer and transferred to 5 mL FACS tubes (Falcon, #352235). Samples were kept on ice and analysed immediately on a 3 – Laser Cytek Aurora Spectral Analyser (Cytek Biosciences). The number of events recorded per sample are detailed in subsequent chapters. FCS files were analysed using FlowJo Version 10.8 (BD Biosciences) with gating strategies detailed in subsequent chapters. For convenience, details of common reagents used for flow cytometry are provided in table 2.1.

2.9 Spectral unmixing workflow

Spectral unmixing of flow cytometry samples was performed on the Cytek 3-Laser Aurora cytometer (Cytek Biosciences). Prior to analysis, a quality control run was performed to ensure that cytometer was at optimal performance. Firstly, in the experiment template, individual fluorescent tags were selected for reference controls, then sample groups including reference

group were created with the number of events recorded per sample. Unstained and reference samples were run first to adjust forward and side scatter gates and to ensure that voltages were on scale and below intensity values of 1 x 10^6. Reference samples were then recorded. To perform spectral unmixing of reference samples, a gate was made on the population of interest on the FSC vs SSC plot, then positive and negative interval gates were made on the respective histogram plot. Then live unmixing was performed and samples were recorded. The MitoSOX Red marker was excited by the violet laser at peak channel of V10, whilst the MitoTracker Deep Red marker was excited by the red laser at peak channel of R2. This ensured that autofluorescence spill over was avoided.

2.10 Bio-Plex Assay

To measure plasma cytokine markers, the Bio-Plex Pro Human Cytokine 27-Plex Panel Kit (Bio-Rad, # M50-0KCAF0Y) was used. Plasma samples were thawed and diluted 1:4 with Bio-Plex sample diluent. A 96 well plate (Bio-Rad, #171-025001) was prewet with 100 μL of assay buffer, liquid was then thoroughly removed with vacuum filtration and by blotting on clean paper towel. Coupled beads were vortexed for 30 sec at medium speed and poured into a reagent reservoir. Using a multichannel pipette, 50 μL of coupled beads were then transferred into each well. The plate was then washed twice with 100 μL Bio-Plex wash buffer. Diluted plasma samples were gently vortexed for 5 sec and 50 μL of plasma sample was then added into each well of the assay plate. The assay plate was then covered with sealing tape and shielded from light using aluminium foil and incubated with shaking at 850 rpm at room temperature for 30 min. Plates were then washed 3 x 100 μL with wash buffer. Detection antibody was vortexed gently for 5 sec and poured into reagent reservoir. 25 μL of detection antibody was then transferred into each well using a multichannel pipette, which was covered with foil and incubated with shaking at 850 rpm for 30 min at room temperature. Following

incubation, plates were washed 3 x 100 μL with wash buffer. Streptavidin-PE was vortexed at medium speed for 5 sec and 50 μL of diluted streptavidin-PE was pipetted into each well. Plate was covered with new sealing tape, shielded from light using aluminium foil and left to incubate with shaking at 850 rpm for 10 min at room temperature. Following incubation sealing tape was gently removed and plate was then washed 3 x 100 μL with wash buffer. For plate reading beads were resuspended with 125 μL of assay buffer to each well. Plate was covered with new sheet of sealing plate and then shook for 30 sec at 850 rpm at room temperature. Sealing plate was then carefully removed and plates were immediately read using a Bio-Plex MAGPIX Multiplex Reader instrument, (Bio-Rad, #171-015001). Cytokine concentrations were then extracted using the standard curve and Bio-Plex Manager™ software (Bio-Rad).

2.11 MSD S-Plex Proinflammatory Kit

To measure serum inflammatory cytokine markers, the Meso Scale Discovery (MSD) S-Plex Proinflammatory Kit (MSD, #K15396S) was used. Serum samples were thawed, centrifuged at 2000 x g for 3 min at 4°C and kept on ice while assay reagents were bought to room temperature. S-Plex 96-Well plates were washed 3 x with 150 μL 1 x MSD wash buffer. Each well was then coated with 50 μL of the coating solution, plates were gently sealed and incubated with shacking at 700 rpm for 1 h at room temperature. During this incubation period, calibrators were prepared as per the manufacturer instructions. After incubation, plates were again washed 3 x 150 μL /well with 1 x MSD wash buffer. Then, 25 μL of blocking solution was added per well and gently tapped on all sides. 25 μL of serum sample was added per well, plates carefully sealed with adhesive, and incubated with shacking at 700 rpm for 1.5 h at room temperature. Plates were then washed again with 3 x 150 μL /well 1 x MSD wash buffer, and 50 μL of TURBO-BOOST antibody solution was added per well. Plates were sealed and incubated with shacking at 700 rpm for 1 h at room temperature. During this incubation step,

then S-PLEX enhance reagents E1, E2 and E3 were thawed and prepared as per manufacturer instructions, 30 min prior to use. Plates were again washed with 3 x 150 µL / well of 1 x MSD wash buffer. 50 µL of enhance solution that contains enhance reagents E1, E2 and E3 was added per well and plates sealed to incubate with shacking at 700 rpm for 30 min at room temperature. TURBO-TAG detection solution was then prepared as per manufacturer instructions, plates washed with 3 x 150 µL / well of 1 x MSD wash buffer and 50 µL of TURBO-TAG added per well. Plates were then sealed and incubated with shacking at 700 rpm at strictly 27°C. A final wash of 3 x 150 µL / well of 1 x MSD wash buffer was carefully conducted and 150 µL of MSD GOLD Read Buffer B was added to each well. Plates were then immediately read on MSD reader instrument (MESO QuickPlex SQ 120MM).

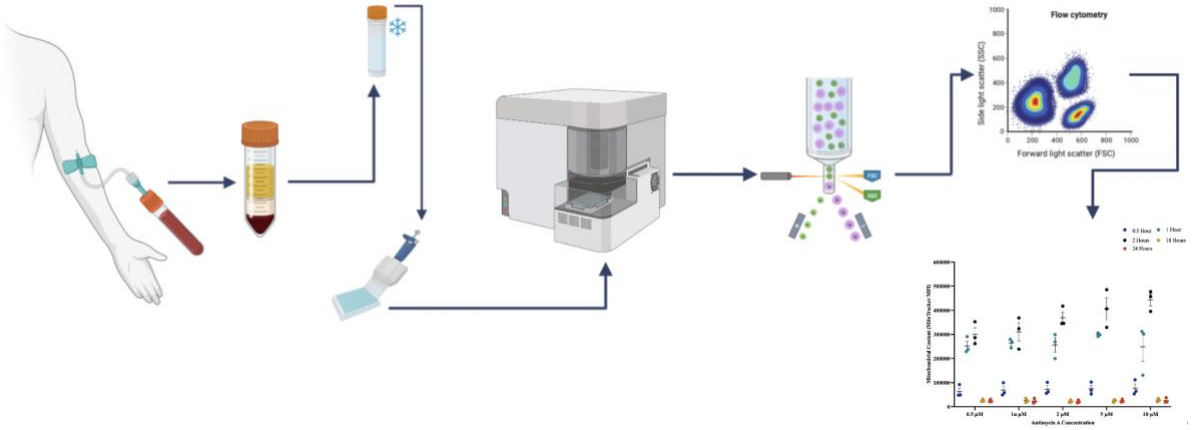


Figure 2.1 Workflow of mitochondrial function flow cytometry assay. Bloods were drawn from patients and PBMCs / monocytes were isolated and cryopreserved. Cells were treated, then stained with mitochondrial probes and surface stain markers. Live cells were analysed on Cytek 3-Laser Aurora cytometer, monocytes were gated, and FCS files were analysed on FlowJo version 10.08. Analyses were performed on IBM SPSS statistics 27 and GraphPad Prism 9 with data presented in the form of individualised dot plot graphs that included mean ± standard error of the mean (SEM). Created with BioRender.com.

Reagent	Concentration / Dilution	Company	Catalogue number
Antimycin A	specific to chapter	Sigma-Aldrich	A8674
DMSO	-	Sigma-Aldrich	D2438
FcR Blocking Reagent	1:20	Miltenyi Biotec	130-059-901
FVS700	1:1000	BD BioSciences	564997
FVS780	1:1000	BD BioSciences	565388
HI-FBS	-	ThermoFisher	10100-147
RPMI 1640	-	ThermoFisher	11875093
L-Glutamine	1 % of total volume	ThermoFisher	25030081
MitoSOX RED	5 μM	ThermoFisher	M36008
MitoTracker Deep Red	100 nm	ThermoFisher	M22426
PBS	-	ThermoFisher	10010023
PE-Cy7 conjugated primary antibody	1:40	BD BioSciences	557742
Penicillin Streptomycin	1 % of total volume	ThermoFisher	15140122
Trypan Blue Stain (0.4%)	1:1 ratio	ThermoFisher	T10282
UltraComp Plus Beads		ThermoFisher	01-3333-41

Table 2.1 List of common reagents used in experimental research.

2.12 Statistical analysis

Data and statistical analyses were performed using IBM SPSS Statistics 27 software and GraphPad Prism v. 9.4.0, with statistical significance determined at p value < 0.05. To assess differences between groups, two-way analysis of variance (ANOVA) with Tukey's post-hoc tests or parametric T-Tests were applied as indicated. With normal distribution assessed using the Anderson-Darling test with normality accepted at p value > 0.05. All tests passed normality therefore no data transformations were required. Two-way ANOVA was applied to assess differences between demographic and clinical data for chapters 4 and 5 and Mann-Whitney U Test was applied for chapter 6. Spearman correlation analyses were applied to assess

relationships between mitochondrial markers, inflammatory measures and clinical variables. Graphs were generated through GraphPad Prism v. 9.4.0 and depict mean ± standard error of the mean (SEM) with dots representing individual values.

CHAPTER THREE

Establishing a Flow Cytometry Assay to Measure Mitochondrial Function

3.1 Introduction

The incidence and prevalence of Parkinson's disease (PD) identify it as the fastest-growing neurological condition (Dorsey et al., 2018) and one of the most prevalent neurodegenerative diseases (Deliz et al., 2024). Though considerable progress on the differential diagnosis of PD has developed (Munhoz et al., 2024), misdiagnosis of PD remains significantly high (Rizzo et al., 2016) and clinical diagnosis remains challenging (Tolosa et al., 2021). The diagnosis of PD is based upon the presentation of combination of motor symptoms (Kouli et al., 2018), which usually manifest at the degeneration of ~ 50% of dopaminergic neurons (Lopez-Aguirre et al., 2023) and for the vast majority a definitive diagnosis can only be provided post-mortem (Signaevsky et al., 2022). The development of sensitive and specific blood-based diagnostic biomarkers that support early accurate diagnosis and therapeutic intervention presents as an urgent pressing need (Tönges et al., 2022).

Mitochondrial dysfunction and elevated mitochondrial reactive oxygen species (ROS) have identified as key hallmarks in the aetiology of both familial and sporadic PD (Henrich et al., 2023). Mitochondria are highly dynamic multifunctional organelles (Monzel et al., 2023), that are essential to the normal functioning of neurons (Duarte et al., 2023). Dopaminergic neurons are heavily dependent on mitochondrial bioenergetics for the maintenance of neuronal homeostasis and survival and therefore are vulnerable to mitochondrial dysfunction (Gao et al.,

2022). Impaired mitochondria are associated with increased levels of ROS (Chen et al., 2019). Maintained levels of mitochondrial ROS molecules have significant role in the normal physiology of cells due to their contribution in signalling and immune responses. Excessive ROS generation that surpasses the cell's ability to detoxify, results in oxidative stress that leads to cellular damage, inflammation, elevated cellular stress response and impaired mitochondrial function (Sun et al., 2024). Further oxidative stress promotes the aggregation of alpha-synuclein (α-syn) in dopaminergic neurons, causing further increased production of intracellular ROS (Xiang et al., 2013). Consequently, mitochondrial dysfunction and elevated levels of mitochondrial ROS are central factors in the degeneration of dopaminergic neurons (Liu et al., 2017). Indeed, epidemiological, histopathological, preclinical, and clinical trial data have suggested mitochondrial dysfunction as being a significant contributing factor to the pathogenesis of PD (Henrich et al., 2023). Therefore, impaired mitochondrial function and increased mitochondrial ROS have risen as potential biomarkers of PD. Significantly, preliminary studies suggest that mitochondrial dysfunction is also evident in peripheral blood mononuclear cells (PBMCs) of PD patients (Qadri et al., 2018; Smith et al., 2018). PBMCs are easily accessible and may be reflective of deeper tissue pathology, therefore, their use as a sample source for exploring promising potential early diagnostic and prognostic biomarkers of disease has notably risen (Alexovic et al., 2023).

Flow cytometry technique allows for single cell measurements and characterisation of heterogeneous cell populations. Due to the advantage of this technique, it has been recently widely applied in translational biomarker research (Ullas & Sinclair, 2024). To further the development of mitochondrial peripheral biomarkers, in this chapter I have set up and optimised the flow cytometry technique reported by Smith et al. (2018) in order to assess peripheral mitochondrial dysfunction in an Australian PD cohort.

3.2 Hypothesis

That flow cytometry can be employed for the measurement of mitochondrial function in peripheral blood mononuclear cells.

3.3 Aims

1. Determine the optimal incubation time-points of mitochondrial probes MitoSOX Red and MitoTracker Deep Red.
2. To validate mitochondrial probe ability to measure mitochondrial ROS and mitochondrial content.
3. To observe the effect of varied timepoints and concentrations of Antimycin A on cell viability.
4. Determine the optimal concentration of antimycin A to induce ROS.

3.4 Chapter Specific Methods

3.4.1 Isolation of peripheral blood monocular cells

Isolation of peripheral blood mononuclear cells is as described in methods chapter, section 2.3.

3.4.2 Flow cytometry

Mitochondrial flow cytometry assay was performed as described in methods chapter, section 2.8 without CD14 and FcR block surface staining. Cells were plated at a cell density of 1 x 10^6 / mL and left to recover from thawing for up to 2 h in a 37°C tissue culture incubator. Samples receiving treatment were stimulated with 0.5 or 1 μM of antimycin A for 1, 2, 3 ,4 or 24 h. For live cell analysis spectral unmixing was performed as described in methods chapter, section 2.9 with 100,000 events recorded.

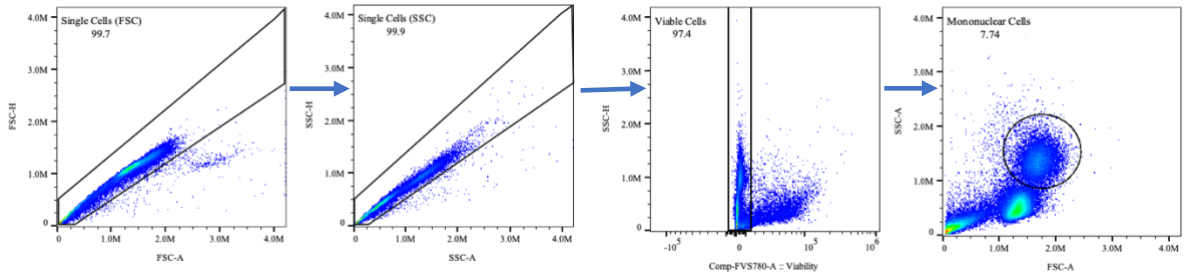


Figure 3.1 Flow cytometry gating strategy for mononuclear cells isolated from healthy donor PBMCs. Initially two singlet gates of FSC-A x FSC – H and SSC-A x SSC-H were made. Following to gate on viable cells Comp-FVS780 x SSC-H gate was created, then mononuclear cells were gated on using FSC-A x SSC-A gate. The median fluorescent intensity (MFI) of MitoSOX Red and MitoTracker Deep red is stratified from mononuclear cells.

3.5 Results

3.5.1 Detection of mitochondrial ROS production and content is optimal at 30-min incubation

Specific flow cytometry mitochondrial probes were applied for live cell measurement of mitochondrial ROS and content. To measure mitochondrial ROS, the MitoSOX Red (ThermoFisher, #M36008) probe was utilised. MitoTracker Deep Red (ThermoFisher, #M22426) was applied for the measurement of mitochondrial content. For the identification of the most optimal time point for the dual measurement of mitochondrial ROS and mitochondrial content in human mononuclear cells, a flow cytometry time course assessment was first conducted. The concentration of MitoSOX Red probe was kept constant at 5 μM, and the concentration of MitoTracker Deep Red probe was kept constant at 100 nm based on the study by Smith et al. (2018). The median fluorescent intensity (MFI) values of MitoSOX Red (Figure 3.2A) and MitoTracker Deep Red (Figure 3.2B) probes at 15, 30, 60 and 120 min time points were then measured. Measurement of monocyte mitochondrial ROS was considered optimal at 30 min incubation timepoint, as the signal was in the middle of the linear range allowing for any increases or decreases in intensity to be measured. Additionally, significant MFI signal for mitochondrial content was only observed at 30 min time point.

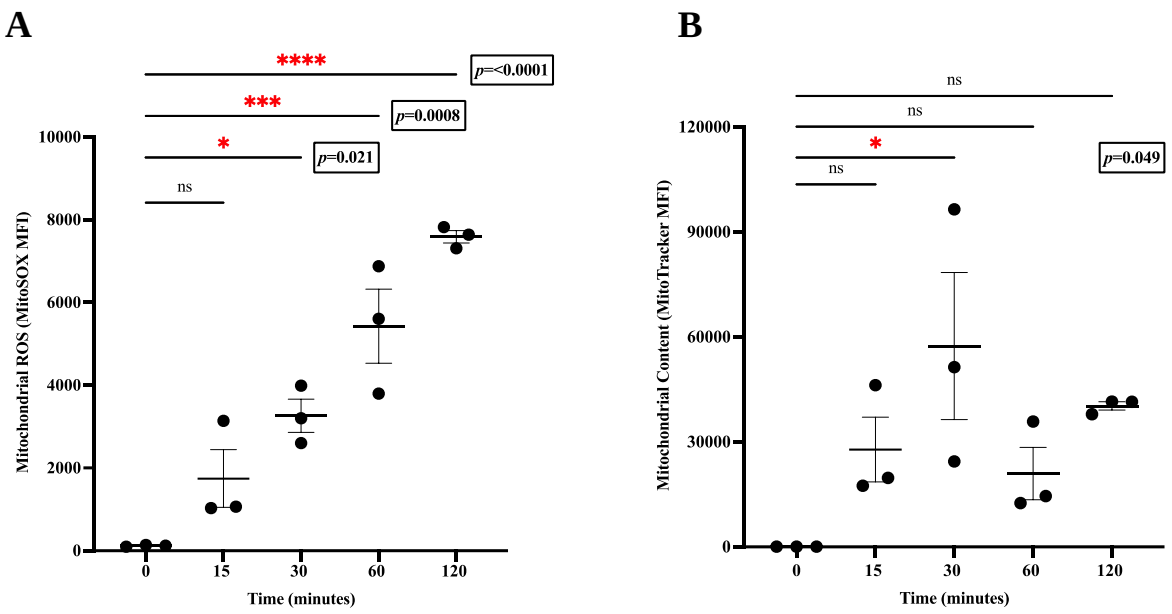


Figure 3.2 Assessment for optimal incubation time-point for median fluorescence intensity (MFI) measure of MitoSOX Red and MitoTracker Deep Red. MFI of Mitochondrial Specific markers, MitoSOX Red (Figure 3.2A) and MitoTracker Deep Red (Figure 3.2B) measured over 2 h, for the detection of optimal incubation time point. Human PBMCs (N=3) were isolated using Ficoll Paque and assessed for mitochondrial ROS and mitochondrial content via flow cytometry. Two-Way ANOVA with Tukey's post-hoc test. Graphs display mean ± SEM with dots representing individual value. Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$.

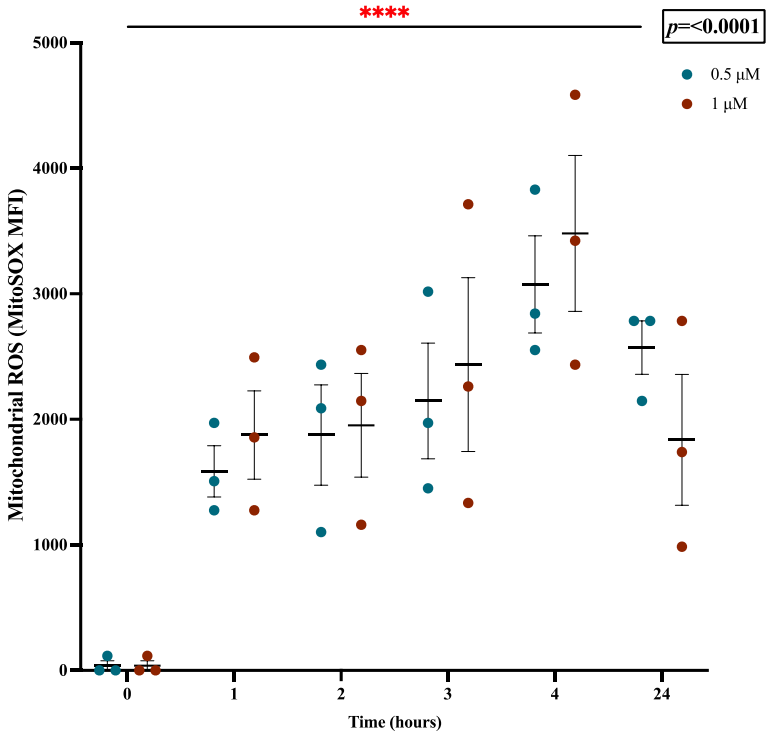
3.5.2 Validation of mitochondrial assay

To validate that the flow cytometry mitochondrial probes can detect increased mitochondrial ROS levels, healthy donor PBMCs were stimulated with 0.5 µM or 1 µM with mitochondrial respiratory chain inhibitor, antimycin A (Sigma-Aldrich, #A8674) for 1, 2, 3, 4 and 24 h time points. Treatment of PBMCs with antimycin A should increase ROS due to the inhibition of mitochondrial complex III through blockage of electron flow of cytochromes b and c. 0.5 µM and 1 µM of antimycin A concentrations were chosen based on the study conducted by Polster

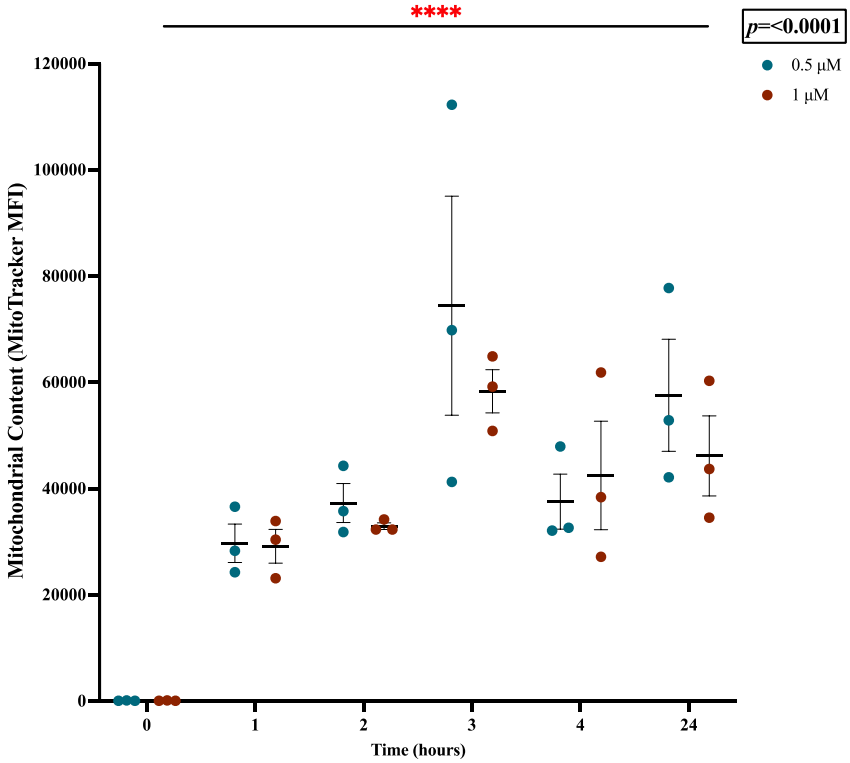
et al. (2014), that suggested antimycin A concentration should not exceed 2 μM if mitochondrial ROS is measured through MitoSOX probe.

The overall effect of antimycin A on mitochondrial ROS in healthy control PBMCs presented a near linear increase of mitochondrial ROS MFI till the 4 h time point, followed by a decrease of mitochondrial ROS MFI at 24 h time point (p=<0.0001) (Figure 3.3A). This may potentially be due to cellular apoptosis, due to prolonged treatment time. Mitochondrial content displayed increased MFI levels with increased incubation of antimycin A, as 0.5 μM of antimycin A presented increased MFI read out at time points 1, 2, 3 and 24 h (p=<0.0001) (Figure 3.3B). The data suggests that antimycin A incubation time has a greater influence on mitochondrial content MFI levels than antimycin A concentration. Viability of cells decreased with increased incubation time points compared to the 0 time point. Similar to mitochondrial content, increased incubation time had a significant effect on viability as MFI measures of 0.5 μM or 1 μM of antimycin A were relatively similar at single time points (p=<0.0001) (Figure 3.3C).

A


****
p=<0.0001
0.5 µM
1 µM
Mitochondrial ROS (MitoSOX MFI)
5000
4000
3000
2000
1000
0
0
1
2
3
4
24
Time (hours)

B


****
p=<0.0001
0.5 µM
1 µM
Mitochondrial Content (MitoTracker MFI)
120000
100000
80000
60000
40000
20000
0
0
1
2
3
4
24
Time (hours)

C

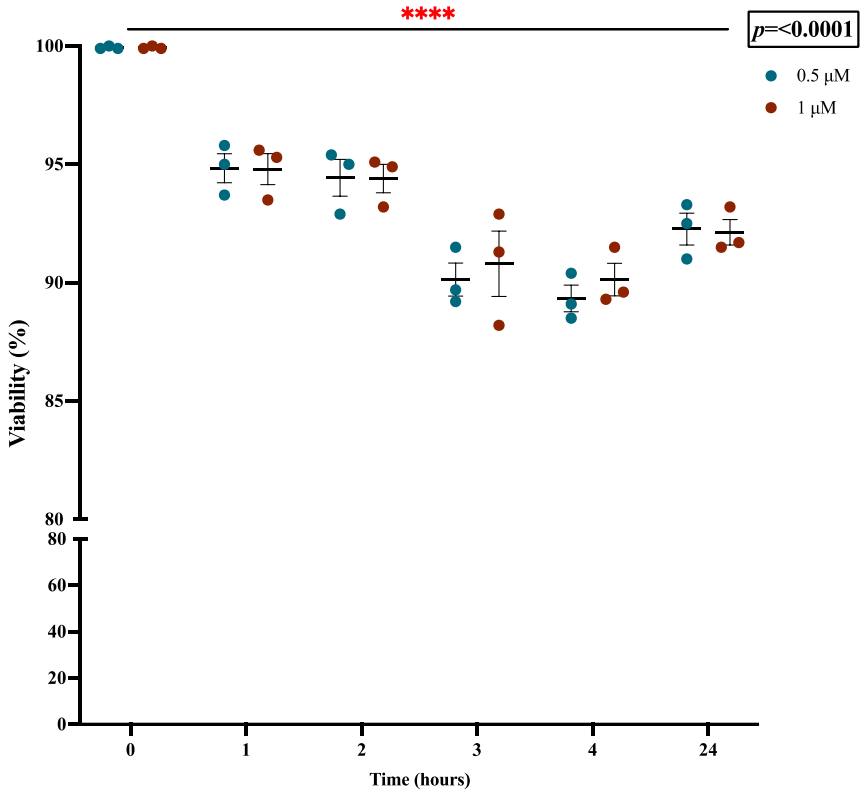


Figure 3.3 Validation of mitochondrial probes MitoSOX Red and MitoTracker Deep Red in measuring mitochondrial ROS and mitochondrial content. Flow cytometry MFI measures of healthy donor PBMCs (N=3) with 0.5 µM or 1 µM of antimycin A inhibitor for 1, 2, 3, 4, and 24 h. Difference of mitochondrial ROS MFI measures of PBMCs untreated, PBMCs incubated with 5 µM MitoSOX Red probe for 30 min, or PBMCs stimulated with 0.5 µM or 1 µM antimycin A for 1, 2, 3, 4 and 24 h and then incubated with 5 µM of MitoSOX for 30 min (Figure 3.3A). Difference of mitochondrial content MFI measures of PBMCs untreated, PBMCs incubated with 100 nm MitoTracker Deep Red probe for 30 min, or PBMCs stimulated with 0.5 µM or 1 µM antimycin A for 1, 2, 3, 4 and 24 h and then incubated with 100 nm of MitoTracker Deep Red probe for 30 mins (Figure 3.3B). Flow cytometry viability of healthy donor PBMCs untreated or treated with 0.5 µM or 1 µM antimycin A for 1, 2, 3, 4, and 24 h and then incubated with mitochondrial probes (Figure 3.3C). Two-Way ANOVA with Tukey's post-hoc test. Graphs display mean ± SEM with dots representing individual value. Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$.

3.6 Discussion

Fluorescent probes have influenced the understanding of mitochondrial biology and have provided insight to the efficacy of potential disease modifying therapeutic interventions (Chu et al., 2021). For the development of peripheral biomarkers for the clinical diagnosis and monitoring of PD fluorescent probes have emerged as robust tools (Chu et al., 2021; Sun et al., 2024). Fluorescent probes provide live, sensitive and selective detection of altered mitochondrial biology. Furthermore, they are non-invasive and have sufficient usability whilst being cost effective. For the application of fluorescent probes in routine clinical practice further research is required into the practical utility of fluorescent technology. The first being to void the risk of toxicity from probes impacting cell biology and the biocompatibility of fluorescent probes with different cell types. Further the stabilisation of maintained fluorescence, for long term clinical application (Sun et al., 2024). Longitudinal, multicentre studies, with diverse PD population will assist in the validation and transition of mitochondrial fluorogenic probes as biomarkers into clinical practice.

The use of fresh or cryopreserved mononuclear cells for the assessment of mitochondrial function for biomarker development is dependent on experimental research design. Experimental application on fresh or cryopreserved mononuclear cells each have their own advantages and limitations. Fresh mononuclear cells provide the advantage of more accurate representation of mitochondrial function and membrane potential, however, have very limited shelf life required for experimental application (< 24 h). Cryopreserved mononuclear cells provide the advantage of standardisation, as studies can be performed in batches within specific time frame and controlled conditions, therefore reducing variability, which is of significance for the biomarker discovery studies. Study conducted by Korandová et al. (2024) assessed the utility of cryopreserved PBMCs as a diagnostic tool for mitochondrial disease through the

measurement of mitochondrial function of 47 patients with confirmed or suspected mitochondrial disease. The study found that 72% of patients presented with asserted diagnosis of mitochondrial disease, while 13% of patients presented false positive diagnosis. The finding of the study conducted by Korandová et al. (2024) and the studies conducted by Smith et al. (2018) and Qadri et al. (2018) that are specific to PD patients present the promising potential of cryopreserved PBMCs for mitochondrial biomarkers. Furthermore, to reduce the impact of cryopreservation, standardised cryopreservation and thawing protocols is essential to maintain the integrity of mitochondrial function.

In this chapter, healthy donor cryopreserved PBMCs were utilised for the optimisation and validation of a flow cytometry assay to assess mitochondrial function. Mitochondrial function was assessed using the MFI signal of mitochondrial ROS (MitoSOX Red) and mitochondrial content (MitoTracker Deep Red) probes. Assessing the MFI of the probes over a time course resulted in the selection of 30 min as the optimal incubation time for the flow cytometry assay, I aimed to establish. This finding is also supported by the study of Smith et al. (2018) who also reported using a 30 min incubation time when assessing mitochondrial function using the same mitochondrial ROS and mitochondrial content probes used in this study (Smith et al., 2018).

To validate the mitochondrial function assay, PBMC samples were then stimulated with 0.5 μM or 1 μM of antimycin A for 1, 2, 3, 4 and 24 h. Results showed an increase in mitochondrial ROS up until the 4 h time point. This suggests that stimulating PBMCs with antimycin A for the generation of ROS is ideal within this time range. A prior study using antimycin A to stimulate ROS production in primary human RPE cells also reports a 4 h incubation as being sufficient to stimulate ROS production (Hytii et al., 2019). This result was attributed to disturbed mitochondrial membrane potential due to the inhibition mitochondrial complex III of the electron transport chain (Hytii et al., 2019).

Stimulation of healthy donor PBMCs with antimycin also showed increased mitochondrial content with increased antimycin A incubation time. Antimycin A was used to model mitochondrial dysfunction observed in PBMCs of PD patients. This observation is possibly reflective of impaired mitophagy leading to increased accumulation of damaged mitochondria in these cells. In addition to impaired mitophagy being observed in PD models (Liu et al., 2019), stimulation of human glioblastoma H4 cells exposed with antimycin A has been found to inhibit the autophagosome process through inhibition of complex III of the mitochondrial electron transport chain (Ma et al., 2011); a shared mechanism that could also be attributed to mitophagy. Together this supports the aim of having established a working model that reflects mitochondrial dysfunction in PD (positive control) where stimulation of antimycin A is associated with elevated mitochondrial ROS and mitochondrial content. Similar results were observed in both the 0.5 μM and 1 μM antimycin A treatment groups, suggesting that alterations of antimycin A concentration within this range did not impact these findings. Furthermore, though inhibition of mitochondrial complex III generated mitochondrial dysfunction, the combined inhibition of mitochondrial complex I with rotenone and mitochondrial complex III with antimycin A may have better modelled mitochondrial dysfunction PD pathology in healthy donor PBMCs.

Overall, this chapter determined the optimal time point for the staining of PBMCs with the mitochondrial probes. This chapter also established working conditions by which antimycin A is able to induce mitochondrial ROS and increased content in healthy donor PBMCs; a positive control to be further optimised and used in the following chapter.

CHAPTER FOUR

Elevated Levels of Mitochondrial ROS and Mitochondrial Content in Monocytes of Parkinson's Disease Patients

4.1 Introduction

Previous studies have suggested that mitochondrial dysfunction can be detected in peripheral blood mononuclear cells (PBMCs) from Parkinson's disease (PD) patients (Qadri et al., 2018; Smith et al., 2018). However, how robust this finding is across multiple cohorts, including in Australia is still unknown. In the previous chapter I therefore established a flow cytometry assay to assess mitochondrial function in PD patients from an Australian cohort. As PD likely initiates 15-20 years prior to the onset of clinical symptoms (Jankovic & Tan, 2020), there is much interest in investigating biomarkers in prodromal cohorts. The best-established prodromal PD cohort is patients that have been diagnosed with REM sleep behaviour disorder (RBD) (Tan et al., 1996). More than 80% of RBD patients develop neurodegenerative synucleinopathy conditions within 10 years (Zhang et al., 2020), with ~50% of iRBD patients developing PD (Postuma et al., 2019). A recent study conducted by Ongari et al. (2024) assessed mitochondrial function in fibroblasts from 10 RBD patients, 8 RBD-PD patients (previous RBD patients) and 10 healthy controls, and found that RBD patients displayed impaired bioenergetics profile, that worsened in RBD-PD patients (Ongari et al., 2024). RBD-PD patients also displayed significant reduction in mitochondrial complex III and V levels (Ongari et al., 2024). The findings suggest that impaired mitochondrial function potentially predisposes during RBD disease phase and promote progression of RBD to PD. Deficiency in

mitochondrial bioenergetic profiles of PD patient monocytes was manifest in a study conducted by Smith et al. (2018), in which the PD patients presented significantly elevated levels of mitochondrial ROS compared to controls (Smith et al., 2018). The findings from Ongari et al. (2024) and Smith et al. (2018) suggest that mitochondrial function in RBD and PD present as potential diagnostic and prognostic biomarkers, as well as a biomarker for monitoring the conversion of RBD to PD.

Antimycin A is an antibiotic produced from several species of streptomyces that is commonly used for the study of mitochondrial function due to its powerful inhibitor properties. Treatment of cells with antimycin A inhibits the flow of electrons to mitochondrial complex III, causing the inhibition of the electron transport chain (ETC) (Ogita et al., 2009). This leads to decreased mitochondrial membrane potential and increased generation of mitochondrial ROS. In the application of research, antimycin A is applied to cells as a positive control to generate increased mitochondrial ROS production (Hytii et al., 2019). This allows for the study of molecular and cellular processes of mitochondrial function and oxidative stress for identification of biomarkers and disease modifying therapies. In previous studies for the validation of potential PD pharmacodynamic biomarkers, PD patient derived neurons were treated with antimycin A to induce mitochondrial stress (Hsieh et al., 2019). The study found that treatment of neurons with antimycin A for 6 h resulted in neuronal cell death and that treatment with Miro1 reducer rescued neuronal degeneration, induced by antimycin A treatment (Hsieh et al., 2019). Specific to this chapter, antimycin A was optimised to model mitochondrial dysfunction present in PD; the optimised model was applied to assess differences between mitochondrial ROS levels and mitochondrial content levels at baseline and at stimulation.

In the current chapter I have therefore employed the mitochondrial flow cytometry assay developed in chapter 3 and assessed mitochondrial ROS and mitochondrial content in monocytes from PD patients and patients diagnosed with iRBD. Moreover, cells were stimulated with and without the mitochondrial respiratory chain inhibitor antimycin A, to determine if mitochondrial dysfunction can be potentiated in the disease groups.

4.2 Hypothesis

That monocyte mitochondrial dysfunction can be measured in PD and iRBD patients compared to controls, and this dysfunction will be further potentiated following treatment of cells with antimycin A.

4.3 Aims

1. To further optimise conditions of antimycin A inhibitor to induce mitochondrial dysfunction in PBMCs through inhibition of mitochondrial complex III.
2. To determine if mitochondrial ROS or content are altered in iRBD and PD patient monocytes at baseline or with antimycin A stimulation compared to controls.
3. Determine any correlations present between mitochondrial measures and PD or iRBD clinical markers.

4.4 Chapter Specific Methods

4.4.1 PBMC isolation

PBMC isolation from whole blood is as described in methods chapter, section 2.2.

4.4.2 Live Flow cytometry assay for measurement of Mitochondrial function

Mitochondrial flow cytometry assay was performed as described in methods chapter, section 2.8. Cells were plated at a cell density of 1 x 10^6 / mL and left to recover from thawing for up to 2 h in a 37°C tissue culture incubator. Samples receiving treatment were stimulated with 0.5, 1, 2, 5 or 10 μM of antimycin A for 0.5, 1, 2, 18 or 24 h. For live cell analysis spectral unmixing was performed as described in methods chapter, section 2.9 with 100,000 events recorded.

4.4.3 Statistical Analysis

Data and statistical analyses were performed using GraphPad Prism v. 9.4.0 and IBM SPSS Statistics 27 software, with statistical significance determined at p value < 0.05. To assess differences between groups two-way analysis of variance (ANOVA) and parametric T-Tests with normal distribution applied for figures 4.2 A-C and figures 4.3 A-C. Two-way ANOVA was applied to assess differences between demographic and clinical data. Spearman correlation analyses were applied to assess relationship between PBMC mitochondrial markers and clinical variables, in the iRBD and PD cohort. Graphs were generated through GraphPad Prism v. 9.4.0 and depict mean ± standard error of the mean (SEM) with dots representing individual values.

4.5 Results

4.5.1 Evaluation of control, iRBD and PD demographic and clinical data

Demographic and clinical data of control, iRBD and PD patients is provided in Table 1. To eliminate the potential cofounding effects of age and sex on the results of the study, all participants were age and sex matched. Significant differences between control and iRBD patients were found in in the Sniffin Sticks test for olfactory function. Several significant

clinical differences were observed between the control and PD group, specifically being the Movement Disorder Society - Unified Parkinson's Disease Rating Scale (MDS – UPDRS) III for motor symptom severity, body mass index (BMI) scale, Sniffin Sticks test, Farnsworth-Munsell test for colour vision impairment, Epworth Sleepiness Scale (EES) and cell viability. The significant differences observed between control and PD patients was also observed between iRBD and PD patients in addition to the depression hospital anxiety and depression scale (HADS).

	Control	**iRBD**	**PD**	***p*-value (Control & iRBD)**	***p*-value (Control & PD)**	***p*-value (iRBD & PD)**
Age (years)	67 ±1.48	69.7	72 ± 1.80	0.48	0.12	0.69
Sex (%M)	57%	60.6%	55.9 %	0.99	0.97	0.93
Disease duration	N/A	3.03 ± 0.73	9.21± 1.34	N/A	N/A	** 0.0013
MDS - UPDRS III	4.97 ± 0.94	8.09 ± 1.33	34.64 ± 2.90	0.40	**** < 0.0001	**** < 0.0001
MMSE	28.41 ± 0.29	28.6 ± 0.21	27.53 ± 0.60	0.98	0.37	0.24
MoCA	26.82 ± 0.45	26.73 ± 0.39	25.06 ± 0.85	0.99	0.09	0.13
LEDD	N/A	N/A	771.09 ± 143.373	N/A	N/A	N/A
H & Y	N/A	N/A	2.08 ± 0.19	N/A	N/A	N/A

Sniffin Sticks Test	9.75 ± 0.31	7.4 ± 0.52	5.12 ± 0.42	** 0.0025	*** <0.0001	*** 0.0007
Farnsworth-Munsell Test	93.83 ± 7.62	104.93 ± 10.93	161.33 ± 21.51	0.85	** 0.006	* 0.012
EES	4.78 ± 0.548.	4.79 ± 0.61	7.53 ± 0.94	0.99	** 0.004	** 0.003
BMI	28 ± 0.96	27.37 ± 0.62	24.47 ± 0.63	0.63	* 0.02	0.25
Statins	1.41 ± 0.11	1.31 ± 0.11	1.43 ± 0.09	0.77	0.86	0.46
Anxiety HADS	3.32 ± 0.49	4.45 ± 0.79	5.76 ± 0.62	0.72	0.06	0.25
Depression HADS	3.08 ± 0.44	2.72 ± 0.47	4.68 ± 0.64	0.91	0.09	* 0.02
Family history	0.069	0.12 ± 0.05	0.27 ± 0.08	0.81	0.18	0.44
Viability	81.45 ±2.01	77.82 ± 2.24	68.15 ± 4.00	0.28	** 0.009	0.31

Table 4.1 Demographic and clinical biomarker table for iRBD, PD and controls. Data are mean ± standard error with range shown in parenthesis. (N= 35 control, 33 iRBD and 34 PD). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$.

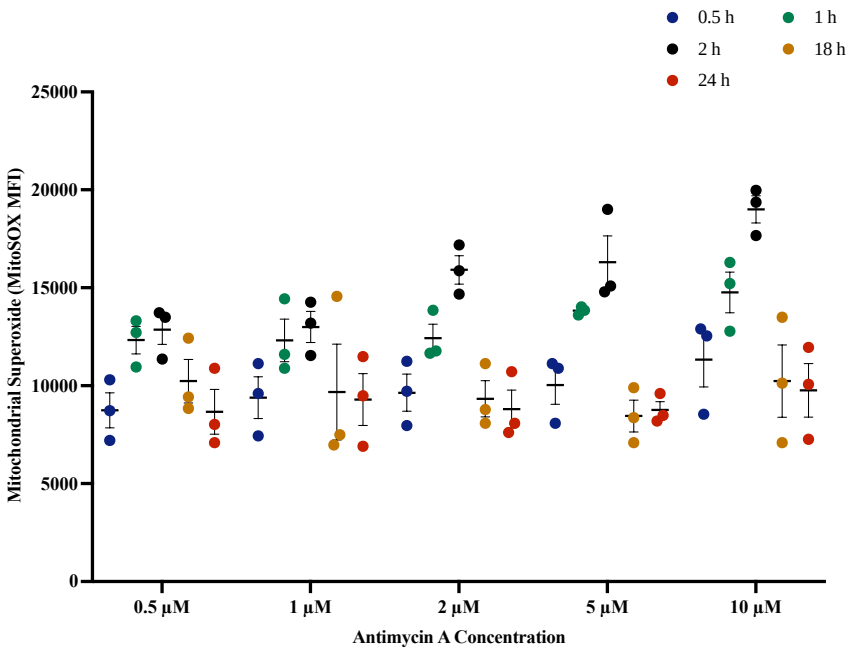
Impaired mitochondria have a multi-faceted role in the development of PD due to the complexity of the organelle and its significance in maintaining cellular homeostasis and normal functioning of neuronal cells (Trinh et al., 2021). To analyse and compare mitochondrial dysfunction in control, iRBD and PD, specific mitochondrial markers measured levels of mitochondrial ROS and mitochondrial content at baseline and at stimulation via flow

cytometry. Stimulation was induced through antimycin A, potent inhibitor of complex III of the ETC, to consequently impact mitochondrial dynamics to mirror PD pathology.

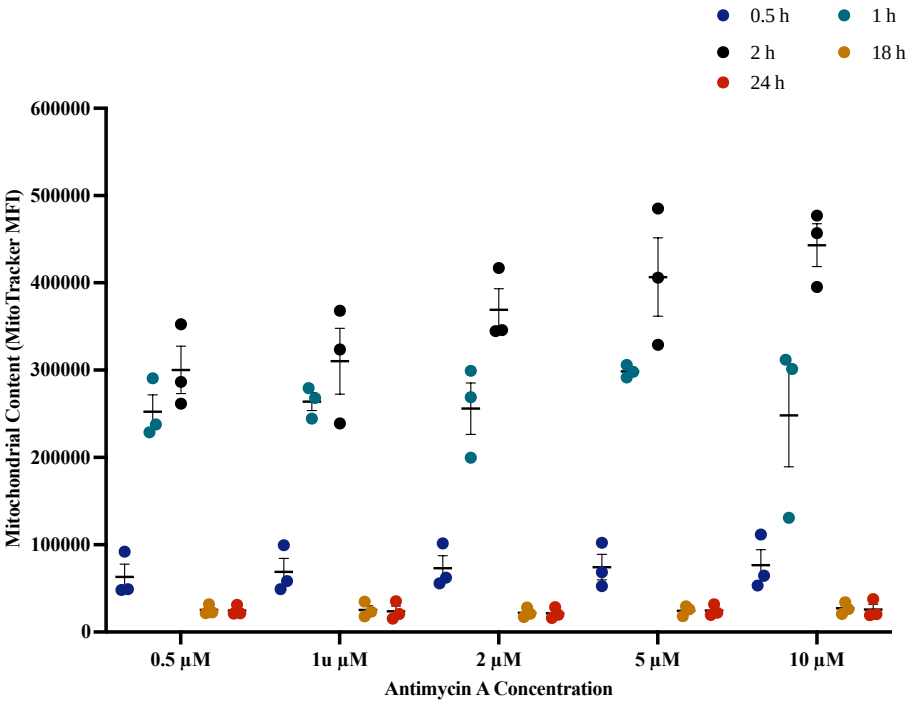
4.5.2 Optimisation of antimycin A stimulation

To determine optimal stimulation conditions of PBMCs with antimycin A, experiments with different antimycin A concentrations and time points were performed. PBMCs from healthy donors were used along with mitochondrial probes MitoSOX Red to measure ROS production and MitoTracker Deep Red to measure mitochondrial content in $CD14^+$ monocytes (Figure 4.1A and 4.1B). Further, the effect of antimycin A concentrations and time points on cell viability was measured via the MFI of FVS780 dye (Figure 4.1C). The optimal concentration for inhibition was selected on the principle of inducing mitochondrial dysfunction whilst maintaining cell viability. 2 μM of antimycin A at 2 h incubation generated elevated mitochondrial ROS and mitochondrial content signal, whilst maintained decent cell viability. Though 0.5 μM of antimycin A had higher cell viability, the MitoSOX MFI and MitoTracker MFI were low regardless of concentration. Therefore, 2 μM of antimycin A for 2 h at 37°C in a tissue culture incubator was chosen. Gating strategy of PBMCs on $CD14^+$ monocytes, population of interest is shown in Figure 4.1D.

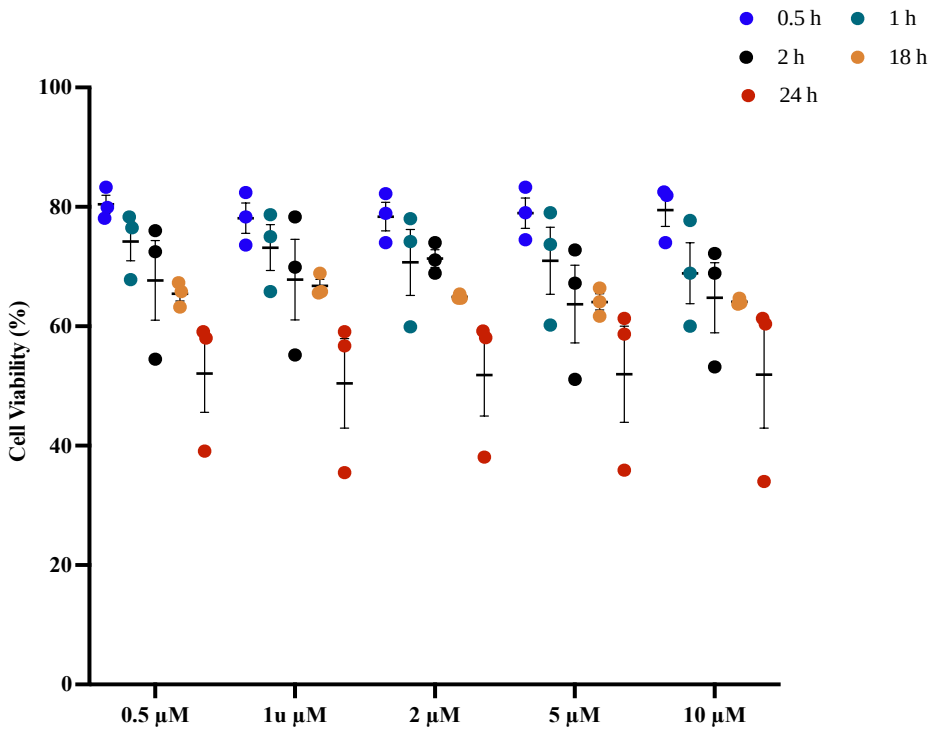
A


0.5 h
1 h
2 h
18 h
24 h
Mitochondrial Superoxide (MitoSOX MFI)
25000
20000
15000
10000
5000
0
0.5 µM
1 µM
2 µM
5 µM
10 µM
Antimycin A Concentration

B


0.5 h
1 h
2 h
18 h
24 h
Mitochondrial Content (MitoTracker MFI)
600000
500000
400000
300000
200000
100000
0
0.5 µM
1u µM
2 µM
5 µM
10 µM
Antimycin A Concentration

C


0.5 h
1 h
2 h
18 h
24 h
Cell Viability (%)
100
80
60
40
20
0
0.5 µM
1u µM
2 µM
5 µM
10 µM

D

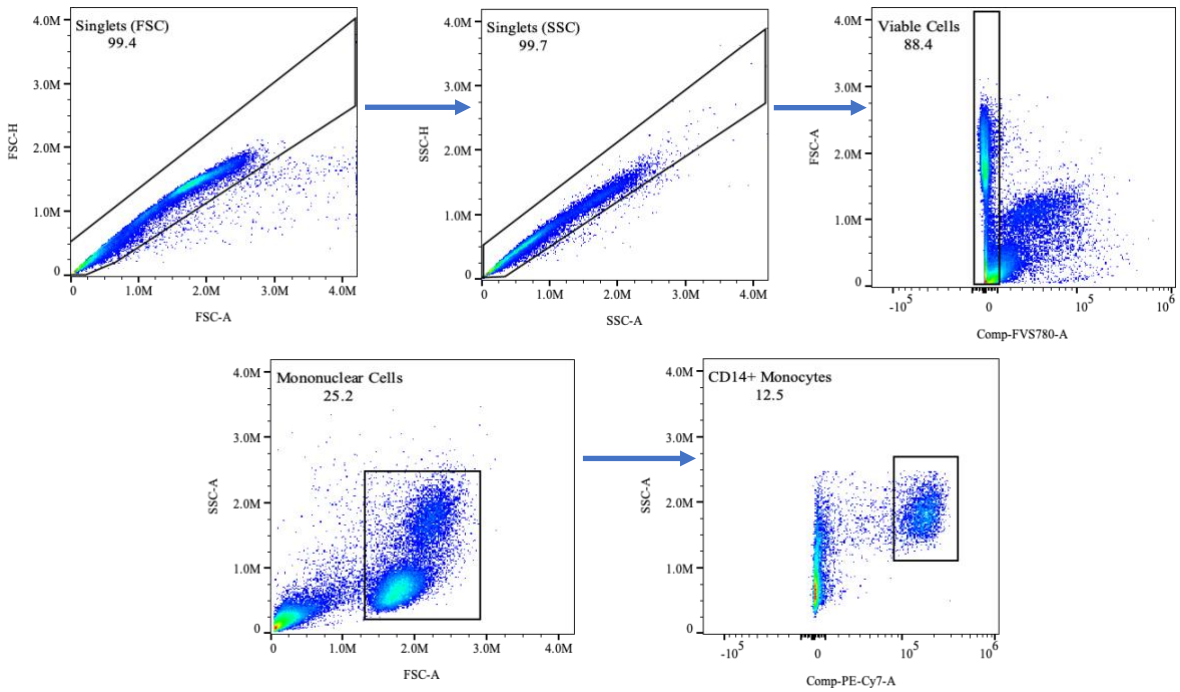
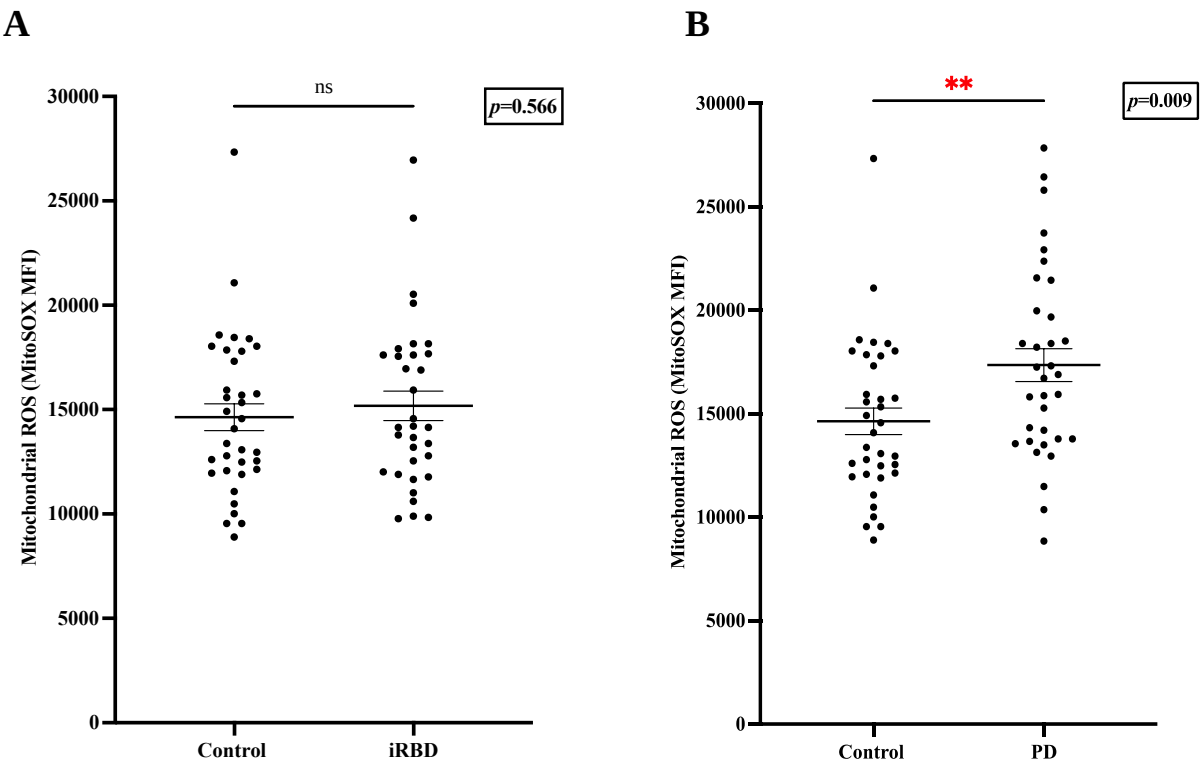

Singlets (FSC)
99.4
FSC-H
FSC-A
Singlets (SSC)
99.7
SSC-H
SSC-A
Viable Cells
88.4
FSC-A
Comp-FVS780-A
Mononuclear Cells
25.2
SSC-A
FSC-A
CD14+ Monocytes
12.5
SSC-A
Comp-PE-Cy7-A

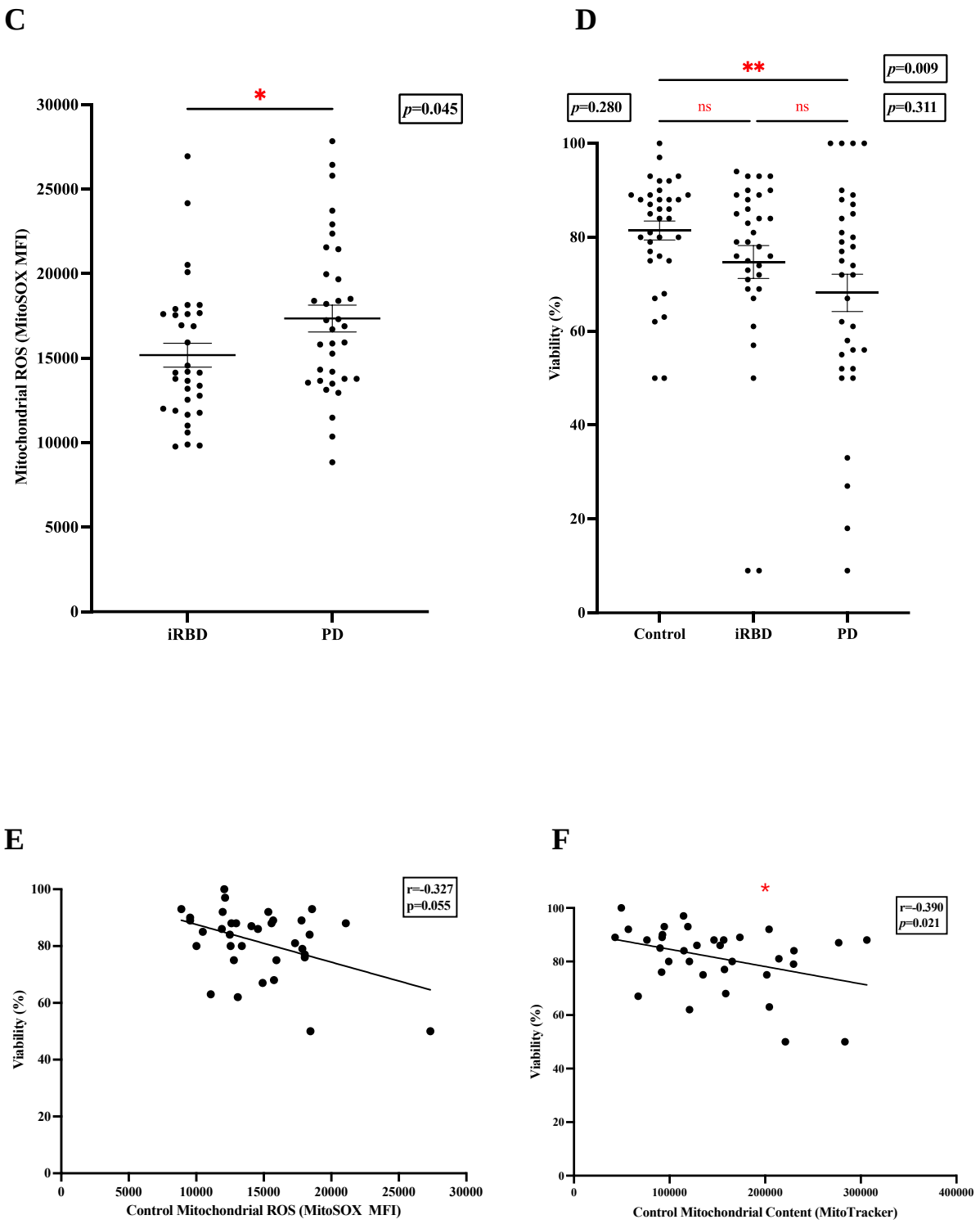
Figure 4.1 Flow cytometry to determine optimal stimulation conditions. Effect of different time points (0.5, 1, 2, 18 and 24 h) and various antimycin A concentrations (0.5 μM, 1 μM, 2 μM, 5 μM and 10 μM) on ROS release (Figure 4.1A). Effect of different time points (0.5, 1, 2, 18 and 24 h) and various antimycin A concentrations (0.5 μM, 1 μM, 2 μM, 5 μM and 10 μM) on mitochondrial content (Figure 4.1B). Effect of concentration and time points of antimycin A on monocyte viability (Figure 4.1C). Mitochondrial function assay to measure mitochondrial median fluorescence intensity through flow cytometry. Applied gating strategy. To exclude doublets, FSC – A x FSC – H and SSC-A and SSC- H parameters were applied. Viable cells were then gated on followed by two subset gates on mononuclear cells and $CD14^+$ monocytes populations (Figure 4.1D). Graphs depict means ± standard error of mean (SEM) (N= 3).

4.5.3 Significant difference in oxidative stress in Parkinson's patient monocytes

To assess differences in mitochondrial ROS levels in control, iRBD and PD patient monocytes at baseline, MitoSOX MFI was measured. Parametric unpaired t-test showed no significant difference between control and iRBD patients at baseline (p=0.566) (Figure 4.2A). However, significant differences in mitochondrial ROS levels were observed between control and PD patients (p=0.009) **(**Figure 4.2B**)** and between iRBD and PD patients' monocytes (p=0.045) (Figure 4.2C). Two-way ANOVA to assess cell viability differences among groups revealed no difference between control and iRBD patient monocyte viability (p=0.280), however significant differences between control and PD patient monocytes (p=0.009) and no significant differences in viability between iRBD and PD patient monocytes (p=0.311) (Figure 4.2D). To determine if cell viability influenced monocyte mitochondrial ROS and mitochondrial content levels of control, iRBD and PD patients at baseline, Spearman correlation analyses were performed. The data revealed no significant effect of viability on control monocyte mitochondrial ROS (Figure 4.2E). However, control monocytes presented significantly negative association between viability and mitochondrial content (r=-0.390, p=0.021) (Figure

4.2F). No significant association was observed between viability and iRBD monocyte mitochondrial ROS (Figure 4.2G) or mitochondrial content (Figure 4.2H). There was also no significant correlation observed between viability and PD monocyte mitochondrial ROS (Figure 4.2I) and mitochondrial content (Figure 4.2J).




C
*
p=0.045
Mitochondrial ROS (MitoSOX MFI)
30000
25000
20000
15000
10000
5000
0
iRBD
PD
D
**
p=0.009
p=0.280
ns
ns
p=0.311
Viability (%)
100
80
60
40
20
0
Control
iRBD
PD
E
r=-0.327
p=0.055
Viability (%)
Control Mitochondrial ROS (MitoSOX MFI)
0
5000
10000
15000
20000
25000
30000
F
*
r=-0.390
p=0.021
Viability (%)
Control Mitochondrial Content (MitoTracker)
0
100000
200000
300000
400000

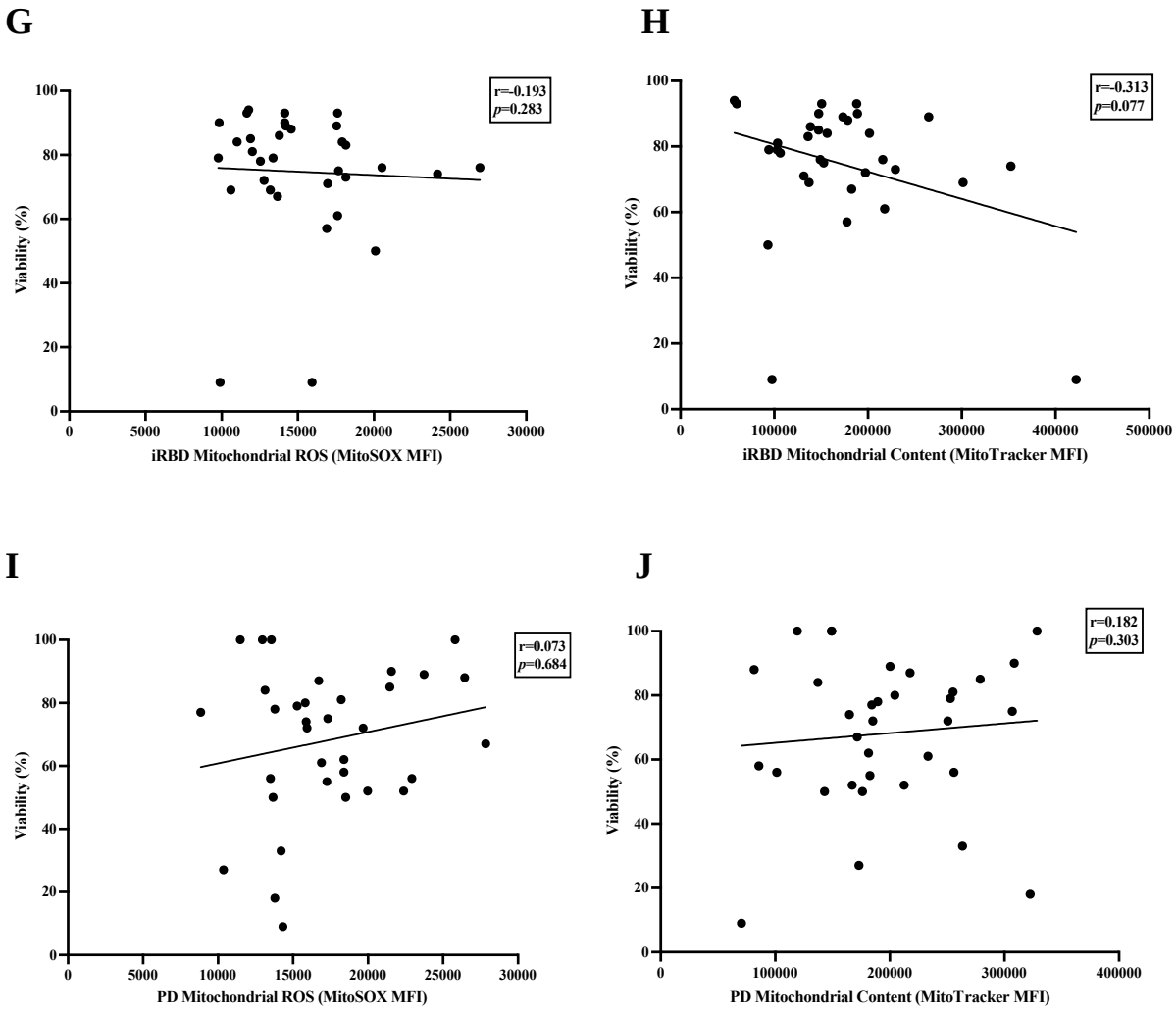
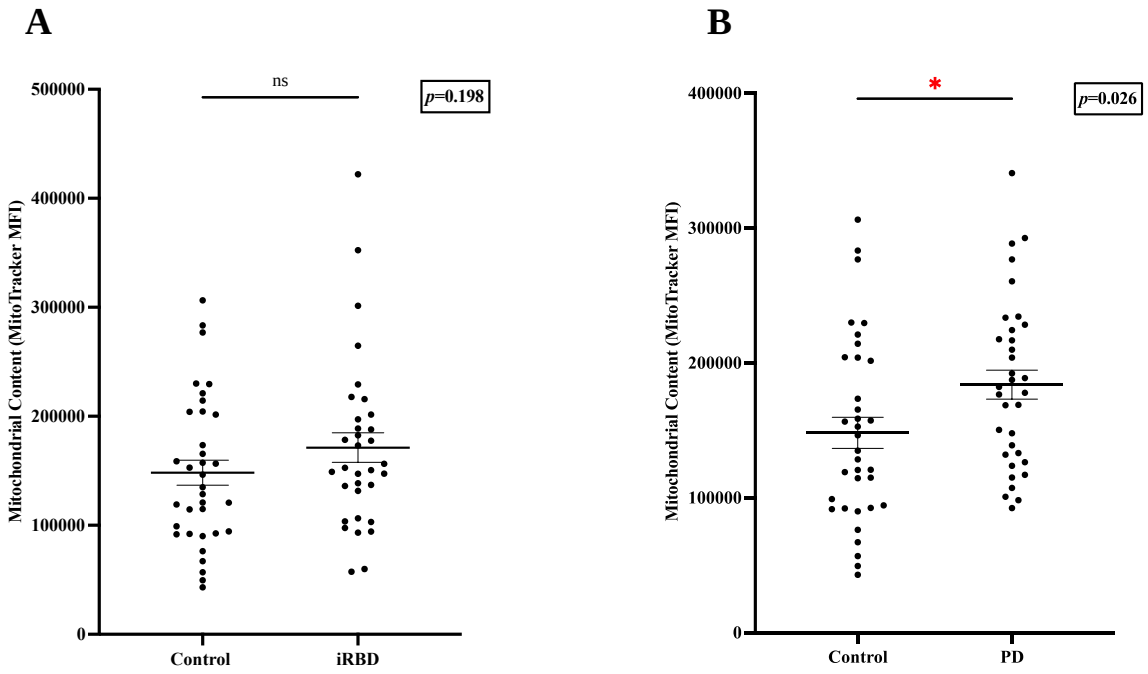


Figure 4.2 Mitochondrial ROS MFI levels at baseline from PBMC samples of control, iRBD and PD patients. Individual value plots display MFI of mitochondrial ROS levels of control, iRBD and PD in figures 4.2A, 4.2B and 4.2C. Parametric unpaired T-Test of control and iRBD displays no significant differences (Figure 4.2A). Parametric unpaired T-Test of control and PD display significant differences of mitochondrial ROS between control and PD (Figure 4.2B) and iRBD and PD (Figure 4.2C). Viability of control, iRBD and PD patient PBMC samples (Figure 4.2D). Spearman correlations analyses to assess association between viability and monocyte mitochondrial ROS and mitochondrial content of control, iRBD and PD patients (Figures 4.2E – J). No significant association was observed between viability and control monoctye mitochondrial ROS (Figure 4.2E), significant negative correlation present between control viability and mitochondrial content (Figure 4.2F). Association between viability and iRBD monocyte mitochondrial ROS (Figure 4.2G) and mitochondrial content (Figure 4.2 H) was not observed. Correlation of PD cell viability and monocyte mitochondrial

ROS (Figure 4.2I) and mitochondrial content (Figure 4.2J) revealed no singificant associations. Graphs 4.2A-D depict means ± SEM. (N= 35 control, 33 iRBD and 34 PD). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$.

4.5.4 Significant difference in mitochondrial content in Parkinson's patients' monocytes

No significant difference was found in mitochondrial content levels between controls and iRBD (p=0.198) (Figure 4.3A). A statistically significant difference was observed for mitochondrial content levels between control and PD patient monocytes (p=0.026) (Figure 4.3B), but again no statistical difference was found between iRBD and PD patient monocytes (p=0.463) (Figure 4.3C).



C

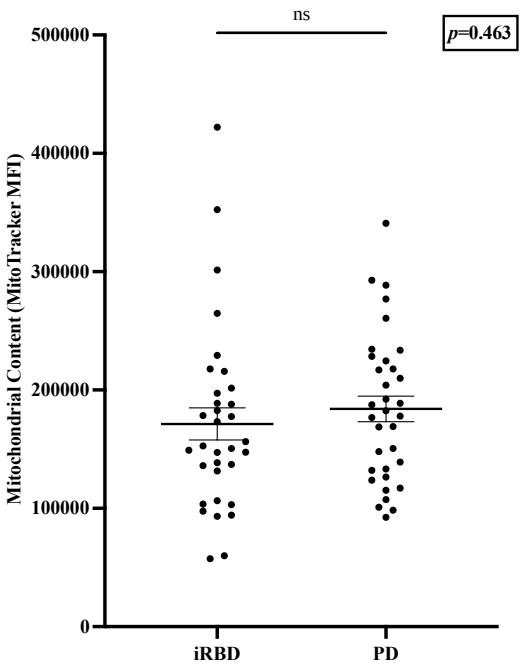


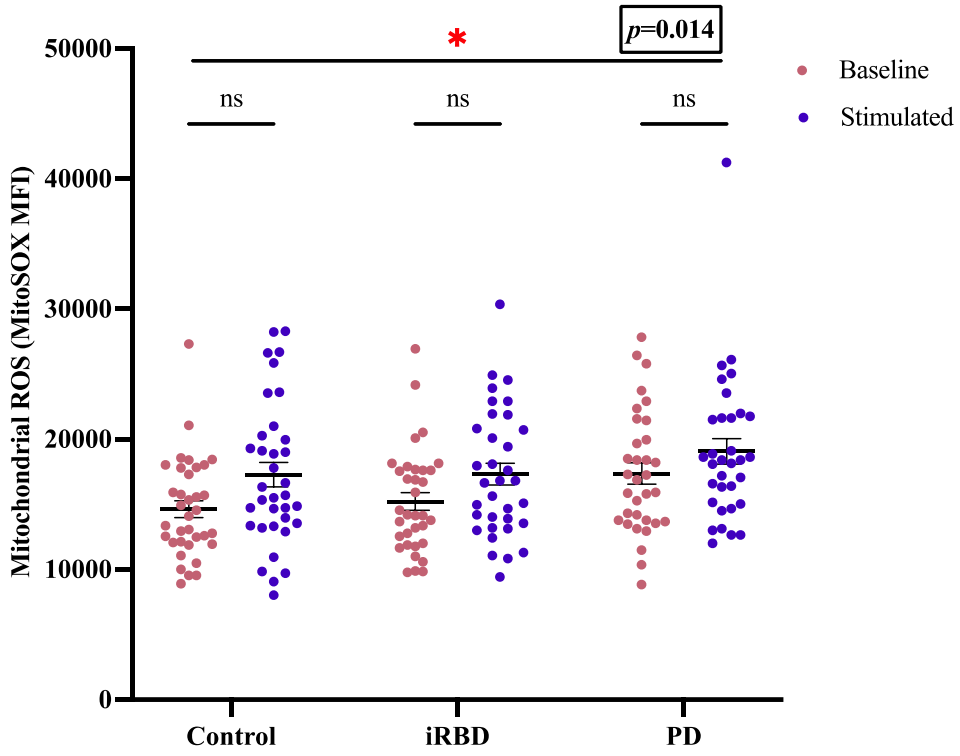
Figure 4.3 Mitochondrial content MFI levels at baseline from CD14$^+$ monocyte samples of control, iRBD and PD patients. Individual value plots display MFI of mitochondrial content level of control, iRBD and PD in figures 4.3A, 4.3B and 4.3C. Parametric unpaired T-Test of control and iRBD present no statistical significance (Figure 4.3A), statistically significant difference present between control and PD (Figure 4.3B) and no significant difference between iRBD and PD group (Figure 4.3C). Graphs depict means ± SEM (N= 35 control, 33 iRBD and 34 PD). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$.

4.5.5 Mitochondrial function of PBMCs at Stimulation

To determine the effect of stimulation on control, iRBD and PD patient monocytes, Two-Way ANOVA analysis was applied. The overall effect of stimulation on control, iRBD and PD monocytes presented statistically significant difference between CD14$^+$ monocyte samples at baseline and at stimulation for mitochondrial ROS release (p=0.014). Though an overall effect of stimulation was statistically significant for monocyte mitochondrial ROS, the data presented no significant difference between groups for mitochondrial ROS release; controls (p=0.06),

iRBD (p=0.21) and PD (p=0.39) (Figure 4.4A). Similar to mitochondrial ROS, stimulation of mitochondrial content presented an overall significant effect of stimulation on control, iRBD and PD monocytes (p=0.027). The data presented no significant effect of stimulation on mitochondrial content between groups; controls (p=0.65), iRBD (p=0.98) and PD (p=0.87) (Figure 4.4B).

A



B

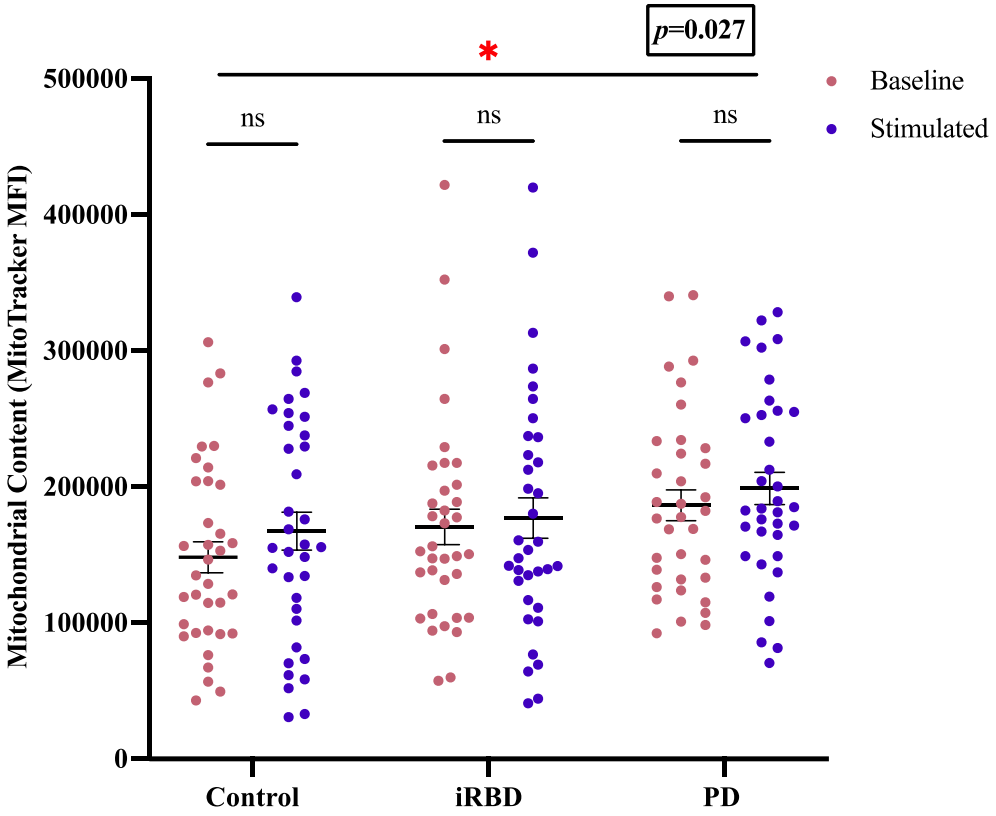


Figure 4.4 Effect of stimulation on mitochondrial ROS and mitochondrial content levels. Individual value plots display MFI of mitochondrial ROS and mitochondrial content level of control, iRBD and PD at baseline and at stimulation in A and B. Two-Way ANOVA analysis, reveal effect of row in mitochondrial ROS release (Figure 4.4A) and mitochondrial content levels (Figure 4.4B) across cohort. Following Šídák's multiple comparison test was performed to assess differences between groups for mitochondrial ROS and mitochondrial content levels. No significant differences were observed. Graphs depict means ± SEM (N= 35 control, 35 iRBD and 35 PD). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$.

4.5.6 Mitochondrial measures correlate to PD and iRBD clinical variables at baseline

To determine if association between $CD14^{+}$ monocytes mitochondrial ROS, mitochondrial content, MitoSOX:Tracker ratio and clinical variables are present in the current PD and iRBD

cohort, spearman correlation analyses were performed. Mitochondrial ROS release to mitochondrial content was measured to normalise mitochondrial ROS levels. Mitochondrial ROS at baseline of PD patients positively correlated to age (r=0.416, *p*=0.015), BMI (r=0.389, *p*=0.045), and mitochondrial content to statins medication (r=0.403, *p*=0.022). Further positive correlations to clinical variables were observed in the iRBD cohort in which, mitochondrial ROS release also correlated to age (r=0.380, *p*=0.029) and Mini Mental State Examination (MMSE) (r=0.381, *p*=0.038). Positive correlation of iRBD patients' mitochondrial content to the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) III (r=0.507, *p*=0.003) was also observed. Negative correlation was observed between iRBD patients' mitochondrial content and the Montreal cognitive assessment (MoCA) scale (r=-0.375, *p*=0.035). MitoSOX:MitoTracker ratio of $CD14^+$ monocytes at baseline of PD and iRBD patients did not reveal any positive correlations to clinical variables. However, a negative correlation was observed between MitoSOX:MitoTracker ratio at baseline and the MDS-UPDRS III score (r=-0.383, *p*=0.030) in the iRBD cohort.

4.5.7 Stimulation of PD and iRBD patients PBMCs positively correlates to clinical markers

Mitochondrial ROS levels in stimulated PD PBMCs positively correlated to the Hoehn and Yahr (H&Y) scale (r=0.354, *p*=0.040) and to the MDS-UPDRS III score (r=0.344, *p*=0.046). A negative association was observed between MitoSOX:MitoTracker ratio at stimulation and the MMSE (r=-0.406, *p*=0.021) in the PD group.

Clinical variable	Mitochondrial ROS (Baseline)	Mitochondrial Content (Baseline)	Mitochondrial ROS (Stimulated)	Mitochondrial Content (Stimulated)	MitoSOX: MitoTracker (Baseline)	MitoSOX: MitoTracker (Stimulated)
Age	*r=0.416 p=0.015	r=0.183 p=0.300	r=0.209 p=0.236	r=0.005 p=0.977	r= 0.083 p=0.400	r=0.127 p=0.474

Sex	*r=-0.368 p=0.032	r=-0.160 p=0.366	*r=-0.347 p=0.044	r=-0.109 p=0.541	r=-0.063 p=0.722	r=-0.045 p=0.799
Disease Duration	r=0.109 p=0.548	r=-0.095 p=0.597	r=0.210 p=0.241	r=-0.114 p=0.529	r=0.215 p=0.230	r=0.328 p=0.062
MDS-UPDRS III	r=0.196 p=0.267	r=-0.019 p=0.917	*r=0.344 p=0.046	r=0.011 p=0.952	r= 0.117 p= 0.511	r=0.227 p=0.197
MMSE	r=-0.008 p=0.964	r=0.103 p=0.574	r=-0.231 p=0.204	r=0.232 p=0.202	r=-0.143 p=0.436	*r=-0.406 p=0.021
MoCA	r=-0.179 p=0.311	r=-0.12 p=0.944	r=-0.257 p=0.143	r=0.001 p=0.997	r=-0.093 p=0.600	r=-0.277 p=0.196
LEDD	r=0.080 p=0.654	r=-0.267 p=0.127	r=0.115 p=0.516	r=-0.184 p=0.297	r=0.302 p=0.083	r=0.321 p=0.064
BMI	*r=0.389 p=0.045	r=0.197 p=0.325	r=0.089 p=0.659	r=0.087 p=0.666	r=0.055 p=0.785	r=-0.124 p=0.537
H & Y	r=0.196 p=0.267	r=-0.075 p=0.671	*r=0.354 p=0.040	r=-0.106 p=0.552	r=0.163 p=0.357	*r=0.385 p=0.024
Statins	r=0.167 p=0.360	*r=0.403 p=0.022	r=-0.195 p=0.286	r=0.143 p=0.434	r=-0.239 p=0.188	r=-0.266 p=0.141
RBDSQ	r=0.155 p=0.388	r=-0.128 p=0.477	r=-0.042 p=0.818	r=-0.196 p=0.274	r=0.164 p=0.361	r=0.078 p=0.665

Table 4.2 PD patient's mitochondrial measures correlate to clinical measures. Spearman correlations at baseline and at stimulation between mitochondrial ROS production, mitochondrial content, MitoSOX:MitoTracker ratio and PD clinical data (N=34). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$.

Clinical variable	Mitochondrial ROS (Baseline)	Mitochondrial Content (Baseline)	Mitochondrial ROS (Stimulated)	Mitochondrial Content (Stimulated)	MitoSOX: MitoTracker (Baseline)	MitoSOX: MitoTracker (Stimulated)
Age	*r=0.380	r=0.337	r=0.297	r=0.155	r=-0.186	r=0.012
	p=0.029	p=0.055	p=0.094	p=0.389	p=0.300	p=0.947
Sex	r=-0.130	r=-0.091	r=-0.085	r=-0.072	r=0.052	r=0.065
	p=0.470	p=0.614	p=0639	p=0.692	p=0.773	p=0.719
Disease	r=0.015	r=-0.019	r=0.094	r=0.033	r=-0.063	r=0.088
Duration	p=0.935	p=0.917	p=0.607	p=0.859	p=0.734	p=0.630
MDS -	r=0.235	*r=0.507	*r=0.360	r=0.329	*r= -0.383	r=-0.204
UPDRS	p=0.196	p=0.003	p=0.043	p=0.066	p= 0.030	p=0.263
III						
MMSE	*r=0.381	r=0.126	r=0.348	r=0.060	r=0.143	r=0.156
	p=0.038	p=0.508	p=0.059	p=0.753	p=0.452	p=0.411
MoCA	r=-0.206	*r=-0.373	r=-0.329	r=-0.262	r=0.253	r=0.074
	p=0.258	p=0.035	p=0.066	p=0.147	p=0.162	p=0.688
RBDSQ	*r=-0.378	r=-0.304	*r=-0.437	r=-0.348	r=0.142	r=0.125
	p=0.043	p=0.109	p=0.018	p=0.064	p=0.463	p=0.519
BMI	r=-0.377	r=-0.206	r=-0.112	r=-0.209	r=-0.080	r=0.049
	p=0.111	p=0.397	p=0.647	p=0.391	p=0.745	p=0.842
Statins	r=-0.070	r=-0.060	r=-0.239	r=-0.100	r=0.000	r=-0.179
	p=0.777	p=0.808	p=0.324	p=0.685	p=1.000	p=0.463

Table 4.3 iRBD patients' clinical measures positively associate to mitochondrial measures. Spearman correlations at baseline and at stimulation between mitochondrial ROS production, mitochondrial content, MitoSOX:MitoTracker ratio and iRBD clinical data (N=33). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$.

4.6 Discussion

Mitochondrial dysfunction is characterised as a key disease hallmark for the pathogenesis of both familial and sporadic PD (Ryan et al., 2015). The potential development of peripheral mitochondrial biomarkers will provide objective and quantitative measures, that will aim to reflect disease pathogenesis ultimately supporting accurate and earlier diagnosis and disease monitoring. This study has revealed differences of mitochondrial parameters in PBMCs of control, iRBD and PD patients. Furthermore, positive correlations of mitochondrial ROS and content levels to clinical variables of iRBD and PD patient's monocyte samples ascertains the association of mitochondrial dysfunction to PD and iRBD pathogenesis.

Determination of optimal concentration and time point of antimycin A application is not fixed rather dependent, to the applied tissue and required outcome (Wang et al., 2015). In previous studies antimycin A concentration has varied from 0.1 μM to 100 μM with range of time points (Han et al., 2008; Mukhopadhyay et al., 2007). However, minimal studies have explored optimal concentration and time point of the inhibition of mitochondrial complex III in $CD14^+$ monocytes. In this current chapter the concentration of antimycin A and timepoint was optimised to inhibit complex III of the mitochondrial electron transport chain to deplete mitochondrial molecular and cellular functions to model impaired mitochondrial function. Therefore, the optimal concentration and time point for antimycin A to induce mitochondrial dysfunction through elevated levels of mitochondrial ROS production whilst maintaining cell viability was determined. Optimisation demonstrated that inhibition of mitochondrial complex III with 2 μM of antimycin A for 2 h maintained cell viability and effectively induced mitochondrial dysfunction. This concentration is consistent with a study conducted by Accardi et al. (2014) that analysed the effects of 0.5 μM to 10 μM of antimycin A on the biophysical properties of cerebellar stellate cells, found that 2 μM presented timely effect of increased

mitochondrial ROS without detrimentally impacting cell health. Further, Polster et al. (2014) recommended that antimycin A should not exceed 2 μM, if mitochondrial ROS is measured through MitoSOX probe. Therefore, the combination of the optimised time point and concentration of antimycin A for the inhibition of mitochondrial complex III of PBMCs from this study may provide a basis for future research.

Analysis of mitochondrial ROS revealed elevated levels of mitochondrial ROS in PD patient monocytes when compared to control and iRBD patient monocytes. The findings of this study elucidate the current literature of the association of oxidative stress and PD pathogenesis (Prigione et al., 2006; Trist et al., 2019), in which PD patients presented significantly increased levels of ROS. Significant difference in ROS levels was not observed between control and iRBD patients. Comparably, the study conducted by Smith et al. (2018) assessed mitochondrial ROS and content in PD and iRBD patient PBMCs. Mitochondrial ROS and content of PD monocytes (N=25) was compared to rotenone-treated positive controls, the study found increased mitochondrial ROS and decreased mitochondrial content in the monocytes of the PD group. iRBD patients that were highly susceptible to developing PD (N=18) were also compared to controls, but no significant differences in monocyte mitochondrial ROS or content was observed between the prodromal PD and control group (Smith et al., 2018). This suggests that the progressive generation of mitochondrial ROS in PD is gradual. Indeed, this was manifest from the current iRBD and PD groups, in which mitochondrial ROS positively correlated with age, reflective of age being a core risk factor (Reeve et al., 2014).

Though iRBD is a well-established precursor to prodromal disease phase of PD, iRBD patient monocytes may not necessarily present with significant levels of mitochondrial ROS. Furthermore, the average years of diagnosis of the iRBD cohort was 3 years whilst 9 years for

the PD cohort. The difference in disease duration is also reflective of disease pathology. Therefore, though the iRBD patients of this current cohort did not present with significant elevated levels of mitochondrial ROS, this does not elucidate the potential of detection of mitochondrial dysfunction in iRBD, but rather reflective of the early disease duration / pathology. In addition, statistically significant difference in monocyte viability was present between PD and control group, with no significant difference in viability measures between iRBD and controls PBMC samples. Decreased cell viability is potentially indicative of the significant impairment in mitochondrial morphology (Osellame, et al., 2012), present in PD PBMCs. Whilst insignificant differences in viability between control and iRBD patient PBMCs may be suggestive of unhindered mitochondrial function, however further analysis of the mitochondrial morphology is needed. Measurement of mitochondrial content levels in controls, iRBD and PD patients PBMC samples, revealed elevated levels of mitochondrial content in PD samples when compared to controls. This is consistent with the findings of Navarro et al. (2009) in which the frontal cortex of post-mortem PD brains presented significantly increased levels of mitochondrial mass. Mitochondrial content levels present in cells is determined by numerous factors pertinent to mitophagy, mitochondrial biogenesis, dynamics, trafficking, turnover and ROS. Elevated levels of mitochondrial content can be due to several factors such as impaired mitophagy, disturbed mitochondrial function and homeostasis, being primary features of PD pathogenesis and present in profiles of RBD-PD patients (Jagtap et al., 2023; Ongari et al., 2024). Mitophagy is the primary process of selective removal of damaged mitochondria, assisting in the maintenance of normal mitochondrial homeostasis. Impaired mitophagy, leading to the accumulation of defective mitochondria has been identified in accelerating neuronal death and neurodegeneration in PD pathogenesis. (Li et al., 2022; Liu et al., 2019). Though mitophagy has been identified to have key role in the pathogenesis of PD, literature on impaired mitophagy in PD monocytes is limited. To determine the role of impaired

mitophagy in PD monocytes it is critical to understand the key processes of mitochondrial morphology involved in the modulation of damaged mitophagy.

iRBD presents as a strong risk precursor to PD, manifesting non-motor features of PD prior to the clinical diagnosis of PD (Van Laar & Jain, 2004). Positive correlation between mitochondrial ROS and MMSE in the iRBD group was observed. Mitochondrial ROS has been suggested to have critical role in the signalling process of synaptic plasticity and memory, therefore, defective ROS are perpetuated to impact the necessary processing for normal cognitive function (Massaad & Klaan, 2011). Core hallmark of iRBD is the decline of muscle atonia that allows for motor manifestations during sleep (Pham et al., 2024). Significant positive correlation of the MDS-UPDS III score and mitochondrial content of the iRBD group was also observed. Mitochondrial content accumulation is indicative of mitochondrial dysfunction, which has also presented in motor diseases (Jurcau & Jurcau, 2022). Furthermore, mitochondrial content at baseline positively associated to statin use in the PD group. Statin is a common form of prescription medication for cholesterol management through the inhibition of hydroxymethylglutaryl-CoA reductase enzyme. To observe if any association is present between statin medication and mitochondrial variables, statin was considered as a variable. The effect of statins in managing or reducing the risk of PD is debateable, with studies reporting significant reduction to PD risk through long term use whilst other studies reporting no effect (Wu et al., 2022). Correlation between mitochondrial content of PD patients at baseline and statins medication could be indicative to the accumulation of damaged mitochondria, in which histochemical studies have revealed statin – associated mitochondrial dysfunction (Broniarek & Jarmuszkiewicz, 2016).

In summary, the objective of this study aimed to determine if mitochondrial dysfunction can utilise as a diagnostic and / or prognostic biomarker for iRBD and PD. Analysis of mitochondrial function in control, iRBD and PD patient monocytes revealed the combination of elevated levels of ROS and mitochondrial content deposition in PD patients PBMCs, core markers of mitochondrial impairment. No significant difference in mitochondrial function parameters was shown between control and iRBD patient monocytes; this may be relative to early disease stage and pathology. As analysis of viability of control and iRBD patient PBMCs revealed no significant difference, however revealed significant differences between control and PD patient PBMCs. Positive correlation of mitochondrial ROS and MMSE in iRBD patient monocytes, elucidates that though elevated levels of ROS were not evident, it may be involved in disease progression. In addition, mitochondrial ROS and mitochondrial content levels of control, iRBD and PD patient monocytes was not affected by reduced cell viability in limited number of cells.

Limitation of this study was the lack of disease duration of the iRBD group. Greater diversity of disease duration particularly of iRBD patients with prolonged disease duration, that are in the conversion period of iRBD to PD may have provided a reflection of mitochondrial dysfunction disease pathology in the iRBD group. Furthermore, it is critical to highlight that the progression of iRBD is not exclusive to the conversion of PD, but also to Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA). Hence, proportion of the current iRBD cohort may potentially be in the prodromal phase of DLB or MSA and not PD. Reproducibility is a critical contributor to the validation of a biomarker, as it provides indication to consistency, quality and efficacy of the measured marker as a diagnostic tool (Zhuang et al., 2019). Assessment of reproducibility of measured mitochondrial functions of this current cohort was not feasible due to limited biospecimen volume. To validate the

biological and clinical efficacy of mitochondrial function as a PD biomarker repeated cohort measures that provide consistent findings is essential.

Mitochondrial dysfunction identifies as a promising PD diagnostic biomarker. For the transition of mitochondrial dysfunction as diagnostic and monitoring biomarkers in PD and potentially iRBD in clinical practice, longitudinal studies that incorporate all PD and iRBD types need to be employed. To better determine and quantify mitochondrial dysfunction application as diagnostic and monitoring biomarkers. It would also be of interest to explore if / how the methodology used has been employed in other neurodegenerative conditions. This would also be important for determining the specificity of proposed biomarkers for PD.

CHAPTER FIVE

Peripheral Pro-inflammatory Cytokine Markers Positively Correlate to Clinical Parkinson's Disease and idiopathic REM Sleep Behaviour Disorder Markers

5.1 Introduction

Exactly how mitochondrial dysfunction contributes to Parkinson's disease (PD) pathogenesis is unclear, however one potential mechanism is through the induction of inflammation. The association of the innate immune system in the pathophysiology of PD has been established in recent decades (Dzamko, 2023; Kannarkat et al., 2013). Inflammation linked to PD was first described in the original 'An Essay on the Shaking Palsy' (Parkinson, 1817). Since then, the involvement and impact of inflammation on the pathogenesis and phenotypical manifestation of PD has been recognised, in which, alterations in peripheral blood immune profile have been observed in PD patients (Abdi et al., 2022).

The contribution of the peripheral innate immune system in the pathophysiology of PD has been increasingly researched with a particular focus on the role of cytokines and monocytes (Munoz-Delgado et al., 2023). The gut-brain axis theory suggests that changes in gut microbiota influence the aggregation of α-syn, which in turn induces peripheral inflammatory responses and modification to cytokine secretion. The gut-brain axis theory explains that α-syn aggregation reaches the brain from the gut via the vagus nerve and consequently triggers further molecular and cellular changes including alpha-synuclein (α-syn) aggregation and

accumulation, mitochondrial dysfunction and elevated levels of reactive oxygen species (ROS). Mitochondria are significant contributors to the innate immune system due to their key roles in signalling and activation of the innate immune response (Chen et al., 2023; Missiroli et al., 2020). Mitochondrial ROS signalling molecules are thought to prompt increased pro-inflammatory cytokine response (Naik & Dixit, 2011). Furthermore, upregulated ROS production is also suggested to drive an inflammatory response (Liu et al., 2023).

Aberrations in pro-inflammatory cytokine levels in PD patients have been observed with upregulated inflammatory cytokine markers often being associated with PD (Dzamko, 2023). A meta-analysis on peripheral blood inflammatory cytokine levels in PD patients that included 2654 PD participants found upregulated levels of interleukin cytokines, IL-6, IL-1β, IL-2, IL-10, and cytokines tumour necrosis factor (TNF), C- reactive protein, and RANTES (Qin et al., 2016). Furthermore, a case control study of 77 iRBD patients and 64 healthy controls found upregulated levels of IL-10 and TNF-α in plasma of iRBD patients (Zhang et al., 2020). Zhang et al. (2020) suggested that this finding was indicative of iRBD phenoconversion to neurodegenerative alpha-synucleinopathies, suggesting the significant contribution of the peripheral immune system in the prodromal phase of neurodegenerative disease. Additionally, peripheral innate inflammation is suggested to peak during the prodromal PD phase (Zimmermann & Brockmann, 2022), therefore potentially manifesting as an advantageous inflammatory peripheral biomarker for early PD diagnosis. However, inconsistency in the association of peripheral blood and inflammatory markers has also been observed and not all studies observe an increase in cytokine levels (Jun & Kim, 2023). Moreover, if changes in peripheral inflammation are associated with change in monocyte mitochondrial function is not clear.

Therefore, the purpose of this chapter is to measure pro-inflammatory cytokines in the serum of PD and iRBD patients and to determine if such measures are distinctive from the control group. Further, this chapter explores the potential link of pro-inflammatory serum cytokines to disease symptomatology, and association of cytokine measures to mitochondrial measures obtained in the previous chapter.

5.2 Hypothesis

That inflammatory serum cytokine levels will be elevated in iRBD and PD patients compared to controls and correlate to increased monocyte ROS levels.

5.3 Aims

1. Measure pro-inflammatory serum cytokine levels in control, iRBD and PD patient samples.
2. Determine if cytokine measures are significantly different amongst control, iRBD and PD levels.
3. Determine if there is association between pro-inflammatory serum cytokines and iRBD and PD clinical markers.
4. Determine any correlations present between PD or iRBD serum inflammatory markers and mitochondrial measures.

5.4 Chapter Specific Methods

5.4.1 Collection of serum samples

Collection of serum samples is as described in methods chapter, section 2.6.

5.4.2 Measurement of pro-inflammatory serum cytokines

Measurement of pro-inflammatory serum cytokines is as described in methods chapter, section 2.11.

5.4.3 Live Flow cytometry assay for measurement of Mitochondrial function

To correlate $CD14^{+}$ monocyte mitochondrial measures to serum pro-inflammatory cytokines of control, iRBD and PD patient samples, mitochondrial measures were obtained from chapter 4 of this thesis. Mitochondrial flow cytometry assay was performed as described in methods chapter, section 2.8. For live cell analysis spectral unmixing was performed as described in methods chapter, section 2.9 with 100,000 events recorded obtained from chapter from chapter 4 of this thesis.

5.4.4 Statistical analysis

To assess if pro-inflammatory serum cytokine levels differ between control, iRBD and PD patient samples, spearman multivariate analysis of variance (MANCOVA) with covarying for age and sex was performed. Following MANCOVA, Tukey's post-hoc tests were performed (IBM SPSS Statistics 27 software). To determine any significant differences between demographic and clinical data across the groups, a Mann Whitney U Test was applied. To determines and significant association between iRBD and PD pro-inflammatory serum cytokine levels and clinical markers, Spearman correlations were performed. Where indicated, cytokine measures were log transformed to achieve normality. Significance was accepted at p value < 0.05. To reduce the possibility of false positive Bonferroni significance threshold was applied to correlation analyses. Significance at p value <0.05 were indicated by a red asterisk "*****", results that upheld Bonferroni significance threshold were indicated using bold font. For

analysis of the association of pro-inflammatory cytokines to iRBD clinical markers, a Bonferroni corrected alpha of 0.00069 was applied (0.05/72 variables) for analysis of the association of pro-inflammatory cytokines to PD clinical markers a Bonferroni corrected alpha of 0.00055 was applied (0.05/90 variables) and for correlation of pro-inflammatory cytokines in the iRBD and PD group a Bonferroni corrected alpha of 0.00062 was applied (0.05/81 variables). Graphs were generated using GraphPad Prism v. 9.4.0 and depict mean ± standard error of the mean (SEM).

5.5 Results

5.5.1 Evaluation of control, iRBD and PD patient's demographic and clinical biomarkers

Demographic and clinical data of PD patients (N=34), iRBD patients (N=65) and controls (N=35) are provided in Table 5.1. Additional iRBD patients were available for inclusion in this chapter compared to the previous chapter. No significant differences were observed between age and sex of PD and iRBD patients compared to controls, however significant difference in age was observed between iRBD and PD patients. Compared to controls, a significant difference was found in the iRBD group for the Sniffin Sticks olfactory test and the REM sleep behaviour disorder screening questionnaire (RBDSQ). Significant differences in clinical measures were observed in the PD group, being, the Movement Disorders Society - Unified Parkinson's Disease Rating Scale (MDS-UPDRS) III, body mass index (BMI), hospital anxiety depression scale (HADS) for anxiety, Sniffin Sticks test, Farnsworth-Munsell test for colour vision deficiencies and the RBDSQ scale compared to controls.

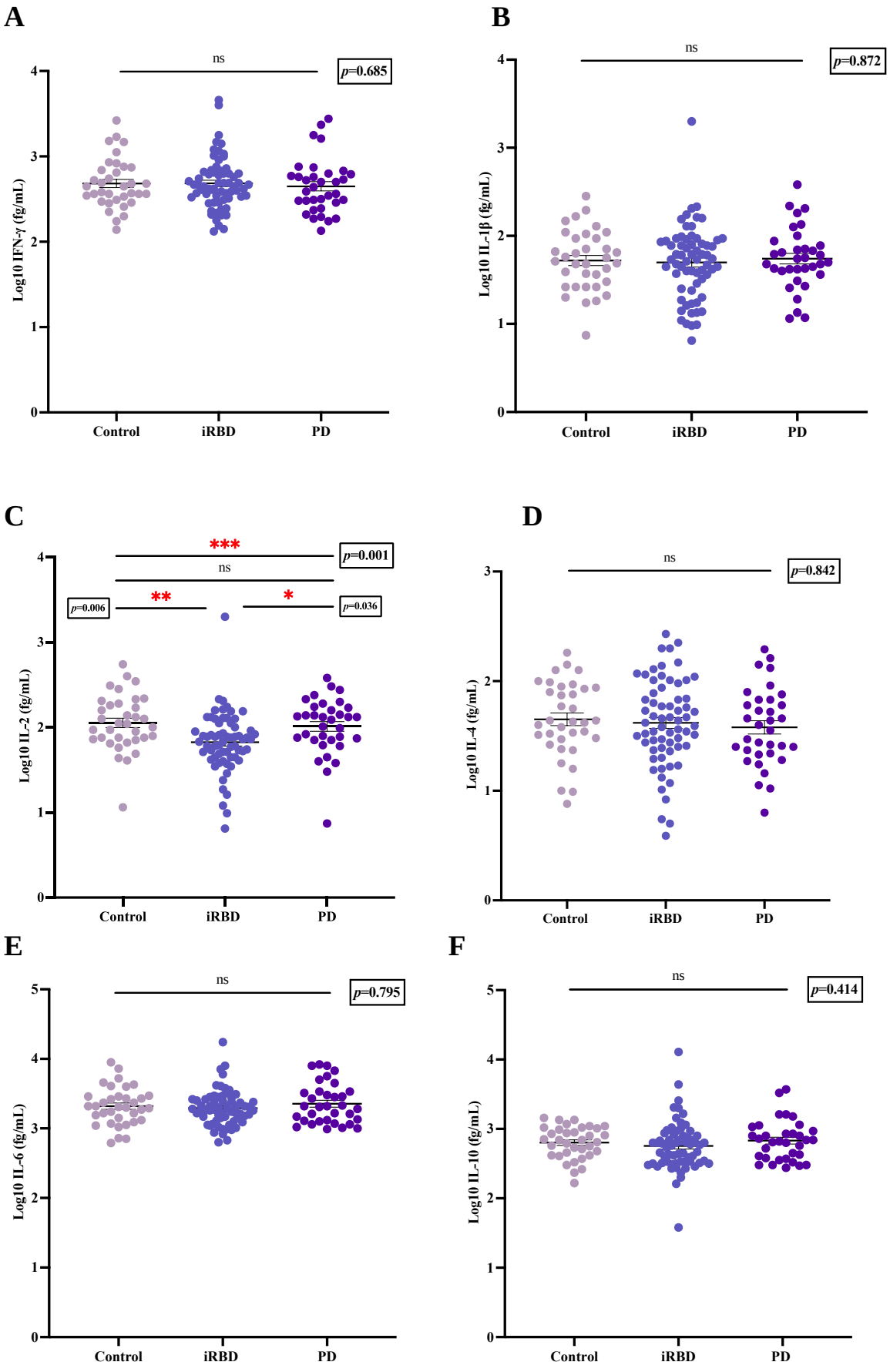
	Control	iRBD	PD	*p*-value (control & iRBD)	*p*-value (control & PD)	*p*-value (iRBD & PD)
Age (years)	67 ± 1.48	67.63 ± 1.21	71.76 ± 1.77	0.78	0.11	* 0.02
Sex (%M)	57.14 %	75 %	55.88 %	0.17	0.99	0.13
Disease duration	N/A	2.64 ± 0.48	9.21 ± 1.34	N/A	N/A	**** <0.0001
MDS - UPDRS III	4.97 ± 0.93	7.60 ± 0.99	34.65 ± 2.89	>0.99	**** <0.0001	**** <0.0001
MMSE	28.41 ± 0.31	28.33 ± 0.19	27.53 ± .0.60	0.72	0.34	0.80
MoCA	26.82 ± 0.45	26.65 ± 0.30	25.06 ± 0.85	0.99	0.88	0.07
LEDD	N/A	N/A	771.09 ± 141.39	N/A	N/A	N/A
H & Y	N/A	N/A	2.09 ± 0.18	N/A	N/A	N/A
Sniffin Sticks Test	9.75 ± 0.31	7.46 ± 0.39	5.12 ± 0.42	*** 0.0006	**** <0.0001	** 0.0095
Farnsworth-Munsell Test	93.83 ± 7.62	101.82 ± 7.61	161.33 ± 21.51	0.22	** 0.005	0.26
EES	4.78 ± 0.54	5.70 ± 0.57	7.53 ± 0.94	0.79	0.09	0.35
RBDSQ	2.44 ± 0.45	7.86 ± 0.47	6.72 ± 0.62	**** <0.0001	**** <0.0001	** 0.0064
BMI	28.35 ± 0.95	27.07 ± 0.48	24.27 ± 0.63	0.20	* 0.01	0.99
Statins	1.41 ± 0.11	1.56 ± 1.20	1.44 ±0.09	0.99	0.95	0.99

Anxiety HADS	3.32 ± 0.49	4.22 ± 0.47	5.76 ± 0.62	0.32	* 0.04	0.65
Depression HADS	3.08 ± 0.44	3.16 ± 0.41	4.68 ± 0.64	0.68	0.21	0.70
Family history	N/A	0.14 ± 0.04	0.27 ± 0.08	NA	N/A	0.10

Table 5.1 Demographic and clinical biomarker table for control, iRBD and PD. Data are mean ± standard error. (N = 35 control, 65 iRBD and 34 PD). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$.

5.5.2 Pro-inflammatory serum cytokine IL-2 is significantly decreased in iRBD patients

To determine if pro-inflammatory serum cytokine measures differ in iRBD and PD groups compared to controls, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A and TNF-α were measured using a sensitive multi-plex assay. There were no significant differences in cytokine levels between control, iRBD and PD groups for IFNγ (Figure 5.1A) and IL-1β (Figure 5.1B). IL-2 was significantly reduced in the iRBD group (Figure 5.1C). There was no other significant findings for IL-4 (Figure 5.1D), IL-6 (Figure 5.1E), IL-10 (Figure 5.1F), IL-12p70, (Figure 5.1G), IL-17A (Figure 5.1H) or TNF-α (Figure 5.1G).


A
ns
p=0.685
Log10 IFN-γ (fg/mL)
Control
iRBD
PD
B
ns
p=0.872
Log10 IL-1β (fg/mL)
Control
iRBD
PD
C
***
p=0.001
ns
**
p=0.006
*
p=0.036
Log10 IL-2 (fg/mL)
Control
iRBD
PD
D
ns
p=0.842
Log10 IL-4 (fg/mL)
Control
iRBD
PD
E
ns
p=0.795
Log10 IL-6 (fg/mL)
Control
iRBD
PD
F
ns
p=0.414
Log10 IL-10 (fg/mL)
Control
iRBD
PD

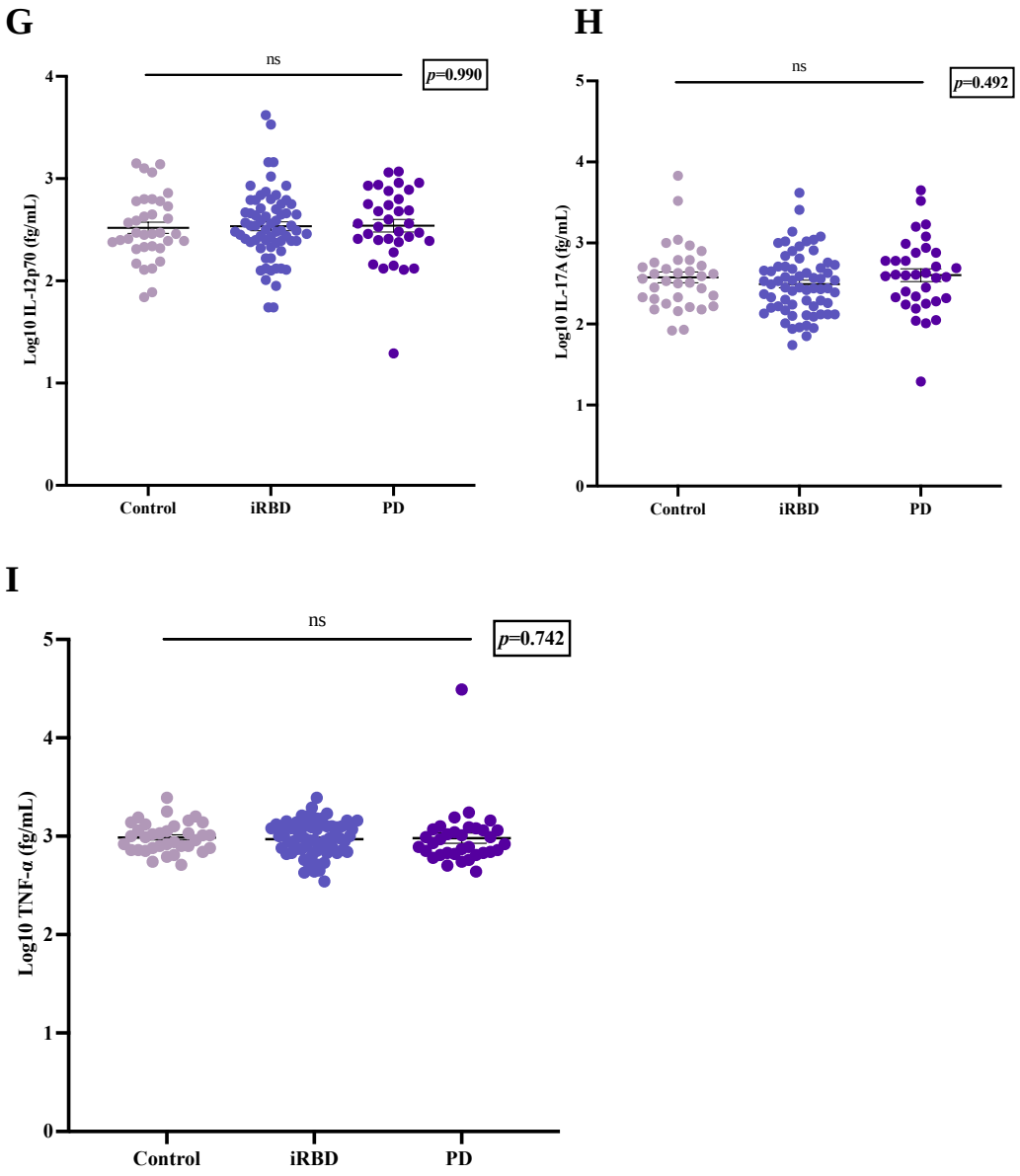


Figure 5.1 Pro-inflammatory serum cytokine measures of control, iRBD and PD samples. MSD S-Plex assay was utilised to measure pro-inflammatory serum cytokine levels of IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A and TNF-α in control (N=35), iRBD (N=65) and PD (N=34) samples. MANCOVA test to assess pro-inflammatory cytokine differences among groups and to covary for age and sex with Tukey's HSD post-hoc test. Graphs display mean ± SEM with dots showing individual values. Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$.

5.5.3 Pro-inflammatory serum cytokines positively correlate to iRBD clinical markers

To determine if pro-inflammatory serum cytokines of iRBD patients are associated with clinical markers, correlation analyses were performed. IL-6 correlated to age (r=0.313, *p*=0.011) and BMI (r=0.344, *p*=0.046), IL-10 also correlated to age (r=0.274, *p*=0.027) and sex (r=0.267, *p*=0.032). IL-17A positively correlated to statins medication (r=0.414, *p*=0.015). The MoCA scale negative correlated to IFN-γ (r=-0.258. *p*=0.041) and TNF-α (r=-0.326, *p*=0.009)

	IFN-γ	**IL-1β**	**IL-2**	**IL-4**	**IL-6**	**IL-10**	**IL-12 p70**	**IL-17A**	**TNF-α**
					*	*			
Age	r=0.163 p=0.194	r=0.039 p=0.758	r=0.190 p=0.129	r=-0.110 p=0.383	r=0.313 p=0.011	r=0.274 p=0.027	r=0.112 p=0.373	r=0.073 p=0.561	r=0.188 p=0.133
						*			
Sex	r=0.086 p=0.497	r=0.116 p=0.357	r=0.077 p=0.542	r=-0.065 p=0.609	r=0.010 p=0.940	r=0.267 p=0.032	r=0.059 p=0.641	r=0.000 p=1.000	r=0.187 p=0.137
MDS - UPDRS III	r=-0.028 p=0.830	r=-0.015 p=0.905	r=-0.243 p=0.055	r=0.026 p=0.841	r=-0.108 p=0.398	r=0.123 p=0.337	r=0.002 p=0.988	r=0.030 p=0.816	r=0.089 p=0.489
MMSE	r=-0.048 p=0.725	r=0.100 p=0.459	r=-0.064 p=0.638	r=0.0460 p=0.732	r=-0.077 p=0.570	r=-0.175 p=0.192	r=0.050 p=0.714	r=-0.060 p=0.656	r=0.021 p=0.877
	*								**
MoCA	r=-0.258 p=0.041	r=-0.059 p=0.645	r=0.128 p=0.319	r=0.024 p=0.850	r=-0.151 p=0.237	r=-0.192 p=0.131	r=-0.207 p=0.103	r=-0.142 p=0.266	r=-0.326 p=0.009
RBDSQ	r=-0.124 p=0.364	r=-0.056 p=0.683	r=0.070 p=0.610	r=0.102 p=0.455	r=-0.182 p=0.180	r=0.187 p=0.169	r=0.025 p=0.858	r=-0.048 p=0.727	r=-0.217 p=0.108

					*				
BMI	r=-0.060 p=0.736	r=0.301 p=0.083	r=0.241 p=0.170	r=0.317 p=0.068	r=0.344 p=0.046	r=0.024 p=0.891	r=0.078 p=0.660	r=0.270 p=0.122	r=0.278 p=0.112
Statins	r=-0.148 p=0.404	r=-0.045 p=0.799	r=0.051 p=0.773	r=0.021 p=0.906	r=0.027 p=0.879	r=0.082 p=0.647	r=-0.033 p=0.852	* r=0.414 p=0.015	r=0.112 p=0.529

Table 5.2 iRBD patients' serum inflammatory measures positively associate to clinical measures. Spearman correlations between pro-inflammatory serum cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A and TNF-α to iRBD clinical data (N=65). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$.

5.5.4 Pro-inflammatory serum cytokines positively correlate to PD clinical markers

IL-17A positively correlated to age (r=0.561, *p*=<0.001), MDS-UPDRS III score (r=0.355, *p*=0.039) and statins use (r=0.375, *p*=0.034). Positive correlation between IL-6 and Hoehn and Yahr (H &Y) scale was also observed (r=0.345, *p*=0.045). Negative correlations were also manifest. IL-12p70 negatively correlated to MMSE (r=-0.0356, *p*=0.045), IL-17A negatively correlated to the Montreal Cognitive Assessment (MoCA) scale (r=-0.489, *p*=0.003) and IL-4 negatively correlated to BMI (r=-0.400, *p*=0.039). No other significant correlation between pro-inflammatory serum cytokines and PD clinical markers were observed.

	IFN-γ	**IL-1β**	**IL-2**	**IL-4**	**IL-6**	**IL-10**	**IL-12 p70**	**IL-17A**	**TNF-α**
								**	
Age	r=0.204 p=0.248	r=0.282 p=0.106	r=0.215 p=0.223	r=-0.031 p=0.862	r=0.223 p=0.205	r=0.186 p=0.292	r=0.202 p=0.253	**r=0.561** **p=<0.001**	r=0.276 p=0.114
Sex	r=-0.088 p=0.622	r=-0.003 p=0986	r=0.033 p=0.852	r=-0.057 p=0.747	r=-0.063 p=0.722	r=-0.015 p=0.932	r=0.063 p=0.722	r=-0.003 p=0.986	r=0.166 p=0.348

								*	
MDS - UPDRS III	r=-0.011 p=0.950	r=0.065 p=0.716	r=0.174 p=0.324	r=-0.217 p=0.219	r=0.057 p=0.749	r=0.232 p=0.182	r=0.243 p=0.166	r=0.355 p=0.039	r=0.152 p=0.390
							*		
MMSE	r=-0.312 p=0.083	r=-0.122 p=0.506	r=-0.195 p=0.286	r=-0.098 p=0.594	r=-0.021 p=0.909	r=-0.079 p=0.668	r=-0.356 p=0.045	r=-0.348 p=0.051	r=-0.221 p=0.225
								**	
MoCA	r=-0.251 p=0.153	r=-0.165 p=0.351	r=-0.213 p=0.226	r=-0.120 p=0.499	r=-0.073 p=0.680	r=-0.242 p=0.168	r=-0.323 p=0.063	r=-0.489 p=0.003	r=-0.303 p=0.082
LEDD	r=0.023 p=0.898	r=0.033 p=0.855	r=0.298 p=0.087	r=-0.105 p=0.555	r=0.296 p=0.089	r=0.103 p=0.563	r=0.280 p=0.109	r=0.173 p=0.328	r=0.148 p=0.405
					*				
H & Y	r=0.077 p=0.664	r=0.264 p=0.132	r=0.084 p=0.636	r=-0.032 p=0.858	r=0.345 p=0.045	r=0.178 p=0.315	r=0.227 p=0.197	r=0.325 p=0.061	r=0.189 p=0.283
RBDSQ	r=0.059 p=0.745	r=0.256 p=0.150	r=0.008 p=0.965	r=-0.002 p=0.993	r=0.123 p=0.494	r=-0.162 p=0.367	r=0.247 p=0.166	r=0.113 p=0.529	r=0.214 p=0.231
				*					
BMI	r=-0.087 p=0.665	r=0.258 p=0.194	r=0.366 p=0.060	r=-0.400 p=0.039	r=-0.068 p=0.734	r=0.150 p=0.455	r=0.141 p=0.482	r=0.291 p=0.140	r=0.148 p=0.405
								*	
Statins	r=0.116 p=0.527	r=0.287 p=0.112	r=0.102 p=0.577	r=0.000 p=1.000	r=0.171 p=0.351	r=-0.096 p=0.603	r=0.082 p=0.656	r=0.375 p=0.034	r=0.225 p=0.215

Table 5.3 PD patients' serum inflammatory measures positively correlate to clinical markers. Spearman correlations between pro-inflammatory serum cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A and TNF-α to PD clinical data (N=34). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$.

5.5.5 Correlation analysis of pro-inflammatory serum cytokines of iRBD patients

Significant positive correlations between pro-inflammatory serum cytokines of iRBD patients (Table 5.4).

	IFN-γ	IL-1β	IL-2	IL-4	IL-6	IL-10	IL-12 p70	IL-17A	TNF-α
IFN-γ	r=1.000 p=.	* r=0.0302 p=0.014	r=0.200 p=0.110	r=0.043 p=0.731	* r=0.256 p=0.040	r=0.231 p=0.064	** **r=0.533** **p=<0.001**	** p=0.351 p=0.004	** r=0.351 p=0.004
IL-1β	r=0.302 p=0.014	r=1.000 p=.	** r=0.420 p=<0.001	r=0.236 p=0.058	r=0.148 p=0.240	r=0.237 p=0.057	** r=0.391 p=0.001	r=0.358 p=0.003	** **r=0.453** **p=<0.001**
IL-2	r=0.200 p=0.110	** **r=0.420** **p=<0.001**	r=1.000 p=.	* r=0.253 p=0.042	** r=0.346 p=0.005	r=0.226 p=0.070	r=0.240 p=0.054	* r=0.309 p=0.012	** r=0.336 p=0.006
IL-4	r=0.043 p=0.731	r=0.236 p=0.058	* r=0.253 p=0.042	r=1.000 p=.	r=0.075 p=0.550	* r=0.316 p=0.010	** **r=0.423** **p=<0.001**	* r=0.279 p=0.024	** r=0.507 p=<0.001
IL-6	r=0.256 p=0.040	r=0.148 p=0.240	** r=0.346 p=0.005	r=0.075 p=0.550	r=1.000 p=.	* r=0.306 p=0.013	r=0.242 p=0.052	* r=0.311 p=0.012	** r=0.461 p=<0.001
IL-10	r=0.231 p=0.064	r=0.237 p=0.057	r=0.226 p=0.070	* r=0.316 p=0.010	* r=0.306 p=0.013	r=1.000 p=.	** r=0.0367 p=0.002	** r=0.434 p=<0.001	** r=0.570 p=<0.001
IL-12 p70	** **r=0.533** **p=<0.001**	** r=0.391 p=0.001	r=0.240 p=0.054	** **r=0.423** **p=<0.001**	r=0.242 p=0.052	** r=0.376 p=0.002	r=1.000 p=.	** **r=0.695** **p=<0.001**	** **r=0.616** **p=<0.001**
IL-17A	** r=0.351 p=0.004	** r=0.358 p=0.003	* r=309 p=0.012	* r=0.279 p=0.024	* r=0.311 p=0.012	** **r=0.434** **p=<0.001**	** **r=0.695** **p=<0.001**	r=1.000 p=.	** **r=0.605** **p=<0.001**
TNF-α	** r=0.351 p=0.004	** **r=0.453** **p=<0.001**	** r=0.336 p=0.006	** **r=0.507** **p=<0.001**	** **r=0.461** **p=<0.001**	** **r=0.570** **p=<0.001**	** **r=0.616** **p=<0.001**	** **r=0.605** **p=<0.001**	r=1.000 p=.

Table 5.4 iRBD patient's serum inflammatory cytokine measures positively correlate. Spearman correlations of pro-inflammatory serum cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A and TNF-α of iRBD patients. (N=65). Statistical significance

determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$.

5.5.6 Correlation analysis of pro-inflammatory serum cytokines of PD patients

PD patients' pro-inflammatory serum cytokines positively associate (Table 5.5).

	IFN-γ	IL-1β	IL-2	IL-4	IL-6	IL-10	IL-12 p70	IL-17A	TNF-α
IFN-γ	r=1.000 p=.	r=0.285 p=0.102	r=0.307 p=0.078	r=0.181 p=0.305	r=0.004 p=0.980	r=0.152 p=0.390	* r=0.378 p=0.027	* r=0.363 p=0.035	* r=0.382 p=0.026
IL-1β	r=0.285 p=0.102	r=1.000 p=.	r=0.039 p=0.828	r=-0.024 p=0.894	r=0.322 p=0.064	r=0.218 p=0.215	r=0.218 p=0.216	* r=0.361 p=0.036	r=0.334 p=0.054
IL-2	r=0.307 p=0.078	r=0.039 p=0.828	r=1.000 p=.	r=-0.130 p=0.464	r=-0.115 p=0.518	** r=0.455 p=0.007	** **r=0.724** **p=<0.001**	** **r=0.635** **p=<0.001**	** r=0.519 p=0.002
IL-4	r=0.181 p=0.305	r=-0.024 p=0.894	r=-0.130 p=0.464	r=1.000 p=.	r=0.282 p=0.106	r=-0.127 p=0.474	r=0.055 p=0.758	r=0.072 p=0.686	r=0.032 p=0.859
IL-6	r=0.004 p=0.980	r=0.322 p=0.064	r=-0.115 p=0.518	r=0.282 p=0.106	r=1.000 p=.	r=-0.126 p=0.479	r=-0.043 p=0.808	r=0.147 p=0.406	r=0.141 p=0.427
IL-10	r=0.152 p=0.390	r=0.218 p=0.215	** r=0.455 p=0.007	r=-0.127 p=0.474	r=-0.126 p=0.479	r=1.000 p=.	** r=0.437 p=0.010	* r=0.415 p=0.015	** **r=0.593** **p=<0.001**
IL-12	* r=0.378 p=0.027	r=0.218 p=0.216	** **r=0.724** **p=<0.001**	r=0.055 p=0.758	r=-0.043 p=0.808	** r=0.437 p=0.010	r=1.000 p=.	** **r=0.687** **p=<0.001**	** **r=0.621** **p=<0.001**
IL-17A	* r=0.363 p=0.035	* r=0.361 p=0.036	** **r=0.635** **p=<0.001**	r=0.072 p=0.686	r=0.147 p=0.406	* r=0.415 p=0.015	** **r=0.687** **p=<0.001**	r=1.000 p=.	** **r=0.724** **p=<0.001**
TNF-α	* r=0.382 p=0.026	r=0.334 p=0.054	** r=0.519 p=0.002	r=0.032 p=0.859	r=0.141 p=0.427	** **r=0.593** **p=<0.001**	** **r=0.621** **p=<0.001**	** **r=0.724** **p=<0.001**	r=1.000 p=.

Table 5.5 PD patients' serum inflammatory cytokine measures positively correlate. Spearman correlations of pro-inflammatory serum cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-6,

IL-10, IL-12p70, IL-17A and TNF-α of PD patients. (N=34). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$.

5.5.7 Mitochondrial measures of PD and iRBD patients do not correlate to inflammatory cytokines

To investigate if PD and iRBD patients' mitochondrial ROS and content correlates to inflammatory cytokine levels, correlation analyses were performed. Positive correlations of mitochondrial measures and inflammatory cytokines were not present. A negative association was present between PD patients' mitochondrial content at baseline and IL-12p70 cytokine (r=-0.042, p=0.012). Further, to identify if monocyte MitoSOX:MitoTracker ratio at baseline and / or at stimulation correlate to serum inflammatory cytokines IFN-γ, IL-10, IL-12, IL-17A, IL-1β, IL-2, IL-4, IL-6 and TNF-α in iRBD and PD, spearman correlation analyses were performed. Positive correlation between PD MitoSOX: MitoTracker ratio at baseline and IL-12p70 cytokine was revealed (r=0.485, p=0.004). No other positive correlations were identified.

Cytokine	MitoSOX (Baseline)	MitoTracker (Baseline)	MitoSOX (Stimulated)	MitoTracker (Stimulated)	MitoSOX / MitoTracker (Baseline)	MitoSOX / MitoTracker (Stimulated)
IFN-γ	r=0.159	r=0.087	r=-0.024	r=-0.022	r=-0.043	r=0.050
	p=0.376	p=0.631	p=0.894	p=0.903	p=0.812	p=0.782
IL-10	r=0.186	r=0.132	r=0.204	r=0.095	r=-0.011	r=0.046
	p=0.300	p=0.465	p=0.256	p=0.599	p=0.954	p=0.801
IL-12p70	r=0.102	r=-0.028	r=0.071	r=-0.035	r=0.096	r=0.152
	p=0.573	p=0.875	p=0.695	p=0.848	p=0.597	p=0.399
IL-17A	r=0.101	r=-0.048	r=0.139	r=-0.087	r=0.114	r=0.272
	p=0.575	p=0.789	p=0.440	p=0.629	p=0.528	p=0.126
IL-1β	r=0.002	r=-0.047	r=0.154	r=-0.075	r=-0.009	r=0.160

	p=0.992	p=0.801	p=0.409	p=0.688	p=0.963	p=0.390
IL-2	r=-0.062	r=-0.107	r=-0.036	r=-0.155	r=0.116	r=0.204
	p=0.742	p=0.568	p=0.847	p=0.404	p=0.534	p=0.271
IL-4	r=-0.092	r=-0.071	r=0.042	r=0.104	r=-0.074	r=-0.154
	p=0.612	p=0.693	p=0.818	p=0.564	p=0.684	p=0.392
IL-6	r=-0.049	r=0.085	r=-0.031	r=-0.021	r=-0.095	r=0.033
	p=0.787	p=0.639	p=0.864	p=0.907	p=0.598	p=0.855
TNF-*α*	r=0.211	r=0.273	r=0.257	r=0.138	r=-0.158	r=0.030
	p=0.239	p=0.124	p=0.148	p=0.442	p=0.381	p=0.869

Table 5.6 iRBD patient's cytokine measures do not correlate to monocyte mitochondrial measures. Spearman correlations at baseline and at stimulation between $CD14^+$ monocytes ROS production, mitochondrial content, MitoSOX:MitoTracker ratio and serum inflammatory cytokine levels in iRBD group (N=33).

Cytokine	MitoSOX (Baseline)	MitoTracker (Baseline)	MitoSOX (Stimulated)	MitoTracker (Stimulated)	MitoSOX / MitoTracker (Baseline)	MitoSOX / MitoTracker (Stimulated)
IFN-γ	r=0.279	r=-0.050	r=-0.134	r=-0.279	r=0.239	r=0.189
	p=0.110	p=0.778	p=0.449	p=0.110	p=0.174	p=0.284
IL-10	r=0.042	r=-0.130	r=0.196	r=-0.083	r=0.160	r=0.190
	p=0.813	p=0.464	p=0.266	p=0.640	p=0.366	p=0.282
IL-12p70	r=0.186	*r=-0.428	r=0.180	r=-0.238	**r=0.485	r=0.324
	p=0.293	p=0.012	p=0.308	p=0.175	p=0.004	p=0.061
IL-17A	r=0.251	r=-0.107	r=0.215	r=-0.026	r=0.258	r=0.171
	p=0.152	p=0.546	p=0.221	p=0.883	p=0.141	p=0.333
IL-1β	r=0.228	r=0.173	r=0.121	r=0.116	r=-0.034	r=-0.041
	p=0.203	p=0.336	p=0.502	p=0.522	p=0.851	p=0.822

IL-2	r=0.112	r=-0.206	r=0.125	r=-0.083	r=0.304	r=0.142
	p=0.529	p=0.243	p=0.483	p=0.640	p=0.080	p=0.422
IL-4	r=-0.104	r=-0.161	r=0.070	r=-0.072	r=0.044	r=0.165
	p=0.560	p=0.362	p=0.695	p=0.686	p=0.804	p=0.350
IL-6	r=0.139	r=-0.025	r=0.063	r=-0.066	r=0.084	r=0.141
	p=0.434	p=0.887	p=0.724	p=0.712	p=0.636	p=0.425
TNF-*α*	r=0.026	r=-0.191	r=0.047	r=-0.238	r=0.215	r=0.268
	p=0.884	p=0.280	p=0.792	p=0.175	p=0.223	p=0.125

Table 5.7 IL-12p70 positively correlates to MitoSOX:MitoTracker ratio. Spearman correlations at baseline and at stimulation between $CD14^+$ monocytes ROS production, mitochondrial content, MitoSOX:MitoTracker ratio and PD serum inflammatory cytokine levels. Positive correlation between MitoSOX:MitoTracker correlation at baseline and IL-12p70 cytokine of PD group (N=34). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$.

5.6 Discussion

The implication of inflammation to the aetiology and pathogenies of PD has been increasingly recognised (Pajeres et al., 2020; Williams et al., 2022). This present study evaluated peripheral inflammation in a cohort that composed of controls, iRBD and PD patients to determine if pro-inflammatory serum cytokine measures can utilise as potential diagnostic PD biomarkers. Specifically pro-inflammatory serum cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A and TNF-α were measured and associated to PD and iRBD clinical markers and monocyte mitochondrial function markers.

Analysis of pro-inflammatory serum cytokines revealed significantly reduced IL-2 levels in the iRBD group. No other significant differences were observed in peripheral immune markers in

the iRBD group. Serum inflammatory profiles of 30 iRBD patients and 12 controls were measured in a longitudinal study to evaluate the potential link of serum inflammatory cytokines to iRBD phenoconversion to alpha-synucleinopathies. Interleukin cytokines IL-1β, IL-2, IL-6, IL-10 and TNF-α were measured. The data revealed no significant differences between PD cytokine measures and control at baseline or significant alterations in cytokine levels at 4 year follow up. The data also revealed that elevated levels of TNF-α in a subgroup of iRBD patients was later associated to increased incidence of phenoconversion to alpha-synucleinopathies (Kim et al., 2020). Upregulated levels of TNF-α were also observed in the plasma of iRBD patients in a longitudinal study conducted by Zhang et al. (2020). Of the 10 plasma cytokines measured, specifically, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α, and IFN-γ, in 77 iRBD and 74 age and sex-matched healthy controls, elevated levels of IL-10 and TNF-α was found in the iRBD group. Further, iRBD patients with elevated levels of IL-10 and TNF-α plasma cytokines had an increased susceptibility of phenoconversion to alpha-synucleinopathies. IL-2 has a significant role in the immune response, in which variations in IL-2 can potentially lead to the aetiology of autoimmune diseases (Rafaqat & Rafaqat, 2023). Pro-inflammatory response is suggested to be prominent during the early PD phase (Taylor et al., 2013). Therefore, reduced IL-2 in iRBD patients compared to both controls and PD patients from this current cohort may be suggestive of the impaired peripheral innate immune system.

Significant alterations in peripheral pro-inflammatory serum cytokine markers of the PD cohort was not observed. The findings of this current study are similar to those of Diaz et al. (2022) that aimed to assess peripheral inflammatory serum cytokines of PD patients and healthy older adults (HOA). Of the 13 cytokines measured, specifically, TNF-*α*, IFN-γ, IL-1β, IL-8, IL-2, IL-7, IL-5, IL-13, IL, 4, IL-10 IL-12p70, GM-CSF, and IL-6, statistically significant difference between the PD group and HOA was only observed in IL-6. In the PD group, IL-6 was upregulated

compared to the HOA group. Nonetheless, previous studies have found increased levels of immune cytokine markers in serum of PD patients compared to controls in the following cytokines : IFN-γ, IL-1β, IL-2, IL-6, IL-10 and TNF-α. Rathnayake et al. (2019) conducted a case control study that constituted of 72 patients and 56 controls to evaluate the usefulness of serum immune markers for preclinical diagnosis of PD. The study found significantly increased levels of IFN-γ and IL-10. Significant increase of IL-1β in serum of PD patients has been observed in several studies (Chatterjee et al., 2020; Roy et al., 2022; Xu et al., 2022). Furthermore, study conducted by Kim et al. (2022) to explore the link between serum inflammatory markers and PD progression and non-motor symptoms also found elevated levels of IL-1β, IL-2 and IL-6. Elevated TNF-α in serum of PD patients was also revealed in case control study of 142 PD patients and 67 controls (Xiromerisiou et al., 2022) and also observed by Chang et al. (2023) that explored the serum inflammatory profiles of 109 PD patients and 113 controls.

Association between peripheral inflammatory cytokines and clinical markers may be indicative of the contribution of inflammatory alterations in disease pathogenesis. Significant positive associations between PD inflammatory markers and disease severity clinical markers were found in this current study. The MDS - UPDRS III for motor movement severity positively correlated to IL-17A, that also correlated to age and statins medication. Furthermore, the H&Y scale for symptom progression positively correlated to IL-6. Pro-inflammatory cytokine IL-6 has significant roles in maintaining the homeostasis of immune cells, due to their crucial role in the neurogenesis and maturation of glial cells (Erta et al., 2012). Diminished signalling pathway and synthesis of IL-6, is suggested to be a contributing factor to the development of PD. Elevated levels of IL-6 were observed in PD group when compared to age and sex matched healthy controls that did not present with family history of inflammation and neurodegenerative diseases

(Kwiatek-Majkusiak et al., 2020). Post-mortem study found significantly increased concentration of IL-6 in dopaminergic and striatal region in parkinsonian brains compared to controls (Mogi et al.,1994). Furthermore, prospective longitudinal PD study revealed possible association of elevated IL-6 levels to increased mortality risk (Dufek et al., 2015). IL-6 and IL-10 also correlated to age in the iRBD group. Interestingly a study conducted by Luporini et al. (2021) found that IL-6 and IL-10 was associated with disease severity and increased comorbidity in older adults. Positive association of plasma IL-17A to non-motor symptoms and IL-6 to motor symptoms severity PD scales was also observed in PD patients in a study performed by Green et al. (2019).

Though a complete understanding of the role of IL-17A in neurodegenerative diseases remains elusive, literature in recent years has suggested the crucial role of IL-17A cytokine in autoimmune diseases and PD (Chen et al., 2020; Ruiz de Morales et al., 2020). Animal PD study model found IL-17A cytokine to have a significant role in the activation of microglial cells that promote neuroinflammation and accelerate the degeneration of dopaminergic neurons (Liu et al., 2019). Positive association between severity of motor and non-motor PD symptoms and plasma cytokines IL-6 and IL-17A was found in study conducted by Holly et al. (2019). Interestingly, IL-17A positively correlated to statins use in both the PD and iRBD group. Statins has an immunomodulatory effect and has been observed to have immunosuppression potency (Zeiser, 2018). The positive correlation of IL-6, IL-10 and IL-17A to clinical markers, age and sex found in this current study and previous studies indicate their potential role in the pathophysiology and symptomology of PD. Further the association of pro-inflammatory cytokines to non-motor symptoms is suggestive of their key role in the prodromal phase of PD.

Bonferroni corrections were applied to correlations of clinical measures and to correlations of pro-inflammatory cytokines in the iRBD and PD group. After the application of Bonferroni corrections, the only clinical marker that upheld significance was the correlation of cytokine IL-17A to age in the PD group. Decent extent of pro-inflammatory cytokine correlations upheld their significance in both the iRBD and PD group. The Bonferroni correction is the stringiest correlation correction, therefore possibly removing true significance of association of clinical markers to pro-inflammatory cytokines.

No significant findings were observed between serum pro-inflammatory cytokines and monocyte mitochondrial function markers. Though peripheral inflammation is suggested to parallel and prompt increased inflammation of the central nervous system in PD (Ferrari & Tarelli, 2011; Lauritsen & Romero-Ramos, 2023) inconsistent findings of peripheral inflammation have been found. Increased serum pro-inflammatory cytokine levels are not consistently observed across studies (Dzamko, 2023). Inflammation involves complex cellular interaction of several cell types including, monocytes, neutrophils and lymphocytes (Chen et al., 2018). As serum inflammatory response is not confined to monocytes, and may constitute of inflammation of other immune cells, therefore correlation of monocyte mitochondrial function to serum inflammatory markers may not present association.

The efficacy of iRBD and PD patient's peripheral inflammatory cytokine levels as diagnostic biomarkers remains elusive due to the inconsistency in findings. Nevertheless, recent findings of upregulated levels of peripheral cytokines and increased susceptibility of phenoconversion to degenerative alpha-syncucleinopathies in iRBD patients provide insight of the role peripheral inflammation in disease progression. To develop a more comprehensive understanding of the involvement of inflammatory cytokines in the pathogenesis of iRBD and

PD, future longitudinal studies should aim to include larger cohort sizes of iRBD and PD patients. It would be ideal to include both familial and sporadic PD patients, equal male to female participants with wide age range and disease duration. To provide indications if the aberration of cytokine levels is relevant to disease type, if changes in cytokine levels is specific to disease phase and ultimately if therapeutic intervention to modify cytokine levels at certain disease phase may ameliorate disease pathogenesis or symptom severity.

In summary, impaired immune system has been linked to the pathogenesis of PD and iRBD, with specific pro-inflammatory markers associated to the pathophysiology of motor and non-motor symptoms. The findings from this study presented significantly decreased IL-2 cytokine in the iRBD group, which may be indicative of depleted peripheral innate immunity. The role pro-inflammatory cytokines IL-6 and IL-17A in PD are increasingly recognised, presenting as potential prospective diagnostic inflammatory markers of PD. Indeed, peripheral inflammatory cytokines, IL-6 and IL-17A positively correlated to PD severity and disease progression scales. The association of specific pro-inflammatory cytokines to core clinical markers of PD, pave the potential to a greater understanding of the linkage between and the impact of impaired immune system and PD pathophysiology. Further, the prospect of immunomodulating therapeutic interventions to reduce disease pathogenesis and symptom severity.

CHAPTER SIX

Monocyte MitoSox:MitoTracker Ratio Correlates with Motor Severity, Plasma Neurofilament Light and Alpha-synuclein in a Parkinson's Disease Cohort

6.1 Introduction

In chapter 4 of this thesis, elevated levels of mitochondrial reactive oxygen species (ROS) and mitochondrial content was observed in monocytes of Parkinson's disease (PD) patients. In this chapter I aim to determine if increased reactive oxygen species (ROS) in PD patient monocytes can be ameliorated with small molecule drug modulators of Glucosylceramidase Beta 1 (GBA1) or Leucine-rich repeat kinase 2 (LRRK2).

Variations in *LRRK2* are a common risk factor for PD. LRRK2 is highly expressed in monocytes and plays a role in the regulation of mitochondrial function and inflammatory pathways (Oun et al., 2022; Singh et al., 2019; Wallings & Tansey, 2019). Mutation in *LRRK2* have been observed to have increased mitochondrial ROS production and increased sensitivity to cell death (Singh et al., 2019). LRRK2 also plays a significant role in regulating mitophagy, which is essential to mitochondrial quality-control mechanism through selective autophagy of damaged mitochondria (Malpartida et al., 2021). Moreover, inhibition of LRRK2 can rescue impaired mitophagy observed in *LRRK2* mutation rodent models (Singh et al., 2021). Due to the therapeutic potential of LRRK2, small molecule inhibitors of *LRRK2* are currently being evaluated as therapeutic target for PD (Taymans et al., 2023), and there is much interest in determining mechanisms by which they may ameliorate PD pathology.

Heterozygous mutations in the *GBA1* gene are the greatest genetic risk factor for PD. *GBA1* encodes the lysosomal enzyme Glucocerebrosidase (GCase), that is responsible for the degradation of glucosylceramide into glucose and ceramide (Dzamko, 2016). Genome wide association studies have shown significant association of *GBA1* mutations and PD, indicating that *GBA1* gene mutation along with age to be one of the strongest risk factors for PD (Avenali et al., 2020; Rizig et al., 2023; Somerville et al., 2024). Diminished GCase activity leads to increased levels of glucosylceramide which promotes the aggregation of alpha-synuclein (α-syn) intermediates (Chatterjee & Krainc, 2023). This leads to bidirectional effect of a positive feedback loop of GCase α-syn pathogenic cycle (Mazzulli et al., 2011). Further, depleted GCase activity has shown to cause mitochondrial dysfunction and oxidative stress (Cleeter et al., 2013), in which decreased GBA levels are associated to *GBA1* mutations (Li et al., 2019). Analysis of post-mortem PD brain tissue carrying heterozygous *GBA1* mutations presented elevated mitochondrial content, increased levels of mitochondrial ROS and impaired autophagy (Li et al., 2019). This finding is indicative of underlying mechanism between heterozygous *GBA1* mutations and mitochondrial dysfunction in PD. Activation of *GBA1* can increase GCase activity leading to reduced glucosylceramide activity and presents as a potential therapeutic modification to reduce α-syn pathology, mitochondrial dysfunction and oxidative stress (Burbulla et al., 2019). Therefore, identification of therapeutic targets that increase GCase activity is of significant importance.

To determine if activation of GCase activity and/or inhibition of LRRK2 kinase activity can ameliorate mitochondrial dysfunction present in PD patient monocytes, my flow cytometry assay was again employed to assess mitochondrial content and ROS levels in isolated monocytes treated with and without small molecule modulators of LRRK2 and GCase.

Correlations of monocyte mitochondrial function to plasma levels of α-syn, GFAP, NfL, and tau, as well as clinical variables and inflammatory cytokines were also conducted.

6.2 Hypothesis

Monocyte mitochondrial dysfunction can be measured in PD monocytes compared to controls and that this dysfunction will be ameliorated with the GCase activator S-181 and / or the LRRK2 inhibitor MLi-2.

6.3 Aims

1. Measure mitochondrial function in monocytes from PD patients and controls using the live cell flow cytometry assay.
2. Determine if mitochondrial ROS or content are increased PD patient monocytes compared to controls.
3. Determine if GCase activation or LRRK2 inhibition can ameliorate increased mitochondrial ROS in PD patient monocytes.
4. Determine any association present between mitochondrial measures and PD clinical markers.
5. Determine any association present between mitochondrial measures and plasma PD biomarkers.

6.4 Chapter Specific Methods

6.4.1 Monocyte isolation and plasma collection

As increased ROS was observed in monocytes in chapter 4, this chapter employed isolated monocytes for the inhibitor treatments. Monocyte isolation is described in methods chapter, section 2.4. Plasma collection is described in methods chapter, section 2.5.

6.4.2Flow cytometry measurement of mitochondrial function

Mitochondrial flow cytometry assay was performed as described in methods chapter, section 2.8. Cells were plated at a cell density of $1x10^5$ per well and left to recover from thawing for up to 2 h in a 37°C tissue culture incubator. Samples were treated with 100 nM of MLi-2 or 20 μM of S-181 small molecule drugs or with equal volume DMSO for 18 hours at 37°C. For live cell analysis spectral unmixing was performed as described in methods chapter, section 2.9 with 30,000 events recorded.

6.4.3 Measurement of plasma inflammatory cytokines

Plasma levels of inflammatory cytokines were obtained using the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad, #M500KCAF0Y) with plasma diluted 1:4 and assay performed as described in methods chapter, section 2.10. Plasma levels of nine inflammatory cytokines that were all below detection (GM-CSF, IL-2, IL-5, IL-6, IL-10, IL-12, IL-15, PDGF-BB and VEGF) were not included in analysis. Only a small proportion of data was missing, 0 values were replaced with half the value of the lowest limit of quantification from the standard curve for each cytokine for both control and patient groups.

6.4.4 Measurement of plasma alpha-synuclein, NfL, GFAP and tau

Plasma levels of α-syn, neurofilament light (NfL), glial fibrillary acidic protein (GFAP) and tau have been assessed previously in this cohort using the U-PLEX Plus Human α-syn kit (Mesoscale Discovery) and the SIMOA neurology 4-plex assay (Quanterix; #102233), (Youssef et al., 2023). Summary data are provided again in Table 6.1 for convenience.

6.4.5 Statistical analysis

Data and statistical analyses were performed using IBM SPSS statistics 27 and GraphPad Prism 9, with p value < 0.05 was considered as statistically significant. Differences between groups were assessed through two-way analysis of variance (ANOVA) for figures 6.1 and 6.2 and through the Kruskal-Wallis Test for figure 6.3. Graphs depict the mean ± standard error of the mean (SEM) with dots representing individual values. Spearman correlation analyses were performed to analyse the association between mitochondrial monocyte markers, clinical variables, and plasma markers from the PD cohort.

6.5 Results

6.5.1 Evaluation of control and PD patient's demographic and clinical biomarkers

Demographic and clinical data of controls (N=25) and PD patients (N=24) is provided in table 6.1. No significant differences were observed between age and sex of PD patients compared to controls, indicating that the groups were matched. As the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) III, Hoehn and Yahr (H&Y) scale and levodopa equivalent daily dose (LEDD) score of controls were not recorded an assessment of the significance between controls and PD patients could not be determined. There were no significant differences between controls and PD patients' plasma α-syn, NfL, GFAP, Tau and viability measures.

	Control	PD	*p*-value
Age (years)	69.08 ± 1.82	68.8 ± 1.61	0.74
Sex (% M)	68%	75%	0.75
Disease duration	N/A	6.83 ± 0.873	
MDS-UPDRS III	N/A	28.25 ± 3.29	
MoCA	26.2 ± 0.51	24.87 ±1.12	0.43
H & Y	N/A	1.94 ± 1.45	
LEDD	N/A	757.52 ± 67.49	
Alpha-synuclein (pg/mL)	12.08 ± 1.04	14.19 ± 2.031	0.88
NfL (pg/mL)	13.38 ± 1.12	16.42 ± 2.41	0.92
GFAP (pg/mL)	104.92 ± 10.53	131.09 ± 16.17	0.26
Tau (pg/mL)	1.86 ± 0.13	1.81 ± 0.13	0.78
Viability	79.48 % ± 2.25	77.83% ± 2.22	0.55

Table 6.1 Demographic, clinical and biomarker data for PD patients and controls. Data are mean ± standard error. Disease severity was recorded using the MDS-UPDRS III. (N= 25 control and 24 PD). N/A = not available.

6.5.2. Mitochondrial content and ROS in PD patient monocytes

Mitochondrial content and mitochondrial ROS levels were measured in isolated monocytes from control and PD patients at baseline and following treatment with the GCase activator S-181 and the LRRK2 inhibitor MLi-2. An example of the gating strategy for assessing mitochondrial ROS and mitochondrial content in $CD14^{+}$ monocytes is shown in Figure 6.1.

A two-way ANOVA was used to determine any significant effect of either PD or drug treatment on the measures of mitochondrial function. An overall significant effect of drugs was present (p=0.018) (Figure 6.2A). There was no significant effect of S-181 treatment on PD monocyte mitochondrial ROS (p = 0.778,) or effect of MLi-2 treatment (p=0.853) compared to controls (Figure 6.2A). There was no overall effect of drugs (p=0.927) or effect of S-181 treatment (p=0.995) or effect of MLi-2 treatment (p=0.944) compared to controls (Figure 6.2B). For MitoSOX:MitoTracker ratio there was no overall effect of drugs (p=0.431) or effect of S-181 drug (p=0.999) or effect of MLi-2 drug (p=0.177) (Figure 6.2C) compared controls (DMSO). No significant difference in viability between control and PD group was observed (p=0.794) or effect of S-181 treatment (p=0.253) or MLi-2 treatment (p=0.253) (Figure 6.2D).

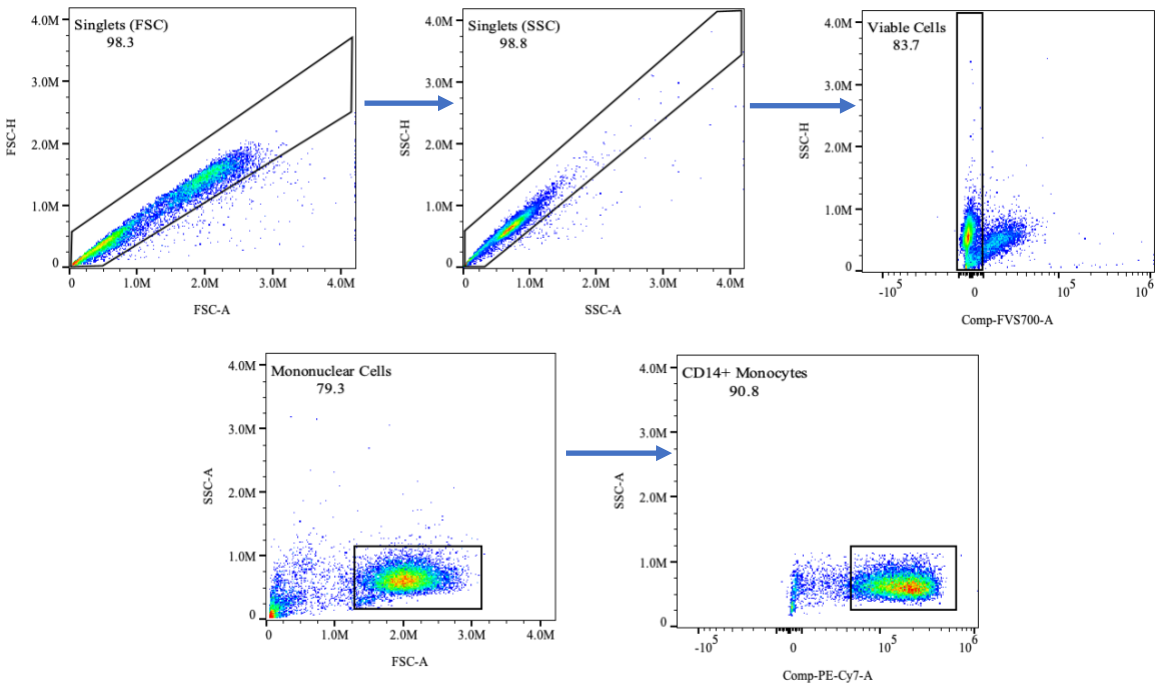


Figure 6.1 Representative flow cytometry gating strategy to assess mitochondrial activity in live monocytes. Initially, two singlet gates are created through FSC and SSC parameters to exclude doublets. Viable monocytes were gated, followed by two subset gates on mononuclear cells and $CD14^{+}$ monocyte populations.

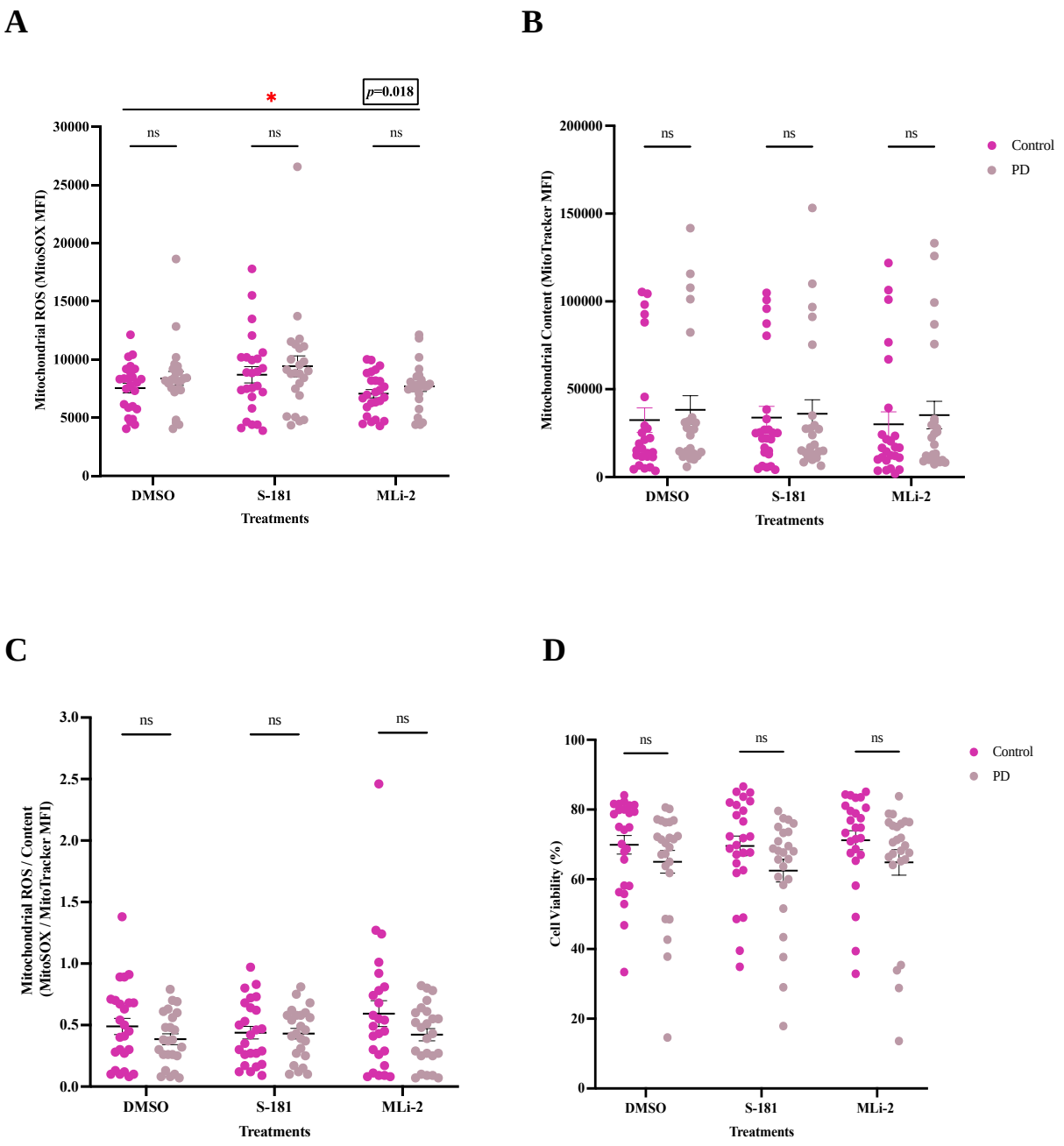


Figure 6.2 Assessment of the effect LRKK-2 inhibitor MLi-2 and GCASE modulator S-181 on monocyte mitochondrial content and ROS production in control and PD groups. Cryopreserved monocytes were plated and treated with 100 nM of MLi-2 or 20 mM of S-181 small molecule drugs or with equal volume DMSO for 18 hours at 37°C. Monocytes were gated and MFI of specific mitochondrial probes was measured. No significant effect of drug was present on PD mitochondrial ROS (Figure 6.2A), mitochondrial content (Figure 6.2B) or on normalised mitochondrial data mitochondrial content / mitochondrial ROS (Figure 6.2C) compared to controls or on viability between control and PD group at baseline or as effect of

treatments (Figure 6.2D). Graphs display mean ± SEM (N= 25 control and 24 PD). Two-way ANOVA with Tukey's Multiple Comparison Test. Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$.

6.5.3 MitoSOX:MitoTracker ratio positively correlates to motor severity in PD patients

Although mitochondrial measures of control and PD were not significantly different, mitochondrial measures displayed a high level of variation. To determine if this variation was associated with clinical variables, correlation analysis was performed. Positive correlation between MitoSOX and MitoTracker at baseline and age, disease duration, MDS – UPDRS III, MoCA, LEDD and Hoehn and Yahr scale (H&Y) were not observed. A single negative correlation between MitoTracker and MDS-UPDRS III was found (r=-0.405, p=0.050). Normalised mitochondrial measures, MitoSOX:MitoTracker ratio positively correlated with the MDS-UPDRS III scores in the PD group (r=0.443, p=0.030). No significant correlations were found between MitoSOX:MitoTracker ratio and age, disease duration, MoCA, LEDD and H&Y scale (Table 6.2).

	Age	**Disease Duration**	**MDS - UPDRS III**	**MoCA**	**H &Y**	**LEDD**
MitoSOX	r=0.198 p=0.353	r=0.026 p=0.905	r=-0.188 p=0.378	r=-0.120 p=0.595	r=-0.055 p=0.797	r=0.012 p=0.957
MitoTracker	r=-0.183 p=0.392	r=-0.198 p=0.353	*r=-0.405 p=0.050	r=-0.253 p=0.257	r=-0.294 p=0.164	r=0.013 p=0.953
MitoSOX: MitoTracker	r=0.249 p=0.241	r=0.314 p=0.135	*r=0.443 p=0.030	r=0.175 p=0.437	r=-0.358 p=0.086	r=0.022 p=0.920

Table 6.2 Monocyte MitoSOX:MitoTracker ratio positively associates to disease progression. Spearman correlations between monocyte ROS production, mitochondrial content, and monocyte MitoSOX:MitoTracker ratio at baseline and PD clinical data (N=24). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$.

6.5.4 MitoSOX:MitoTracker ratio correlates to plasma NfL, GFAP and alpha-synuclein

Plasma collected from the participants in this study has previously been used to measure levels of potential PD biomarkers α-syn, NfL, GFAP, and tau. Thus, we determined if mitochondrial measures also correlated to these potential PD biomarkers. Spearman correlation between MitoSOX and MitoTracker at baseline and plasma α-syn, NfL, GFAP and tau did not present any positive associations. Negative correlations between MitoTracker and α-syn (r=-0.540, *p*=0.006), NfL (r=-0.470, *p*=0.021) and GFAP (r=-0.449, *p*=0.028) were observed. Within the PD group, significant positive correlations were found between the MitoSOX:MitoTracker ratio and plasma levels of α-syn (r=0.582, *p*=0.003), NfL (r=0.473, *p*=0.020) and GFAP (r=0.452, *p*=0.027) (Table 6.3).

	Alpha - Synuclein	**NfL**	**GFAP**	**Tau**
MitoSOX	r=-0.131 p=0.543	r=-0.096 p=0.656	r=-0.121 p=0.574	r=0.057 p=0.791
MitoTracker	**r=-0.540 p=0.006	*r=-0.470 p=0.021	*r=-0.449 p=0.028	r=0.054 p=0.802
MitoSOX: MitoTracker	*r=0.582 p=0.003	*r=0.473 p=0.020	*r=0.452 p=0.027	r=0.032 p=0.882

Table 6.3 Monocyte MitoSOX:MitoTracker ratio positively correlates to plasma markers. Spearman correlations between monocyte ROS production, mitochondrial content

and monocyte MitoSOX:MitoTracker ratio at baseline and PD plasma levels (α-syn, NfL, GFAP and tau) (N=24). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$.

6.5.5 MitoSOX:MitoTracker ratio does not correlate to inflammatory cytokines

Finally, we aimed to determine if ROS in PD patient monocytes is associated with increased inflammation. In the measured cytokines, IL-1ra (p=0.018), IL-1β (p=0.025), 1L-8 (p=0.028), MIP-1α (p=0.003) and basic FGF (p=0.033) were significantly reduced in PD compared to control (Figure 6.3). No significant correlations were observed between cytokines and mitochondrial measures in the PD group (Table 6.4).

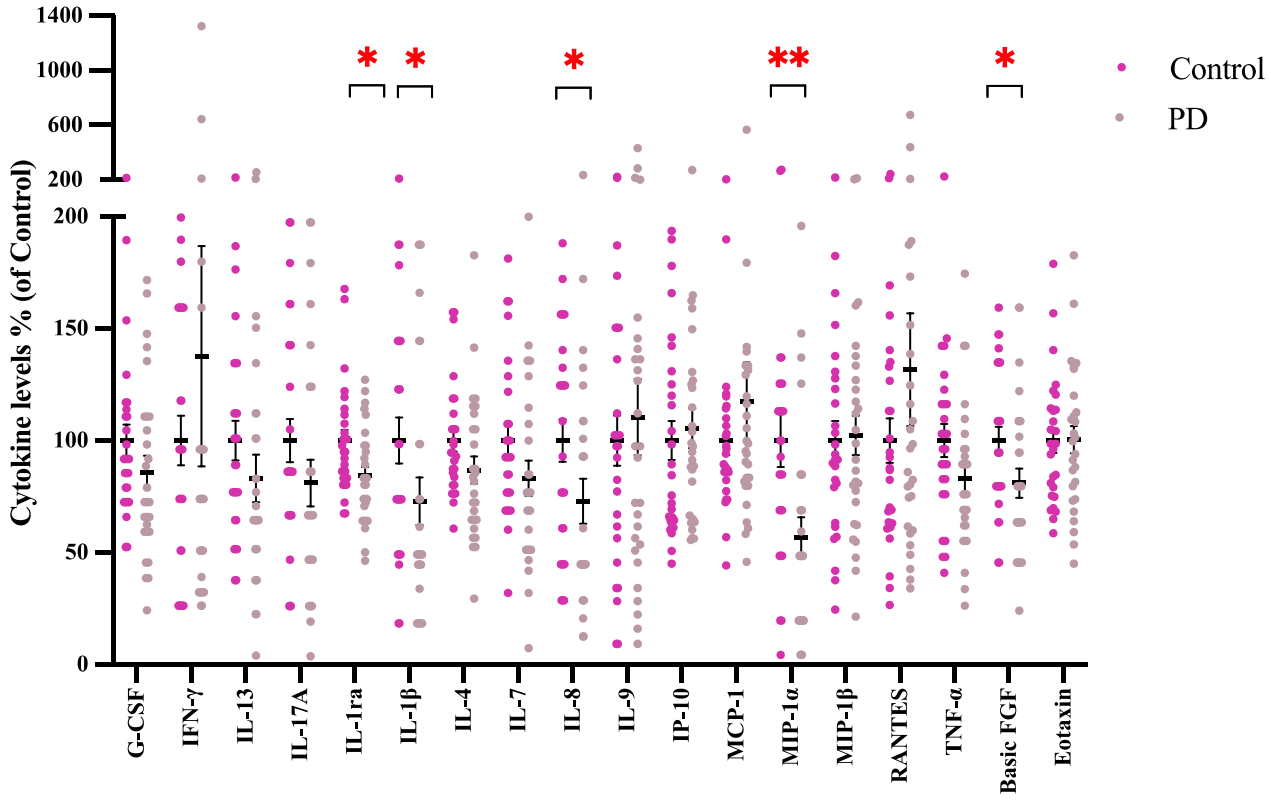


Figure 6.3. Multiplex Cytokine Assay to measure differences in plasma inflammatory cytokine levels between control and PD group. Kruskal-Wallis test to assess differences in cytokine levels between controls and PD patients. Graphs display mean ± SEM. (N= 25 control

and 24 PD). Statistical significance determined at $p = < 0.05$. *Indicates $p < 0.05$, ** indicates $p < 0.01$ compared to control.

	MitoSOX:MitoTracker (DMSO)	
Cytokine	***r* - value**	***p* - value**
G-CSF	-0.021	0.925
IFN-γ	0.151	0.491
IL-13	0.126	0.567
IL-17A	0.158	0.472
IL-1ra	0.090	0.682
IL-1β	0.048	0.828
IL-4	-0.044	0.842
IL-7	0.009	0.968
IL-8	0.073	0.740
IL-9	0.387	0.068
IP-10	-0.097	0.660
MCP-1	0.068	0.759
MIP-1α	0.053	0.810
MIP-1β	0.269	0.215
RANTES	0.256	0.239
TNF-α	0.208	0.340
Basic FGF	0.074	0.736
Eotaxin	0.017	0.939

Table 6.4 PD patient's inflammatory cytokine levels do not correlate to monocytes MitoSOX:MitoTracker ratio. Spearman correlations between monocyte ROS production / mitochondrial content and plasma inflammatory cytokine levels in the PD cohort. (N= 24)

6.6 Discussion

The aim of this study was to assess differences in mitochondrial parameters between PD and control cohort, and to determine if the small molecule drugs S-181 and MLi-2 could ameliorate mitochondrial dysfunction. Correlation analyses of mitochondrial parameters to clinical and inflammatory measures was also performed to identify potential associations.

Significant differences in mitochondrial ROS release and mitochondrial content between control and PD groups was not observed. The difference in findings of this chapter and chapter 4, where elevated levels of mitochondrial ROS and mitochondrial content were observed may potentially be multifactorial. In this current chapter isolated monocytes were used and baseline samples were treated with DMSO at 37°C incubation overnight as control vehicles. DMSO treatment impacts cell structure, mitochondrial integrity and mitochondrial membrane potential. Study conducted by Tuncer et al. (2018) found that small doses of DMSO commonly used for control vehicles has the potential to impact and interfere with cellular function. To determine if mitochondrial function was impacted by DMSO treatment on control vehicles, measurement of mitochondrial function of untreated samples could provide indication. Due to the limited number of cells, it was not feasible in this current chapter to measure monocyte mitochondrial function of PD and control samples untreated. Nevertheless, the measurement of mitochondrial function of untreated samples at baseline without treatment or prolonged incubation as performed in chapter 4 indicates that mitochondrial function of PD patient monocytes can be measured using the mitochondrial flow cytometry assay.

The aetiology of mitochondrial dysfunction in PD has been increasingly linked to *GBA1* and *LRRK2* mutations. This association has been well supported through post-mortem studies that have revealed increased mitochondrial content and oxidative stress in post-mortem PD brains (Li et al., 2019; Singh et al., 2019). Variations in *GBA1* and *LRRK2* have an effect on one

another, therefore the identification of modifying therapeutic targets is of paramount interest (Pang et al., 2022). In this study mitochondrial markers have been utilised to measures the effect of *GBA1* and *LRRK2* modulating drugs on mitochondrial function in control and PD patient monocytes. S-181 is a small molecule modulator that has presented as potential therapeutic target for PD through activation of wild type GCase activity (Burbella et al., 2019). MLi-2 is highly potent and selective brain penetrant inhibitor of LRRK2 (Fell et al., 2015). To determine if S-181 and MLi-2 small molecule drugs ameliorate mitochondrial dysfunction in PD monocytes, control and PD patient monocytes were treated separately with S-181, MLi-2 and DMSO as negative control / baseline measure. The data displayed an overall significant effect of treatments on mitochondrial ROS. No significant effect of treatment on mitochondrial ROS was observed between groups and no effect was observed on mitochondrial content. Application of S-181 small molecule for increasing GCase enzyme activity to improve PD pathology has been previously applied in a study conducted by Burbulla et al. (2019) on PD iPSC-derived DA neurons. The study found that S-181 treatment for 10 consecutive days with increase in concentration from 5 μM to 25 μM in human dopamine neurons, increased activation of GCase activity. The link between LRRK2 and compromised mitochondrial dysfunction in PD has been well observed in which, the most frequent *LRRK2* variation being the *G2019S* mutation (Ilieva et al., 2024; Ren et al., 2019; Williamson et al., 2023). An in-vitro study using characterised cell line HEK 293-T with *LRRK2* genotype, treated WT 293-T, *LRRK2-G2019S*, and LRRK2 knockout (KO) cells with either toxicants alone or toxicants in combination with 1 μM of MLi-2, LRRK2 inhibitor. The data revealed *LRRK2 G2019S* promoted elevated levels of ROS production and that inhibition of LRRK2 reduced oxidative stress (Ilieva et al., 2024). Further impaired mitophagy was observed in WT and G2019S cells, whilst LRRK2 KO cells presented protection from diminished mitophagy. Findings from Burbulla et al. (2019) and Ilieva et al. (2024) are indicative of the effect of GCase and LRRK2

deficiencies in mitochondrial dysfunction pathology in PD. Furthermore, the difference in findings from this current study and previous studies suggest that increased concentration and incubation time of S-181 and MLi-2 has potential therapeutic intervention to ameliorate mitochondrial dysfunction in PD (Burbella et al., 2019; Ilieva et al., 2024). Differentiation of monocytes to macrophages to allow for extended incubation and increased concentration may provide indication to the potential of small molecule drug modulators in ameliorating mitochondrial ROS in PD.

A significant positive association was observed between normalised monocyte mitochondrial measures and plasma markers α-syn, NfL and GFAP in the PD cohort of the current study. Increased levels of plasma α-syn, NfL and GFAP in PD patients has been previously observed (Che et al., 2024; Lin et al., 2017; Lin et al., 2019). Further, a positive correlation was observed between PD patient monocyte ROS levels at baseline and the degree of motor severity, assessed using the MDS-UPDRS III. A positive correlation between mitochondrial ROS and the MDS-UPDRS III has been previously observed in a study that assessed mitochondrial function through fibroblasts of idiopathic PD patients (Milanese et al., 2019). Seahorse Extracellular Flux Analyser and MitoSOX probe assessed the mitochondrial superoxide of 47 idiopathic PD patients' fibroblasts (Milanese et al., 2019). Similar to the observation of this current study, elevated mitochondrial superoxide levels were associated to greater symptom severity. The finding from this current study and of previous studies are indicative that monocyte mitochondrial ROS of PD patients may utilise as motor severity scale. Furthermore, in line with mitochondrial alterations and disease trajectory, Barhoorn et al. (2024) found that peripheral mitochondrial alterations were increasingly more pronounced in PD patients during a 3-year observation period. The association observed between monocyte mitochondrial ROS and the

MDS-UPDRS III motor severity scale may be indicative of clinical disease progression and/or disease severity (Barnhoorn et al., 2024).

An interconnected bidirectional relationship between genetic mutations, inflammatory cytokines and increased ROS levels is constitute to the complex pathology of PD (Magnusen et al., 2021). To determine the association of monocyte mitochondrial measures and plasma inflammatory cytokine markers of PD patients, spearman correlations analyses were performed. The data showed no significant correlations between monocyte mitochondrial measures at baseline and plasma inflammatory cytokines G-CSF, IFN-γ, IL-13, IL-17A, IL-1ra, IL-1β, IL-4, IL-7, IL-8, IL-9, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α, Basic FGF and Eotaxin. Plasma inflammatory cytokine levels of PD patients is often inconsistent, with plethora of studies reporting increased, comparable, or decreased cytokine levels of PD patients compared to controls (Jun & Kim, 2023). To determine if plasma inflammatory cytokine levels could quantify as potential biomarker measures, Multiplex Cytokine Assay was performed to measure differences in plasma cytokine levels between controls and PD and was correlated to clinical markers. The data revealed significant differences in plasma cytokine levels between controls and PD. Compared to controls, PD patients presented decreased cytokines levels of IL-1RA, IL-1β, IL-8, MIP-1α and Basic FGF. The results of this study have been observed in previous studies. Decreased levels IL-1RA in plasma of PD patients was recognised as a potential biomarker (Pochmann et al., 2018). Inflammatory PD studies often report decreased levels IL-1β cytokine in serum and increased levels in peripheral plasma of PD patients (Zimmerman & Brockmann, 2022). Analysis of current plasma IL-1β showed decreased levels in PD patients, distinctive to previous studies that observed elevated IL-1β in plasma extracellular vesicles and elevated serum IL-1β in PD patients (Chan et al., 2021; Kim et al., 2022; Roy et al., 2022; Xu et al., 2022).

In summary, the efficacy of small molecule drugs S-181 and MLi-2 in ameliorating mitochondrial dysfunction in PD cannot be concluded from this study, as an effect on PD to increase dysfunction was not found. However, a positive correlation of monocyte mitochondrial function markers to the MDS-UPRDS III for motor movement severity was found, suggesting mitochondrial function may have utility as a PD prognosis biomarker. Further positive correlation of monocyte mitochondrial function to pro-inflammatory plasma markers α-syn NfL and GFAP further support that mitochondrial dysfunction in monocytes is associated to the pathophysiology of PD. The limitations of this study include the modest cohort size that may have not sufficiently reflected the breadth of mitochondrial dysfunction in PD. A second limitation is the measurement of mitochondrial function at a single time point. Therefore, not indicative of change or trends that may occur over prolonged periods. Single time point measurement could also introduce bias, as mitochondria are highly impacted by activity and diet. Further limited background data of PD patient's lifestyle eliminated the potential of assessing the association or effect of lifestyle on PD patients' mitochondrial measures. Future studies that aim to assess mitochondrial function in PD, should include larger cohort size to better reflect mitochondrial impairment in PD and the effect of small molecules in ameliorating in mitochondrial dysfunction.

CHAPTER SEVEN

General Conclusion and Future Directions

Though substantial progress has been made in PD research, the discovery of biomarkers for the diagnosis and monitoring of disease progression and disease modifying therapeutic treatments remain a significant gap. Although disease severity scales provide an indication of the severity of motor and non-motor PD symptoms, they are susceptible to potential bias and do not provide objective measures of disease pathology. Peripheral blood-based biomarkers present as advantageous biospecimen candidates as being minimally invasive and reflective of deeper pathology (Alexovic et al., 2023; Chahine, et al., 2014).

Mitochondrial dysfunction, increased mitochondrial ROS and aberrations in the innate immune system have risen as having significant roles in the progression in PD. The pivotal involvement of mitochondrial dysfunction in the pathogenesis of sporadic and familial PD is well established (Chen et al., 2019; Exner et al., 2012; Henrich et al., 2023; Malpartida et al., 2020). Further, increasing evidence is suggestive of the contributing role of the innate immune system in the pathogenesis and progression of PD (Lai et al., 2022; Schonhoff et al., 2019; Tansey et al., 2022). This thesis aimed to determine if peripheral mitochondrial and inflammatory readouts may utilise as diagnostic and /or prognostic biomarkers of PD.

Elevated levels of mitochondrial ROS and aberrations in mitochondrial content levels are significant determinants of defective mitochondrial function, therefore constituted as markers of mitochondrial dysfunction. Serum pro-inflammatory and plasma inflammatory cytokine

levels were measured to determine if distinct cytokine variations are present among iRBD or PD groups. Mitochondrial and inflammatory measures were measured in PD, iRBD and healthy control groups and were associated to clinical measures and correlated to each other to determine their feasibility as diagnostic / prognostic biomarkers, and if mitochondrial and inflammatory dysfunction have underlying relationship in the pathogenesis of PD. iRBD is the best defined precursor to PD, therefore mitochondrial and inflammatory read out measures of iRBD were conducted to assess if mitochondrial and inflammatory dysfunction can be identified in prodromal PD disease phase.

To measure peripheral mitochondrial function for biomarkers discovery, flow cytometry was applied as being a high precision and single cell analysis technique. A mitochondrial flow cytometry assay was optimised and validated in chapter 3 to assess the efficacy of mitochondrial probes in measuring fluctuating levels of mitochondrial ROS and mitochondrial content. This was conducted through the stimulation of healthy donor PBMCs, with mitochondrial complex III inhibitor antimycin A for the generation of excessive mitochondrial ROS and mitochondrial content. Inhibition of mitochondrial complex III has been reported to be part of the impaired molecular pathways of PD and generated the expected outcome of mitochondrial dysfunction in healthy donor PBMCs.

The validated mitochondrial flow cytometry assay was utilised in chapter 4 of this thesis to measure mitochondrial ROS and mitochondrial content at baseline and at stimulation in age and sex matched PD, iRBD and healthy control PBMC samples. Elevated levels of mitochondrial content were in line with increased mitochondrial content observed in the established positive control – antimycin A treated healthy donor PBMCs. Significant mitochondrial differences were not observed in iRBD patients; this may be associated to early

disease stage or differences in disease pathology. Association analyses of PD and iRBD mitochondrial measures to clinical markers revealed positive correlation of both PD and iRBD mitochondrial measures to clinical markers. Interestingly iRBD patients' mitochondrial ROS at baseline positively correlated to the Mini Mental State Examination (MMSE), while mitochondrial content of iRBD patients at baseline positively correlated to the Movement Disorder Society - Unified Parkinson's Disease Rating Scale (MDS-UPDRS) III. The findings are indicative of mitochondrial dysfunction being associated to both motor and non-motor symptoms of PD. Furthermore, the findings are suggestive of mitochondrial disruptions being potentially present in the iRBD cohort although mitochondrial ROS and mitochondrial content alterations were not significant. To determine if mitochondrial ROS and mitochondrial content may perpetuate as diagnostic biomarkers during the prodromal phase of PD, larger iRBD cohort at an increased disease stage may be a vital factor to assess the utility of mitochondrial markers as diagnostic PD biomarkers, as early disease stage iRBD cohort from this current study may have not reflected mitochondrial dysfunction that occurs in mid to later stages in iRBD. The findings of this chapter have nonetheless provided a platform for further research into the utility of peripheral mitochondrial measures as potential diagnostic biomarkers for PD.

The role of chronic inflammation, mitochondrial dysfunction and elevated levels of ROS are indicated to have a synergistic contribution to the pathogenesis of PD (Hunter et al., 2006). Furthermore, the involvement of the peripheral innate immune system in the progression of PD is increasingly recognised, with an array of studies presenting variation in PD patient cytokine levels. In chapter 5 of this thesis, I aimed to determine if inflammatory measures of PD and iRBD are distinct from healthy controls. Pro-inflammatory cytokines levels in PD, iRBD and healthy controls were measured through an ultrasensitive assay platform that has not been previously used to measure inflammation in PD, iRBD and healthy control serum samples. In

addition, mitochondrial levels measured in chapter 4 were correlated to serum pro-inflammatory cytokine levels to determine if an underlying association between mitochondrial function and inflammation is present in the current cohort. The results revealed that serum pro-inflammatory cytokine, Interleukin (IL)-2 in the iRBD group was significantly reduced in comparison to the control and PD group. No other significant findings were observed in pro-inflammatory cytokine levels of the PD and iRBD group. Alterations in IL-2 cytokine levels has potential to perpetuate to autoimmune diseases (Rafaqat & Rafaqat, 2023) therefore, diminished levels of IL-2 in the iRBD group may be indicative of the role of impaired innate immune system in the pathophysiology of PD. Inflammation in PD is most significant during the prodromal disease phase, then follows trends of maintained inflammation (Zimmermann & Brockmann, 2022). As the mean disease duration of the PD group in chapter 5 was 9 years, therefore may perpetuate as to the lack of significant differences in serum pro-inflammatory cytokine levels between the PD and control group. The absence of association between serum pro-inflammatory cytokines and monocyte mitochondrial measures may be associated to inflammation in serum constituting of several inflammatory cell types and not specific to monocytes. Further, the initial absence of elevated inflammation in serum pro-inflammatory cytokines may consolidate as another factor to the lack of correlation between monocyte mitochondrial markers and serum pro-inflammatory markers. However, positive associations were observed between serum pro-inflammatory cytokines IL-6 and IL-17A and clinical measures in the PD and iRBD group. Serum cytokines IL-6 and IL-17A have been reported to have association to motor and non-motor symptoms respectively (Green et al., 2019). The findings observed in chapter 5 may indicate the efficacy of inflammatory markers as diagnostic biomarkers and are potentially more appropriate during the prodromal disease phase of PD. Therefore, indicating that serum may hold value for the diagnosis of iRBD or prodromal PD disease phase.

As elevated levels of mitochondrial ROS and mitochondrial content were observed in PBMCs of PD patients in chapter 6 of this current thesis; I aimed to assess if mitochondrial ROS and mitochondrial content are elevated in monocytes of PD patients compared to healthy controls. The second aim of chapter 6 was to assess if Glucocerebrosidase (GCase) enzyme activator, S-181 and LRRK2 inhibitor, MLi-2 can ameliorate mitochondrial dysfunction in PD monocytes. The data revealed that no significant differences were observed in mitochondrial ROS and mitochondrial content readouts between healthy controls and PD monocytes. The effectiveness of small molecule drug modulators S-181 and MLi-2 cannot be concluded from this current study as no effect was observed. This was potentially postulated to the initial absence of mitochondrial dysfunction in PD monocytes or potentially due to the decreased treatment time performed in the current study compared to previous studies that observed effect of drug (Burbulla et al., 2019; Ilieva et al., 2024). This may also be potentially due to the different concentrations, to confirm the efficacy of small molecule drug modulators further optimising may be required. Though mitochondrial dysfunction was not observed in PD monocytes this may be due to the modest cohort size of the study, rather than the definite absence of increased mitochondrial ROS levels in PD monocytes. Interestingly normalised monocyte mitochondrial measures, MitoSOX:MitoTracker ratio positively correlated the MDS-UPDRS III score and plasma markers a-syn, NfL and GFAP. This finding is indicative that monocyte MitoSOX: MitoTracker ratio readouts may utilise as indicative measures for monitoring PD progression. However, further larger longitudinal studies are required to assess the association of monocyte mitochondrial dysfunction and plasma inflammation in PD patients for potential biomarker application.

Overall, the optimised mitochondrial flow cytometry assay performed in this thesis has provided a platform for the utility of peripheral mitochondrial readouts as diagnostic and

prognostic biomarkers of PD. Further, the data of this thesis has revealed the potential of peripheral mitochondrial dysfunction measures as diagnostic and prognostic biomarkers of PD. For the validation of peripheral mitochondrial biomarkers in routine clinical practice, longitudinal multi-site clinical trial studies that include different forms of PD and iRBD patients is required. This will allow for the stratification of PD and iRBD patients that will potentially benefit from disease modifying therapies that target mitochondrial dysfunction.

References

Abdi, I. Y., Ghanem, S. S., & El-Agnaf, O. M. (2022). Immune-related biomarkers for Parkinson's disease. *Neurobiology of Disease*, 105771. https://doi.org/10.1016/j.nbd.2022.105771

Alexovič, M., Uličná, C., Sabo, J., & Davalieva, K. (2023). Human peripheral blood mononuclear cells as a valuable source of disease-related biomarkers: Evidence from comparative proteomics studies. *PROTEOMICS - CLINICAL APPLICATIONS*, *18*(2). https://doi.org/10.1002/prca.202300072

Antony, P. M. A., Kondratyeva, O., Mommaerts, K., Ostaszewski, M., Sokolowska, K., Baumuratov, A. S., Longhino, L., Poulain, J. F., Grossmann, D., Balling, R., Krüger, R., & Diederich, N. J. (2020). Fibroblast mitochondria in idiopathic Parkinson's disease display morphological changes and enhanced resistance to depolarization. *Scientific Reports*, *10*(1), 1569. https://doi.org/10.1038/s41598-020-58505-6

Aurora, R. N., Zak, R. S., Maganti, R. K., Auerbach, S. H., Casey, K. R., Chowdhuri, S., Karippot, A., Ramar, K., Kristo, D. A., Morgenthaler, T. I., Standards of Practice Committee, & American Academy of Sleep Medicine. (2010). Best practice guide for the treatment of REM sleep behavior disorder (RBD). *Journal of Clinical Sleep Medicine : JCSM : Official Publication of the American Academy of Sleep Medicine*, *6*(1), 85–95. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2823283/

Avenali, M., Blandini, F., & Cerri, S. (2020). Glucocerebrosidase Defects as a Major Risk Factor for Parkinson's Disease. *Frontiers in Aging Neuroscience*, *12*. https://doi.org/10.3389/fnagi.2020.00097

Baert, F., Vlaemynck, G., Maselyne, J., & Matthys, C. (2022). Odor Identification by Parkinson's Disease Patients Tested by Using Sniffin' Sticks versus Natural Spices. *Parkinson's Disease*, *2022*, 1–10. https://doi.org/10.1155/2022/2272691

Balestrino, R., & Schapira, A. H. V. (2019). Parkinson disease. *European Journal of Neurology*, *27*(1), 27–42. https://doi.org/10.1111/ene.14108

Barnard, R. M. (2012). Flow cytometry: a flexible tool for biomarker research. *Bioanalysis*, *4*(20), 2471–2483. https://doi.org/10.4155/bio.12.225

Barnhoorn, S., Milanese, C., Li, T., Lieke Dons, Mehrnaz Ghazvini, Sette, M., Farina, S., Sproviero, D., Payan-Gomez, C., & Mastroberardino, P. G. (2024). Orthogonal analysis of mitochondrial function in Parkinson's disease patients. *Cell Death and Disease*, *15*(4). https://doi.org/10.1038/s41419-024-06617-6

Bertrand, J.-A., Bedetti, C., Postuma, R. B., Monchi, O., Génier Marchand, D., Jubault, T., & Gagnon, J.-F. (2012). Color discrimination deficits in Parkinson's disease are related to cognitive impairment and white-matter alterations. *Movement Disorders*, *27*(14), 1781–1788. https://doi.org/10.1002/mds.25272

Bivol, S., Mellick, G. D., Gratten, J., Parker, R., Mulcahy, A., Mosley, P. E., Poortvliet, P. C., Campos, A. I., Mitchell, B. L., Garcia-Marin, L. M., Cross, S., Ferguson, M., Lind, P. A., Loesch, D. Z., Visscher, P. M., Medland, S. E., Scherzer, C. R., Martin, N. G., & Rentería, M. E. (2022). Australian Parkinson's Genetics Study (APGS): pilot (n=1532). *BMJ Open*, *12*(2), e052032. https://doi.org/10.1136/bmjopen-2021-052032

Bose, A., & Beal, M. F. (2016). Mitochondrial dysfunction in Parkinson's disease. *Journal of Neurochemistry*, *139*(S1), 216–231. https://doi.org/10.1111/jnc.13731

Braak, H., Tredici, K. D., Rüb, U., de Vos, R. A. I., Jansen Steur, E. N. H., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging*, *24*(2), 197–211. https://doi.org/10.1016/s0197-4580(02)00065-9

Bramich, S., King, A., Kuruvilla, M., Naismith, S. L., Noyce, A., & Alty, J. (2022). Isolated REM sleep behaviour disorder: current diagnostic procedures and emerging new technologies. *Journal of Neurology*, *269*(9), 4684–4695. https://doi.org/10.1007/s00415-022-11213-9

Bratic, A., & Larsson, N.-G. (2013). The role of mitochondria in aging. *Journal of Clinical Investigation*, *123*(3), 951–957. https://doi.org/10.1172/jci64125

Broniarek, I., & Jarmuszkiewicz, W. (2016). [Statins and mitochondria]. *Postepy Biochemii*, *62*(2). https://pubmed.ncbi.nlm.nih.gov/28132458/

Burbulla, L. F., Jeon, S., Zheng, J., Song, P., Silverman, R. B., & Krainc, D. (2019). A modulator of wild-type glucocerebrosidase improves pathogenic phenotypes in dopaminergic neuronal models of Parkinson's disease. *Science Translational Medicine*, *11*(514), eaau6870. https://doi.org/10.1126/scitranslmed.aau6870

Bušková, J., Peřinová, P., Miletínová, E., Dušek, P., Růžička, E., Šonka, K., & Kemlink, D. (2019). Validation of the REM sleep behavior disorder screening questionnaire in the Czech population. *BMC Neurology*, *19*(1). https://doi.org/10.1186/s12883-019-1340-4

Calabresi, P., Mechelli, A., Natale, G., Volpicelli-Daley, L., Di Lazzaro, G., & Ghiglieri, V. (2023). Alpha-synuclein in Parkinson's disease and other synucleinopathies: from overt neurodegeneration back to early synaptic dysfunction. *Cell Death & Disease*, *14*(3), 1–16. https://doi.org/10.1038/s41419-023-05672-9

Calvani, R., Picca, A., Landi, G., Marini, F., Biancolillo, A., Coelho-Júnior, H. J., Gervasoni, J., Persichilli, S., Primiano, A., Arcidiacono, A., Urbani, A., Bossola, M., Rita Bentivoglio, A., Cesari, M., Bernabei, R., Rita, M., & Marzetti, E. (2020). A novel multi-marker discovery approach identifies new serum biomarkers for Parkinson's disease in older people: an EXosomes in PArkiNson Disease (EXPAND) ancillary study. *GeroScience*, *42*(5), 1323–1334. https://doi.org/10.1007/s11357-020-00192-2

Cannon, J. R., Tapias, V., Na, H. M., Honick, A. S., Drolet, R. E., & Greenamyre, J. T. (2009). A highly reproducible rotenone model of Parkinson's disease. *Neurobiology of Disease*, *34*(2), 279–290. https://doi.org/10.1016/j.nbd.2009.01.016

Cantero-Fortiz, Y., & Boada, M. (2024). The role of inflammation in neurological disorders: a brief overview of multiple sclerosis, Alzheimer's, and Parkinson's disease'. *Frontiers in Neurology*, *15*. https://doi.org/10.3389/fneur.2024.1439125

Chahine, L. M., Stern, M. B., & Chen-Plotkin, A. (2014). Blood-based biomarkers for Parkinson's disease. *Parkinsonism & Related Disorders*, *20*, S99–S103. https://doi.org/10.1016/s1353-8020(13)70025-7

Chai, C., & Lim, K.-L. (2014). Genetic Insights into Sporadic Parkinson's Disease Pathogenesis. *Current Genomics*, *14*(8), 486–501. https://doi.org/10.2174/1389202914666131210195808

Chan, L., Chung, C.-C., Chen, J.-H., Yu, R.-C., & Hong, C.-T. (2021). Cytokine Profile in Plasma Extracellular Vesicles of Parkinson's Disease and the Association with Cognitive Function. *Cells*, *10*(3), 604. https://doi.org/10.3390/cells10030604

Chang, L., Dong, W., Luo, B., Qiu, C., Lu, Y., Lin, X., & Zhang, W. (2023). Deep brain stimulation improves central nervous system inflammation in Parkinson's disease: Evidence and perspectives. *CNS Neuroscience & Therapeutics*. https://doi.org/10.1111/cns.14167

Chartier-Harlin, M.-C., Dachsel, Justus C., Vilariño-Güell, C., Lincoln, Sarah J., Leprêtre, F., Hulihan, Mary M., Kachergus, J., Milnerwood, Austen J., Tapia, L., Song, M.-S., Le Rhun, E., Mutez, E., Larvor, L., Duflot, A., Vanbesien-Mailliot, C., Kreisler, A., Ross, Owen A., Nishioka, K., Soto-Ortolaza, Alexandra I., & Cobb, Stephanie A. (2011). Translation Initiator EIF4G1 Mutations in Familial Parkinson Disease. *The American Journal of Human Genetics*, *89*(3), 398–406. https://doi.org/10.1016/j.ajhg.2011.08.009

Chatterjee, D., & Krainc, D. (2023). Mechanisms of Glucocerebrosidase Dysfunction in Parkinson's Disease. *Journal of Molecular Biology*, *435*(12), 168023. https://doi.org/10.1016/j.jmb.2023.168023

Chatterjee, K., Roy, A., Banerjee, R., Choudhury, S., Mondal, B., Halder, S., Basu, P., Shubham, S., Dey, S., & Kumar, H. (2020). Inflammasome and α-synuclein in Parkinson's disease: A cross-sectional study. *Journal of Neuroimmunology*, *338*, 577089. https://doi.org/10.1016/j.jneuroim.2019.577089

Chaudhuri, K. R., Healy, D. G., & Schapira, A. H. (2006). Non-motor symptoms of Parkinson's disease: diagnosis and management. *The Lancet Neurology*, *5*(3), 235–245. https://doi.org/10.1016/s1474-4422(06)70373-8

Chaudhuri, K. R., Yates, L., & Martinez-Martin, P. (2005). The non-motor symptom complex of Parkinson's disease: A comprehensive assessment is essential. *Current Neurology and Neuroscience Reports*, *5*(4), 275–283. https://doi.org/10.1007/s11910-005-0072-6

Che, N., Ou, R., Li, C., Zhang, L., Wei, Q., Wang, S., Jiang, Q., Yang, T., Xiao, Y., Lin, J., Zhao, B., Chen, X., & Shang, H. (2024). Plasma GFAP as a prognostic biomarker of motor subtype in early Parkinson's disease. *Npj Parkinson's Disease*, *10*(1). https://doi.org/10.1038/s41531-024-00664-8

Chen, C., Turnbull, D. M., & Reeve, A. K. (2019). Mitochondrial Dysfunction in Parkinson's Disease—Cause or Consequence? *Biology*, *8*(2), 38. https://doi.org/10.3390/biology8020038

Chen, J., Liu, X., & Zhong, Y. (2020). Interleukin-17A: The Key Cytokine in Neurodegenerative Diseases. *Frontiers in Aging Neuroscience*, *12*. https://doi.org/10.3389/fnagi.2020.566922

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2018). Inflammatory Responses and inflammation-associated Diseases in Organs. *Oncotarget*, *9*(6), 7204–7218. https://doi.org/10.18632/oncotarget.23208

Chen, S., Liao, Z., & Xu, P. (2023). Mitochondrial control of innate immune responses. *Frontiers in Immunology*, *14*. https://doi.org/10.3389/fimmu.2023.1166214

Chen, S.-J., Chi, Y.-C., Ho, C.-H., Yang, W.-S., & Lin, C.-H. (2021). Plasma Lipopolysaccharide-Binding Protein Reflects Risk and Progression of Parkinson's Disease. *Journal of Parkinson's Disease*, *11*(3), 1129–1139. https://doi.org/10.3233/jpd-212574

Cheon, S.-M., Chan, L., Chan, D. K. Y., & Kim, J. W. (2012). Genetics of Parkinson's Disease - A Clinical Perspective. *Journal of Movement Disorders*, *5*(2), 33–41. https://doi.org/10.14802/jmd.12009

Chu, Y., Park, J., Kim, E., & Lee, S. (2021). Fluorescent Materials for Monitoring Mitochondrial Biology. *Materials, 14*(15), 4180. https://doi.org/10.3390/ma14154180

Cleeter, M. W. J., Chau, K.-Y., Gluck, C., Mehta, A., Hughes, D. A., Duchen, M., Wood, N. W., Hardy, J., Cooper, J. M., & Schapira, A. H. (2013). Glucocerebrosidase inhibition causes mitochondrial dysfunction and free radical damage. *Neurochemistry International*, *62*(1), 1–7. https://doi.org/10.1016/j.neuint.2012.10.010

Cohen, E., Bay, A. A., Ni, L., & Hackney, M. E. (2022). Apathy-Related Symptoms Appear Early in Parkinson's Disease. *Healthcare, 10*(1), 91. https://doi.org/10.3390/healthcare10010091

Day, J. O., & Mullin, S. (2021). The Genetics of Parkinson's Disease and Implications for Clinical Practice. *Genes*, *12*(7), 1006.

Deliz, J. R., Tanner, C. M., & Gonzalez-Latapi, P. (2024). Epidemiology of Parkinson's Disease: An Update. *Current Neurology and Neuroscience Reports*, *24*. https://doi.org/10.1007/s11910-024-01339-w

Deshpande, O. A., & Mohiuddin, S. S. (2022). *Biochemistry, Oxidative Phosphorylation*. PubMed; StatPearls Publishing. https://pubmed.ncbi.nlm.nih.gov/31985985/

Diaconu, Ș., Falup-pecurariu, O., Țînț, D., & Falup-Pecurariu, C. (2021). REM sleep behaviour disorder in Parkinson's disease (Review). *Experimental and Therapeutic Medicine*, *22*(2). https://doi.org/10.3892/etm.2021.10244

Diaz, K., Kohut, M. L., Russell, D. W., & Stegemöller, E. L. (2022). Peripheral inflammatory cytokines and motor symptoms in persons with Parkinson's disease. *Brain, Behavior, & Immunity - Health, 21*, 100442. https://doi.org/10.1016/j.bbih.2022.100442

Dorsey, E. R., Sherer, T., Okun, M. S., & Bloem, B. R. (2018). The Emerging Evidence of the Parkinson Pandemic. *Journal of Parkinson's Disease*, *8*(s1), S3–S8. https://doi.org/10.3233/jpd-181474

Doxaki, C., & Palikaras, K. (2021). Neuronal Mitophagy: Friend or Foe? *Frontiers in Cell and Developmental Biology*, *8*. https://doi.org/10.3389/fcell.2020.611938

Duarte, F. V., Ciampi, D., & Duarte, C. B. (2023). Mitochondria as central hubs in synaptic modulation. *Cellular and Molecular Life Sciences*, *80*(6). https://doi.org/10.1007/s00018-023-04814-8

Dufek, M., Rektorova, I., Thon, V., Lokaj, J., & Rektor, I. (2015). Interleukin-6 May Contribute to Mortality in Parkinson's Disease Patients: A 4-Year Prospective Study. *Parkinson's Disease*, *2015*, 1–5. https://doi.org/10.1155/2015/898192

Dzamko, N., Rowe, D. B., & Halliday, G. M. (2016). Increased peripheral inflammation in asymptomatic leucine-rich repeat kinase 2 mutation carriers. *Movement Disorders*, *31*(6), 889–897. https://doi.org/10.1002/mds.26529

Dzamko, N. (2023). Cytokine activity in Parkinson's disease. *Neuronal Signaling*, *7*(4). https://doi.org/10.1042/ns20220063

Erta, M., Quintana, A., & Hidalgo, J. (2012). Interleukin-6, a Major Cytokine in the Central Nervous System. *International Journal of Biological Sciences*, *8*(9), 1254–1266. https://doi.org/10.7150/ijbs.4679

Exner, N., Lutz, A. K., Haass, C., & Winklhofer, K. F. (2012). Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. *The EMBO Journal*, *31*(14), 3038–3062. https://doi.org/10.1038/emboj.2012.170

Faria-Pereira, A., & Morais, V. A. (2022). Synapses: The Brain's Energy-Demanding Sites. *International Journal of Molecular Sciences*, *23*(7), 3627. https://doi.org/10.3390/ijms23073627

Farmen, K., Nissen, S. K., Stokholm, M. G., Iranzo, A., Østergaard, K., Serradell, M., Otto, M., Svendsen, K. B., Garrido, A., Vilas, D., Borghammer, P., Santamaria, J., Møller, A., Gaig, C., Brooks, D. J., Tolosa, E., Pavese, N., & Romero-Ramos, M. (2021). Monocyte markers correlate with immune and neuronal brain changes in REM sleep behavior disorder. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(10). https://doi.org/10.1073/pnas.2020858118

Farrer, M. J., Kristoffer Haugarvoll, Ross, O. A., Stone, J. G., Milkovic, N. M., Cobb, S. A., Whittle, A. J., Lincoln, S., Hulihan, M. M., Heckman, M. G., White, L. R., Aasly, J. O., Gibson, J. M., Gosal, D., Lynch, T., Wszolek, Z. K., Uitti, R. J., & Toft, M. (2006). Genomewide Association, Parkinson Disease, and PARK10. *American Journal of Human Genetics*, *78*(6), 1084–1088. https://doi.org/10.1086/504728

Fell, M. J., Mirescu, C., Basu, K., Cheewatrakoolpong, B., DeMong, D. E., Ellis, J. M., Hyde, L. A., Lin, Y., Markgraf, C. G., Mei, H., Miller, M., Poulet, F. M., Scott, J. D., Smith, M. D., Yin, Z., Zhou, X., Parker, E. M., Kennedy, M. E., & Morrow, J. A. (2015). MLi-2, a Potent, Selective, and Centrally Active Compound for Exploring the Therapeutic Potential and Safety of LRRK2 Kinase Inhibition. *Journal of*

Pharmacology and Experimental Therapeutics, *355*(3), 397–409. https://doi.org/10.1124/jpet.115.227587

Ferrari, C. C., & Tarelli, R. (2011). Parkinson's Disease and Systemic Inflammation. *Parkinson's Disease*, *2011*, 1–9. https://doi.org/10.4061/2011/436813

Fonzo, A. D., Dekker, M. C. J., Montagna, P., Baruzzi, A., Yonova, E. H., Guedes, L. C., Szczerbinska, A., Zhao, T., Dubbel-Hulsman, L. O. M., Wouters, C. H., de Graaff, E., Oyen, W. J. G., Simons, E. J., Breedveld, G. J., Oostra, B. A., Horstink, M. W., & Bonifati, V. (2008). FBXO7 mutations cause autosomal recessive, early-onset parkinsonian-pyramidal syndrome. *Neurology*, *72*(3), 240–245. https://doi.org/10.1212/01.wnl.0000338144.10967.2b

Funayama, M., Ohe, K., Amo, T., Furuya, N., Yamaguchi, J., Saiki, S., Li, Y., Ogaki, K., Ando, M., Yoshino, H., Tomiyama, H., Nishioka, K., Hasegawa, K., Saiki, H., Satake, W., Mogushi, K., Sasaki, R., Kokubo, Y., Kuzuhara, S., & Toda, T. (2015). CHCHD2 mutations in autosomal dominant late-onset Parkinson's disease: a genome-wide linkage and sequencing study. *The Lancet Neurology*, *14*(3), 274–282. https://doi.org/10.1016/s1474-4422(14)70266-2

Gao, F., Yang, J., Wang, D., Li, C., Fu, Y., Wang, H., He, W., & Zhang, J. (2017). Mitophagy in Parkinson's Disease: Pathogenic and Therapeutic Implications. *Frontiers in Neurology*, *8*. https://doi.org/10.3389/fneur.2017.00527

Gao, X.-Y., Yang, T., Gu, Y., & Sun, X.-H. (2022). Mitochondrial Dysfunction in Parkinson's Disease: From Mechanistic Insights to Therapy. *Frontiers in Aging Neuroscience*, *14*. https://doi.org/10.3389/fnagi.2022.885500

Gezen-Ak, D., Alaylıoğlu, M., Genç, G., Şengül, B., Keskin, E., Sordu, P., Güleç, Z. E. K., Apaydın, H., Bayram-Gürel, Ç., Ulutin, T., Yılmazer, S., Ertan, S., & Dursun, E. (2020). Altered Transcriptional Profile of Mitochondrial DNA-Encoded OXPHOS Subunits, Mitochondria Quality Control Genes, and Intracellular ATP Levels in Blood Samples of Patients with Parkinson's Disease. *Journal of Alzheimer's Disease: JAD*, *74*(1), 287–307. https://doi.org/10.3233/JAD-191164

Goedert, M., Spillantini, M. G., Del Tredici, K., & Braak, H. (2012). 100 years of Lewy pathology. *Nature Reviews Neurology*, *9*(1), 13–24. https://doi.org/10.1038/nrneurol.2012.242

Gómez-Benito, M., Granado, N., García-Sanz, P., Michel, A., Dumoulin, M., & Moratalla, R. (2020). Modeling Parkinson's Disease With the Alpha-Synuclein Protein. *Frontiers in Pharmacology*, *11*(356). https://doi.org/10.3389/fphar.2020.00356

Green, H. F., Khosousi, S., & Svenningsson, P. (2019). Plasma IL-6 and IL-17A Correlate with Severity of Motor and Non-Motor Symptoms in Parkinson's Disease. *Journal of Parkinson's Disease*, 1–5. https://doi.org/10.3233/jpd-191699

Greenland, J. C., & Barker, R. A. (2018). The Differential Diagnosis of Parkinson's Disease. *Parkinson's Disease: Pathogenesis and Clinical Aspects*, 109–128. https://doi.org/10.15586/codonpublications.parkinsonsdisease.2018.ch6

Haga, H., Matsuo, K., Yabuki, Y., Zhang, C., Han, F., & Fukunaga, K. (2019). Enhancement of ATP production ameliorates motor and cognitive impairments in a mouse model of MPTP−induced Parkinson's disease. *Neurochemistry International*, *129*, 104492–104492. https://doi.org/10.1016/j.neuint.2019.104492

Halliday, G., Lees, A., & Stern, M. (2011). Milestones in Parkinson's disease-Clinical and pathologic features. *Movement Disorders*, *26*(6), 1015–1021. https://doi.org/10.1002/mds.23669

Han, Y. H., Kim, S. H., Kim, S. Z., & Park, W. H. (2008). Antimycin A as a mitochondria damage agent induces an S phase arrest of the cell cycle in HeLa cells. *Life Sciences*, *83*(9-10), 346–355. https://doi.org/10.1016/j.lfs.2008.06.023

Harms, A. S., Ferreira, S. A., & Romero-Ramos, M. (2021). Periphery and brain, innate and adaptive immunity in Parkinson's disease. *Acta Neuropathologica*, *141*(4), 527–545. https://doi.org/10.1007/s00401-021-02268-5

Hely, M. A., Reid, W. G. J., Adena, M. A., Halliday, G. M., & Morris, J. G. L. (2008). The Sydney multicenter study of Parkinson's disease: The inevitability of dementia at 20 years. *Movement Disorders*, *23*(6), 837–844. https://doi.org/10.1002/mds.21956

Henrich, M. T., Oertel, W. H., D. James Surmeier, & Geibl, F. F. (2023). Mitochondrial dysfunction in Parkinson's disease – a key disease hallmark with therapeutic potential. *Molecular Neurodegeneration*, *18*(1). https://doi.org/10.1186/s13024-023-00676-7

Holden, S. K., Finseth, T., Sillau, S. H., & Berman, B. D. (2017). Progression of MDS-UPDRS Scores Over Five Years in De Novo Parkinson Disease from the Parkinson's Progression Markers Initiative Cohort. *Movement Disorders Clinical Practice*, *5*(1), 47–53. https://doi.org/10.1002/mdc3.12553

Howell, M. J., & Schenck, C. H. (2015). Rapid Eye Movement Sleep Behavior Disorder and Neurodegenerative Disease. *JAMA Neurology*, *72*(6), 707. https://doi.org/10.1001/jamaneurol.2014.4563

Hsieh, C.-H., Li, L., Vanhauwaert, R., Nguyen, K. T., Davis, M. D., Bu, G., Wszolek, Z. K., & Wang, X. (2019). Miro1 Marks Parkinson's Disease Subset and Miro1 Reducer Rescues Neuron Loss in Parkinson's Models. *Cell Metabolism*. https://doi.org/10.1016/j.cmet.2019.08.023

Hsu, L. J., Sagara, Y., Arroyo, A., Rockenstein, E., Sisk, A., Mallory, M., Wong, J., Takenouchi, T., Hashimoto, M., & Masliah, E. (2000). α-Synuclein Promotes Mitochondrial Deficit and Oxidative Stress. *The American Journal of Pathology*, *157*(2), 401–410. https://doi.org/10.1016/s0002-9440(10)64553-1

Hubens, W. H. G., Vallbona-Garcia, A., de Coo, I. F. M., Tienen, F. H. J. van, Webers, C. A. B., & Smeets, H. J. M. (2022). Blood biomarkers for assessment of mitochondrial dysfunction: An expert review. Mitochondrion, 62, 187–204. https://doi.org/10.1016/j.mito.2021.10.008

Hughes, L., Halliday, G., & Dzamko, N. (2020). Flow Cytometry Measurement of Glucocerebrosidase Activity in Human Monocytes. *BIO-PROTOCOL*, *10*(7). https://doi.org/10.21769/bioprotoc.3572

Hytti, M., Korhonen, E., Hyttinen, J. M. T., Roehrich, H., Kaarniranta, K., Ferrington, D. A., & Kauppinen, A. (2019). Antimycin A-Induced Mitochondrial Damage Causes Human RPE Cell Death despite Activation of Autophagy. *Oxidative Medicine and Cellular Longevity*, *2019*, 1–12. https://doi.org/10.1155/2019/1583656

Ilieva, N. M., Hoffman, E. K., Ghalib, M. A., Greenamyre, J. T., & De, B. R. (2024). LRRK2 kinase inhibition protects against Parkinson's disease-associated environmental toxicants. *Neurobiology of Disease*, *196*, 106522–106522. https://doi.org/10.1016/j.nbd.2024.106522

Iranzo, A., Santamaria, J., & Tolosa, E. (2016). Idiopathic rapid eye movement sleep behaviour disorder: diagnosis, management, and the need for neuroprotective interventions. *The Lancet Neurology*, *15*(4), 405–419. https://doi.org/10.1016/s1474-4422(16)00057-0

Jankovic, J., & Tan, E. K. (2020). Parkinson's disease: Etiopathogenesis and treatment. *Journal of Neurology, Neurosurgery & Psychiatry*, *91*(8), 795–808. https://doi.org/10.1136/jnnp-2019-322338

Jenner, P., & Olanow, C. W. (1996). Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology*, *47*(Issue 6, Supplement 3), 161S170S. https://doi.org/10.1212/wnl.47.6_suppl_3.161s

Jun, J.-S., & Kim, R. (2023). Peripheral blood inflammatory cytokines in prodromal and overt α-synucleinopathies: a review of current evidence. *Encephalitis*, *3*(3), 81–86. https://doi.org/10.47936/encephalitis.2023.00031

Jurcau, A., & Jurcau, C. (2022). Mitochondria in Huntington′s disease: implications in pathogenesis and mitochondrial-targeted therapeutic strategies. *Neural Regeneration Research*, *0*(0), 0. https://doi.org/10.4103/1673-5374.360289

Kang, U. J., Boehme, A. K., Fairfoul, G., Shahnawaz, M., Ma, T. C., Hutten, S. J., Green, A., & Soto, C. (2019). Comparative study of cerebrospinal fluid α-synuclein seeding aggregation assays for diagnosis of Parkinson's disease. *Movement Disorders*, *34*(4), 536–544. https://doi.org/10.1002/mds.27646

Kannarkat, G. T., Boss, J. M., & Tansey, M. G. (2013). The Role of Innate and Adaptive Immunity in Parkinson's Disease. *Journal of Parkinson's Disease*, *3*(4), 493–514. https://doi.org/10.3233/jpd-130250

Kasen, A., Houck, C., Burmeister, A. R., Sha, Q., Brundin, L., & Brundin, P. (2022). Upregulation of α-synuclein following immune activation: Possible trigger of Parkinson's disease. *Neurobiology of Disease*, *166*, 105654. https://doi.org/10.1016/j.nbd.2022.105654

Kataoka, H., & Sugie, K. (2021). Association between Fatigue and Hoehn-Yahr Staging in Parkinson's Disease: Eight-Year Follow-Up Study. *Neurology International*, *13*(2), 224–231. https://doi.org/10.3390/neurolint13020023

Kelly, K., & West, A. B. (2020). Pharmacodynamic Biomarkers for Emerging LRRK2 Therapeutics. *Frontiers in Neuroscience*, *14*. https://doi.org/10.3389/fnins.2020.00807

Kim, R., Jun, J.-S., Kim, H.-J., Jung, K.-Y., Shin, Y.-W., Yang, T.-W., Kim, K. T., Kim, T.-J., Byun, J.-I., Sunwoo, J.-S., & Jeon, B. (2019). Peripheral Blood Inflammatory Cytokines in Idiopathic REM Sleep Behavior Disorder. *Movement Disorders: Official Journal of the Movement Disorder Society*, *34*(11), 1739–1744. https://doi.org/10.1002/mds.27841

Kim, R., Kim, H., Shin, J. H., Lee, C. Y., Jeon, S. H., & Jeon, B. (2022). Serum Inflammatory Markers and Progression of Nonmotor Symptoms in Early Parkinson's Disease. *Movement Disorders*. https://doi.org/10.1002/mds.29056

Kim, R., Lee, J.-Y., Kim, H.-J., Kim, Y. K., Nam, H., & Jeon, B. (2020). Serum TNF-α and neurodegeneration in isolated REM sleep behavior disorder. *Parkinsonism & Related Disorders*, *81*, 1–7. https://doi.org/10.1016/j.parkreldis.2020.09.041

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., & Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, *392*(6676), 605–608. https://doi.org/10.1038/33416

Klein, C., & Westenberger, A. (2012). Genetics of Parkinson's Disease. *Cold Spring Harbor Perspectives in Medicine*, *2*(1), a008888–a008888. https://doi.org/10.1101/cshperspect.a008888

Klemmensen, M. M., Borrowman, S. H., Pearce, C. A., Pyles, B., & Chandra, B. (2023). Mitochondrial dysfunction in neurodegenerative disorders. *Neurotherapeutics*, e00292–e00292. https://doi.org/10.1016/j.neurot.2023.10.002

Korandová, Z., Pecina, P., Pecinová, A., Koňaříková, E., Tesařová, M., Houštěk, J., Hansíková, H., Ptáčková, H., Zeman, J., Honzík, T., & Mráček, T. (2024). Cryopreserved PBMCs can be used for the analysis of mitochondrial respiration and serve as a diagnostic tool for mitochondrial diseases. *Analytical Biochemistry*, *698*, 115745. https://doi.org/10.1016/j.ab.2024.115745

Kouli, A., Torsney, K. M., & Kuan, W.-L. (2018). Parkinson's Disease: Etiology, Neuropathology, and Pathogenesis. *Parkinson's Disease: Pathogenesis and Clinical Aspects*, *1*(1), 3–26. https://doi.org/10.15586/codonpublications.parkinsonsdisease.2018.ch1

Krebs, C. E., Karkheiran, S., Powell, J. C., Cao, M., Makarov, V., Darvish, H., Di Paolo, G., Walker, R. H., Shahidi, G. A., Buxbaum, J. D., De Camilli, P., Yue, Z., & Paisán-Ruiz, C. (2013). The Sac1 Domain ofSYNJ1Identified Mutated in a Family with Early-Onset Progressive Parkinsonism with Generalized Seizures. *Human Mutation*, *34*(9), 1200–1207. https://doi.org/10.1002/humu.22372

Kumar, S., Bhatia, M., & Behari, M. (2003). Excessive daytime sleepiness in Parkinson's disease as assessed by Epworth Sleepiness Scale (ESS). *Sleep Medicine*, *4*(4), 339–342. https://doi.org/10.1016/s1389-9457(03)00105-9

Kwiatek-Majkusiak, J., Geremek, M., Koziorowski, D., Tomasiuk, R., Szlufik, S., & Friedman, A. (2020). Serum levels of hepcidin and interleukin 6 in Parkinson's disease. *Acta Neurobiologiae Experimentalis*, *80*(3), 297–304. https://doi.org/10.21307/ane-2020-026

Lai, T. T., Kim, Y. J., Ma, H., & Kim, Y. E. (2022). Evidence of Inflammation in Parkinson's Disease and Its Contribution to Synucleinopathy. *Journal of Movement Disorders*, *15*(1), 1–14. https://doi.org/10.14802/jmd.21078

Langston, J. W. (2017). The MPTP Story. *Journal of Parkinson's Disease*, *7*(s1), S11–S19. https://doi.org/10.3233/jpd-179006

Lauritsen, J., & Romero-Ramos, M. (2023). The systemic immune response in Parkinson's disease: focus on the peripheral immune component. *Trends in Neurosciences*, *46*(10), 863–878. https://doi.org/10.1016/j.tins.2023.07.005

Lee, H., Choi, J., & Suk, K. (2011). Increases of pentraxin 3 plasma levels in patients with Parkinson's disease. *Movement Disorders*, *26*(13), 2364–2370. https://doi.org/10.1002/mds.23871

Lee, Y. H., Kuk, M. U., So, M. K., Song, E. S., Lee, H., Ahn, S. K., Kwon, H. W., Park, J. T., & Park, S. C. (2023). Targeting Mitochondrial Oxidative Stress as a Strategy to Treat Aging and Age-Related Diseases. *Antioxidants*, *12*(4), 934. https://doi.org/10.3390/antiox12040934

Lesage, S., Drouet, V., Majounie, E., Deramecourt, V., Jacoupy, M., Nicolas, A., Cormier-Dequaire, F., Hassoun, S., Pujol, C., Ciura, S., Erpapazoglou, Z., Usenko, T., Maurage, C.-A., Sahbatou, M., Liebau, S., Ding, J., Bilgic, B., Emre, M., Erginel-Unaltuna, N., & Guven, G. (2016). Loss of VPS13C Function in Autosomal-Recessive Parkinsonism Causes Mitochondrial Dysfunction and Increases PINK1/Parkin-Dependent Mitophagy. *The American Journal of Human Genetics*, *98*(3), 500–513. https://doi.org/10.1016/j.ajhg.2016.01.014

Li, H., Ham, A., Ma, T. C., Kuo, S.-H., Kanter, E., Kim, D., Ko, H. S., Quan, Y., Sardi, S. P., Li, A., Arancio, O., Kang, U. J., Sulzer, D., & Tang, G. (2018). Mitochondrial dysfunction and mitophagy defect triggered by heterozygous GBA mutations. *Autophagy*, *15*(1), 113–130. https://doi.org/10.1080/15548627.2018.1509818

Li, Y., Yan, Y., Zhao, A., Luo, N., Niu, M., Kang, W., Xie, A., Lu, H., Chen, L., & Li, Y. (2022). *Parkinson's disease peripheral immune biomarker profile: a multicentre, cross-sectional and longitudinal study*. *19*(1). https://doi.org/10.1186/s12974-022-02481-3

Lin, C.-H., Li, C.-H., Yang, K.-C., Lin, F.-J., Wu, C.-C., Chieh, J.-J., & Chiu, M.-J. (2019). Blood NfL. *Neurology*, *93*(11), e1104–e1111. https://doi.org/10.1212/wnl.0000000000008088

Lin, C.-H., Yang, S.-Y., Horng, H.-E., Yang, C.-C., Chieh, J.-J., Chen, H.-H., Liu, B.-H., & Chiu, M.-J. (2017). Plasma α-synuclein predicts cognitive decline in Parkinson's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *88*(10), 818–824. https://doi.org/10.1136/jnnp-2016-314857

Liu, J., Han, X., Zhang, T., Tian, K., Li, Z., & Luo, F. (2023). Reactive oxygen species (ROS) scavenging biomaterials for anti-inflammatory diseases: from mechanism to therapy. *Journal of Hematology & Oncology*, *16*(1). https://doi.org/10.1186/s13045-023-01512-7

Liu, J., Liu, W., Li, R., & Yang, H. (2019). Mitophagy in Parkinson's Disease: From Pathogenesis to Treatment. *Cells*, *8*(7). https://doi.org/10.3390/cells8070712

Liu, T.-W., Chen, C.-M., & Chang, K.-H. (2022). Biomarker of Neuroinflammation in Parkinson's Disease. *International Journal of Molecular Sciences*, *23*(8), 4148. https://doi.org/10.3390/ijms23084148

Liu, X., Wang, Y., Yu, X., Li, D., & Li, G. (2017). Mitochondria-mediated damage to dopaminergic neurons in Parkinson's disease (Review). *International Journal of Molecular Medicine*. https://doi.org/10.3892/ijmm.2017.3255

Long, H., Zhu, W., Wei, L., & Zhao, J. (2023). Iron homeostasis imbalance and ferroptosis in brain diseases. *MedComm*, *4*(4). https://doi.org/10.1002/mco2.298

López-Aguirre, M., Matarazzo, M., Blesa, J., Monje, M. H. G., Rodríguez-Rojas, R., Sánchez-Ferro, A., Obeso, J. A., & Pineda-Pardo, J. A. (2023). Dopaminergic denervation and associated MRI microstructural changes in the nigrostriatal projection in early Parkinson's disease patients. *Npj Parkinson's Disease*, *9*(1). https://doi.org/10.1038/s41531-023-00586-x

Luporini, R. L., Rodolpho, J. M. de A., Kubota, L. T., Martin, A. C. B. M., Cominetti, M. R., Anibal, F. de F., & Pott-Junior, H. (2021). IL-6 and IL-10 are associated with disease severity and higher comorbidity in adults with COVID-19. *Cytokine*, *143*, 155507. https://doi.org/10.1016/j.cyto.2021.155507

Ma, X., Jin, M., Cai, Y., Xia, H., Long, K., Liu, J., Yu, Q., & Yuan, J. (2011). Mitochondrial Electron Transport Chain Complex III Is Required for Antimycin A to Inhibit Autophagy. *Chemistry & Biology*, *18*(11), 1474–1481. https://doi.org/10.1016/j.chembiol.2011.08.009

Magistrelli, L., Storelli, E., Rasini, E., Contaldi, E., Comi, C., Cosentino, M., & Marino, F. (2020). Relationship between circulating CD4+ T lymphocytes and cognitive impairment in patients with Parkinson's disease. *Brain, Behavior, and Immunity*, *89*, 668–674. https://doi.org/10.1016/j.bbi.2020.07.005

Mahlknecht, P., Marini, K., Werkmann, M., Poewe, W., & Seppi, K. (2022). Prodromal Parkinson's disease: hype or hope for disease-modification trials? *Translational Neurodegeneration*, *11*(1). https://doi.org/10.1186/s40035-022-00286-1

Maldonado, K. A., & Alsayouri, K. (2023, March 17). *Physiology, Brain*. PubMed; StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK551718/

Malpartida, A. B., Williamson, M., Narendra, D. P., Wade-Martins, R., & Ryan, B. J. (2020). Mitochondrial Dysfunction and Mitophagy in Parkinson's Disease: From Mechanism to Therapy. *Trends in Biochemical Sciences*, *46*(4). https://doi.org/10.1016/j.tibs.2020.11.007

Mamikonyan, E., Moberg, P. J., Siderowf, A., Duda, J. E., Have, T. T., Hurtig, H. I., Stern, M. B., & Weintraub, D. (2009). Mild cognitive impairment is common in Parkinson's disease patients with normal Mini-Mental State Examination (MMSE) scores. *Parkinsonism & Related Disorders*, *15*(3), 226–231. https://doi.org/10.1016/j.parkreldis.2008.05.006

Mangia, S., Svátková, A., Mascali, D., Nissi, M. J., Burton, P. J., Bednarik, P., Auerbach, E. J., Giove, F., Eberly, L. E., Howell, M. D., Igor Nestrasil, Tuite, P. J., & Michaeli, S. (2017). Multi-modal Brain MRI in Subjects with PD and iRBD. *Frontiers in Neuroscience*, *11*. https://doi.org/10.3389/fnins.2017.00709

Marinus, J., Leentjens, A. F. G., Visser, M., Stiggelbout, A. M., & van Hilten, J. J. (2002). Evaluation of the Hospital Anxiety and Depression Scale in Patients With Parkinson's Disease. *Clinical Neuropharmacology*, *25*(6), 318–324. https://doi.org/10.1097/00002826-200211000-00008

Masaldan, S., Callegari, S., & Dewson, G. (2022). Therapeutic targeting of mitophagy in Parkinson's disease. *Biochemical Society Transactions*. https://doi.org/10.1042/bst20211107

Massaad, C. A., & Klann, E. (2011). Reactive Oxygen Species in the Regulation of Synaptic Plasticity and Memory. *Antioxidants & Redox Signaling*, *14*(10), 2013–2054. https://doi.org/10.1089/ars.2010.3208

Massacci, G., Venafra, V., Zwiebel, M., Wahle, M., Cerroni, R., Bissacco, J., Perfetto, L., Michienzi, V., Stefani, A., Mercuri, N. B., Schirinzi, T., & Sacco, F. (2024). Stage-dependent phosphoproteome remodeling of Parkinson's disease blood cells. *Neurobiology of Disease*, *200*, 106622–106622. https://doi.org/10.1016/j.nbd.2024.106622

Mazzulli, Joseph R., Xu, Y.-H., Sun, Y., Knight, Adam L., McLean, Pamela J., Caldwell, Guy A., Sidransky, E., Grabowski, Gregory A., & Krainc, D. (2011). Gaucher Disease Glucocerebrosidase and α-Synuclein Form a Bidirectional Pathogenic Loop in Synucleinopathies. *Cell*, *146*(1), 37–52. https://doi.org/10.1016/j.cell.2011.06.001

McWilliams, T. G., & Muqit, M. M. (2017). PINK1 and Parkin: emerging themes in mitochondrial homeostasis. *Current Opinion in Cell Biology*, *45*, 83–91. https://doi.org/10.1016/j.ceb.2017.03.013

Merchant, K., Simuni, T., Fedler, J., Caspell-Garcia, C., Brumm, M. C., Nudelman, K., Tengstrandt, E., Hsieh, F., Alcalay, R. N., Coffey, C. S., Chahine, L. M., Foroud, T., Singleton, A. B., Weintraub, D., Hutten, S. J., Sherer, T., Mollenhauer, B., Siderowf, A., Tanner, C. M., & Marek, K. (2023). LRRK2 and GBA1 variant carriers have higher urinary bis(monacylglycerol) phosphate concentrations in PPMI cohorts. *Npj Parkinson's Disease*, *9*(1). https://doi.org/10.1038/s41531-023-00468-2

Milanese, C., Payán-Gómez, C., Galvani, M., Molano González, N., Tresini, M., Nait Abdellah, S., van Roon-Mom, W. M. C., Figini, S., Marinus, J., van Hilten, J. J., & Mastroberardino, P. G. (2019). Peripheral mitochondrial function correlates with clinical severity in idiopathic Parkinson's disease. *Movement Disorders: Official Journal of the Movement Disorder Society*, *34*(8), 1192–1202. https://doi.org/10.1002/mds.27723

Missiroli, S., Genovese, I., Perrone, M., Vezzani, B., Vitto, V. A. M., & Giorgi, C. (2020). The Role of Mitochondria in Inflammation: From Cancer to Neurodegenerative Disorders. *Journal of Clinical Medicine*, *9*(3), 740. https://doi.org/10.3390/jcm9030740

Mogi, M., Harada, M., Kondo, T., Riederer, P., Inagaki, H., Minami, M., & Nagatsu, T. (1994). Interleukin-1β, interleukin-6, epidermal growth factor and transforming growth factor-α are elevated in the brain from parkinsonian patients. *Neuroscience Letters*, *180*(2), 147–150. https://doi.org/10.1016/0304-3940(94)90508-8

Mollenhauer, B. (2023). Status of Current Biofluid Biomarkers in Parkinson's Disease. *Movement Disorders Clinical Practice*, *10*(S2). https://doi.org/10.1002/mdc3.13753

Monzel, A. S., Antonio Enríquez, J., & Picard, M. (2023). *Multifaceted mitochondria: moving mitochondrial science beyond function and dysfunction*. *5*(4), 546–562. https://doi.org/10.1038/s42255-023-00783-1

Moon, H. E., & Paek, S. H. (2015). Mitochondrial Dysfunction in Parkinson's Disease. *Experimental Neurobiology*, *24*(2), 103. https://doi.org/10.5607/en.2015.24.2.103

Moscovich, M., Heinzel, S., Postuma, R. B., Reilmann, R., Klockgether, T., Jacobi, H., Höglinger, G., & Berg, D. (2020). How specific are non-motor symptoms in the prodrome of Parkinson's disease compared to other movement

disorders? *Parkinsonism & Related Disorders*, *81*, 213–218. https://doi.org/10.1016/j.parkreldis.2020.10.003

Mukhopadhyay, P., Rajesh, M., Haskó, G., Hawkins, B. J., Madesh, M., & Pacher, P. (2007). Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. *Nature Protocols*, *2*(9), 2295–2301. https://doi.org/10.1038/nprot.2007.327

Mukhopadhyay, P., Rajesh, M., Yoshihiro, K., Haskó, G., & Pacher, P. (2007). Simple quantitative detection of mitochondrial superoxide production in live cells. *Biochemical and Biophysical Research Communications*, *358*(1), 203–208. https://doi.org/10.1016/j.bbrc.2007.04.106

Munhoz, R. P., Tumas, V., Pedroso, J. L., & Silveira-Moriyama, L. (2024). The clinical diagnosis of Parkinson's disease. *Arquivos de Neuro-Psiquiatria*, *82*(06), 001-010. https://doi.org/10.1055/s-0043-1777775

Muñoz-Delgado, L., Macías-García, D., Periñán, M. T., Jesús, S., Adarmes-Gómez, A. D., Bonilla Toribio, M., Buiza Rueda, D., Jiménez-Jaraba, M. del V., Benítez Zamora, B., Díaz Belloso, R., García-Díaz, S., Martín-Bórnez, M., Pineda Sánchez, R., Carrillo, F., Gómez-Garre, P., & Mir, P. (2023). Peripheral inflammatory immune response differs among sporadic and familial Parkinson's disease. *Npj Parkinson's Disease*, *9*(1). https://doi.org/10.1038/s41531-023-00457-5

Naik, E., & Dixit, V. M. (2011). Mitochondrial reactive oxygen species drive proinflammatory cytokine production: Figure 1. *The Journal of Experimental Medicine*, *208*(3), 417–420. https://doi.org/10.1084/jem.20110367

Nalls, M. A., Blauwendraat, C., Vallerga, C. L., Heilbron, K., Bandres-Ciga, S., Chang, D., Tan, M., Kia, D. A., Noyce, A. J., Xue, A., Bras, J., Young, E., von Coelln, R., Simón-Sánchez, J., Schulte, C., Sharma, M., Krohn, L., Pihlstrøm, L., Siitonen, A., & Iwaki, H. (2019). Identification of novel risk loci, causal insights, and heritable risk for Parkinson's disease: a meta-analysis of genome-wide association studies. *The Lancet Neurology*, *18*(12), 1091–1102. https://doi.org/10.1016/S1474-4422(19)30320-5

Narayanan, S., Shanker, A., Khera, T., & Subramaniam, B. (2021). Neurofilament light: a narrative review on biomarker utility. *Faculty Reviews*, *10*. https://doi.org/10.12703/r/10-46

Navarro, A., Boveris, A., Bández, M. J., Sánchez-Pino, M. J., Gómez, C., Muntané, G., & Ferrer, I. (2009). Human brain cortex: mitochondrial oxidative damage and adaptive

response in Parkinson disease and in dementia with Lewy bodies. *Free Radical Biology and Medicine*, *46*(12), 1574–1580. https://doi.org/10.1016/j.freeradbiomed.2009.03.007

Nicoletti, V., Palermo, G., Del Prete, E., Mancuso, M., & Ceravolo, R. (2021). Understanding the Multiple Role of Mitochondria in Parkinson's Disease and Related Disorders: Lesson From Genetics and Protein–Interaction Network. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/fcell.2021.636506

Nishikawa, K., Li, H., Kawamura, R., Osaka, H., Wang, Y.-L., Hara, Y., Hirokawa, T., Manago, Y., Amano, T., Noda, M., Aoki, S., & Wada, K. (2003). Alterations of structure and hydrolase activity of parkinsonism-associated human ubiquitin carboxyl-terminal hydrolase L1 variants. *Biochemical and Biophysical Research Communications*, *304*(1), 176–183. https://doi.org/10.1016/s0006-291x(03)00555-2

Nissen, S. K., Farmen, K., Carstensen, M., Schulte, C., Goldeck, D., Brockmann, K., & Romero-Ramos, M. (2022). Changes in CD163+, CD11b+, and CCR2+ peripheral monocytes relate to Parkinson's disease and cognition. *Brain, Behavior, and Immunity*, *101*, 182–193. https://doi.org/10.1016/j.bbi.2022.01.005

Ogita, M., Ogita, A., Usuki, Y., Fujita, K., & Tanaka, T. (2009). Antimycin A-induced cell death depends on AIF translocation through NO production and PARP activation and is not involved in ROS generation, cytochrome c release and caspase-3 activation in HL-60 cells. *The Journal of Antibiotics*, *62*(3), 145–152. https://doi.org/10.1038/ja.2009.2

Olagunju, A. S., Ahammad, F., Alagbe, A. A., Otenaike, T. A., Teibo, J. O., Mohammad, F., Alsaiari, A. A., Omotoso, O., & Talukder, M. E. K. (2023). Mitochondrial dysfunction: A notable contributor to the progression of Alzheimer's and Parkinson's disease. *Heliyon*, *9*(3), e14387. https://doi.org/10.1016/j.heliyon.2023.e14387

Olesen, M. A., Villavicencio-Tejo, F., & Quintanilla, R. A. (2022). The use of fibroblasts as a valuable strategy for studying mitochondrial impairment in neurological disorders. *Translational Neurodegeneration*, *11*(1). https://doi.org/10.1186/s40035-022-00308-y

Olgiati, S., Quadri, M., Fang, M., Rood, J. P. M. A., Saute, J. A., Chien, H. F., Bouwkamp, C. G., Graafland, J., Minneboo, M., Breedveld, G. J., Zhang, J., Verheijen, F. W., Boon, A. J. W., Kievit, A. J. A., Jardim, L. B., Mandemakers, W., Barbosa, E. R., Rieder, C. R. M., Leenders, K. L., & Wang, J. (2016). D NAJC 6 Mutations Associated With Early-Onset Parkinson's Disease. *Annals of Neurology*, *79*(2), 244–256. https://doi.org/10.1002/ana.24553

Ongari, G., Ghezzi, C., Di Martino, D., Pisani, A., Terzaghi, M., Avenali, M., Maria Valente, E., Cerri, S., & Blandini, F. (2023). Impaired Mitochondrial Respiration in REM-Sleep Behavior Disorder: A Biomarker of Parkinson's Disease? *Movement Disorders*, *39*(2), 294–304. https://doi.org/10.1002/mds.29643

Osellame, L. D., Blacker, T. S., & Duchen, M. R. (2012). Cellular and molecular mechanisms of mitochondrial function. *Best Practice & Research. Clinical Endocrinology & Metabolism*, *26*(6), 711–723. https://doi.org/10.1016/j.beem.2012.05.003

Oun, A., Soliman, A., Trombetta-Lima, M., Tzepapadaki, A., Tsagkari, D., Kortholt, A., & Dolga, A. M. (2022). LRRK2 protects immune cells against erastin-induced ferroptosis. *Neurobiology of Disease*, *175*, 105917. https://doi.org/10.1016/j.nbd.2022.105917

Padmanabhan, S., Lanz, T. A., Gorman, D., Wolfe, M., Joyce, A., Cabrera, C., Lawrence-Henderson, R., Levers, N., Joshi, N., Ma, T. C., Liong, C., Narayan, S., Alcalay, R. N., Hutten, S. J., Baptista, M. A. S., & Merchant, K. (2020). An Assessment of LRRK2 Serine 935 Phosphorylation in Human Peripheral Blood Mononuclear Cells in Idiopathic Parkinson's Disease and G2019S LRRK2 Cohorts. *Journal of Parkinson's Disease*, *10*(2), 623–629. https://doi.org/10.3233/jpd-191786

Paisan-Ruiz, C., Bhatia, K. P., Li, A., Hernandez, D., Davis, M., Wood, N. W., Hardy, J., Houlden, H., Singleton, A., & Schneider, S. A. (2008). Characterization of PLA2G6 as a locus for dystonia-parkinsonism. *Annals of Neurology*, *65*(1), 19–23. https://doi.org/10.1002/ana.21415

Pajares, M., I. Rojo, A., Manda, G., Boscá, L., & Cuadrado, A. (2020). Inflammation in Parkinson's Disease: Mechanisms and Therapeutic Implications. *Cells*, *9*(7), 1687. https://doi.org/10.3390/cells9071687

Pang, S. Y.-Y., Lo, R. C. N., Ho, P. W.-L., Liu, H.-F., Chang, E. E. S., Leung, C.-T., Malki, Y., Choi, Z. Y.-K., Wong, W. Y., Kung, M. H.-W., Ramsden, D. B., & Ho, S.-L. (2022). LRRK2, GBA and their interaction in the regulation of autophagy: implications on therapeutics in Parkinson's disease. *Translational Neurodegeneration*, *11*(1). https://doi.org/10.1186/s40035-022-00281-6

Parkinson, J. (1817). An Essay on the Shaking Palsy. *The Journal of Neuropsychiatry and Clinical Neurosciences*, *14*(2), 223–236. https://doi.org/10.1176/jnp.14.2.223

Patel, S., Shah, R. J., Coleman, P., & Sabbagh, M. (2011). Potential Peripheral Biomarkers for the Diagnosis of Alzheimer's Disease. *International Journal of Alzheimer's Disease*, *2011*, 1–9. https://doi.org/10.4061/2011/572495

Payne, T., Burgess, T., Bradley, S., Roscoe, S., Sassani, M., Dunning, M. J., Hernandez, D., Scholz, S., McNeill, A., Taylor, R., Su, L., Wilkinson, I., Jenkins, T., Mortiboys, H., & Bandmann, O. (2023). Multimodal assessment of mitochondrial function in Parkinson's disease. *Brain*, *147*(1), 267–280. https://doi.org/10.1093/brain/awad364

Peggion, C., Tito Calì, & Brini, M. (2024). Mitochondria Dysfunction and Neuroinflammation in Neurodegeneration: Who Comes First? *Antioxidants*, *13*(2), 240–240. https://doi.org/10.3390/antiox13020240

Peng, G., Qiu, J., Liu, H., Zhou, M., Huang, S., Guo, W., Lin, Y., Chen, X., Li, Z., Li, G., Zhang, W., Zhang, Y., Li, X., Wu, Z., Wei, L., Yang, X., Zhu, X., Mo, M., & Xu, P. (2019). Analysis of Cerebrospinal Fluid Soluble TREM2 and Polymorphisms in Sporadic Parkinson's Disease in a Chinese Population. *Journal of Molecular Neuroscience*, *70*(2), 294–301. https://doi.org/10.1007/s12031-019-01424-7

Perner, C., Perner, F., Gaur, N., Zimmermann, S., Witte, O. W., Heidel, F. H., Grosskreutz, J., & Prell, T. (2019). Plasma VCAM1 levels correlate with disease severity in Parkinson's disease. *Journal of Neuroinflammation*, *16*(1), 94. https://doi.org/10.1186/s12974-019-1482-8

Pham, C. K., Sankari, A., & Slowik, J. M. (2022). *Rapid Eye Movement Sleep Behavior Disorder*. PubMed; StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK555928/

Pochmann, D., Peccin, P. K., da Silva, I. R. V., Dorneles, G. P., Peres, A., Nique, S., Striebel, V., & Elsner, V. R. (2018). Cytokine modulation in response to acute and chronic aquatic therapy intervention in Parkinson disease individuals: A pilot study. *Neuroscience Letters*, *674*, 30–35. https://doi.org/10.1016/j.neulet.2018.03.021

Postuma, R. B., Gagnon, J.-F., Pelletier, A., & Montplaisir, J. (2013). Prodromal autonomic symptoms and signs in Parkinson's disease and dementia with Lewy bodies. *Movement Disorders*, *28*(5), 597–604. https://doi.org/10.1002/mds.25445

Postuma, R. B., Gagnon, J.-F., Vendette, M., Desjardins, C., & Montplaisir, J. Y. (2011). Olfaction and color vision identify impending neurodegeneration in rapid eye movement sleep behavior disorder. *Annals of Neurology*, *69*(5), 811–818. https://doi.org/10.1002/ana.22282

Postuma, R. B., Iranzo, A., Hu, M., Högl, B., Boeve, B. F., Manni, R., Oertel, W. H., Arnulf, I., Ferini-Strambi, L., Puligheddu, M., Antelmi, E., Cochen De Cock, V., Arnaldi, D., Mollenhauer, B., Videnovic, A., Sonka, K., Jung, K.-Y., Kunz, D., Dauvilliers, Y., & Provini, F. (2019). Risk and predictors of dementia and parkinsonism in idiopathic

REM sleep behaviour disorder: a multicentre study. *Brain*, *142*(3), 744–759. https://doi.org/10.1093/brain/awz030

Prigione, A., Begni, B., Galbussera, A., Beretta, S., Brighina, L., Garofalo, R., Andreoni, S., Piolti, R., & Ferrarese, C. (2006). Oxidative stress in peripheral blood mononuclear cells from patients with Parkinson's disease: Negative correlation with levodopa dosage. *Neurobiology of Disease*, *23*(1), 36–43. https://doi.org/10.1016/j.nbd.2006.01.013

Qadri, R., Namdeo, M., Behari, M., Goyal, V., Sharma, S., & Mukhopadhyay, A. K. (2018). Alterations in mitochondrial membrane potential in peripheral blood mononuclear cells in Parkinson's Disease: Potential for a novel biomarker. *Restorative Neurology and Neuroscience*, *36*(6), 719–727. https://doi.org/10.3233/rnn-180852

Qi, R., Sammler, E., Gonzalez-Hunt, C. P., Barraza, I., Pena, N., Rouanet, J. P., Naaldijk, Y., Goodson, S. D., Fuzzati, M., Blandini, F., Erickson, K. I., Weinstein, A. M., Lutz, M. W., Kwok, J. B., Halliday, G. M., Dzamko, N., Padmanabhan, S., Alcalay, R. N., Waters, C., & Hogarth, P. (2023). A blood-based marker of mitochondrial DNA damage in Parkinson's disease. *Science Translational Medicine*, *15*(711). https://doi.org/10.1126/scitranslmed.abo1557

Qin, X.-Y., Zhang, S.-P., Cao, C., Loh, Y. P., & Cheng, Y. (2016). Aberrations in Peripheral Inflammatory Cytokine Levels in Parkinson Disease. *JAMA Neurology*, *73*(11), 1316. https://doi.org/10.1001/jamaneurol.2016.2742

Qu, Y., Li, J., Qin, Q., Wang, D., Zhao, J., An, K., Mao, Z., Min, Z., Xiong, Y., Li, J., & Xue, Z. (2023). A systematic review and meta-analysis of inflammatory biomarkers in Parkinson's disease. *Npj Parkinson's Disease*, *9*(1). https://doi.org/10.1038/s41531-023-00449-5

Rafaqat, S., & Rafaqat, S. (2023). Role of IL-2/IL-2 receptor in pathogenesis of autoimmune disorders: Genetic and therapeutic aspects. *World Journal of Medical Genetics*, *11*(3), 28–38. https://doi.org/10.5496/wjmg.v11.i3.28

Ramirez, A., Heimbach, A., Gründemann, J., Stiller, B., Hampshire, D., Cid, L. P., Goebel, I., Mubaidin, A. F., Wriekat, A.-L., Roeper, J., Al-Din, A., Hillmer, A. M., Karsak, M., Liss, B., Woods, C. G., Behrens, M. I., & Kubisch, C. (2006). Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. *Nature Genetics*, *38*(10), 1184–1191. https://doi.org/10.1038/ng1884

Rathnayake, D., Chang, T., & Udagama, P. (2019). Selected serum cytokines and nitric oxide as potential multi-marker biosignature panels for Parkinson disease of varying durations: a case-control study. *BMC Neurology*, *19*(1). https://doi.org/10.1186/s12883-019-1286-6

Ravenhill, S., Evans, A. H., & Crewther, S. G. (2023). Escalating Bi-Directional Feedback Loops between Proinflammatory Microglia and Mitochondria in Ageing and Post-Diagnosis of Parkinson's Disease. *Antioxidants*, *12*(5), 1117–1117. https://doi.org/10.3390/antiox12051117

Rees, R. N., Noyce, A. J., & Schrag, A. E. (2024). Identification of Prodromal Parkinson Disease. *Neurology*, *102*(11). https://doi.org/10.1212/wnl.0000000000209394

Reeve, A., Simcox, E., & Turnbull, D. (2014). Ageing and Parkinson's disease: Why Is Advancing Age the Biggest Risk factor? *Ageing Research Reviews*, *14*(100), 19–30. https://doi.org/10.1016/j.arr.2014.01.004

Ren, C., Ding, Y., Wei, S., Guan, L., Zhang, C., Ji, Y., Wang, F., Yin, S., & Yin, P. (2019). G2019S Variation in LRRK2: An Ideal Model for the Study of Parkinson's Disease? *Frontiers in Human Neuroscience*, *13*. https://doi.org/10.3389/fnhum.2019.00306

Rizig, M., Bandrés-Ciga, S., Makarious, M. B., Ojo, O. O., Wild Crea, P., Abiodun, O. V., Levine, K. S., Atta Abubakar, S., Achoru, C., Vitale, D., Adeniji, O., Agabi, O. P., Koretsky, M. J., Agulanna, U., Hall, D. A., Akinyemi, R., Xie, T., Wulgo Ali, M., Shamim, E. A., & Ani-Osheku, I. (2023). Identification of genetic risk loci and causal insights associated with Parkinson's disease in African and African admixed populations: a genome-wide association study. *Lancet Neurology*, *22*(11), 1015–1025. https://doi.org/10.1016/s1474-4422(23)00283-1

Rizzo, G., Copetti, M., Arcuti, S., Martino, D., Fontana, A., & Logroscino, G. (2016). Accuracy of clinical diagnosis of Parkinson disease. *Neurology*, *86*(6), 566–576. https://doi.org/10.1212/wnl.0000000000002350

Roy, A., Banerjee, R., Choudhury, S., Chatterjee, K., Mondal, B., Dey, S., & Kumar, H. (2022). Novel inflammasome and oxidative modulators in Parkinson's disease: A prospective study. *Neuroscience Letters*, *786*, 136768–136768. https://doi.org/10.1016/j.neulet.2022.136768

Ruiz de Morales, J. M. G., Puig, L., Daudén, E., Cañete, J. D., Pablos, J. L., Martín, A. O., Juanatey, C. G., Adán, A., Montalbán, X., Borruel, N., Ortí, G., Holgado-Martín, E., García-Vidal, C., Vizcaya-Morales, C., Martín-Vázquez, V., & González-Gay, M. Á. (2020). Critical role of interleukin (IL)-17 in inflammatory and immune disorders: An

updated review of the evidence focusing in controversies. *Autoimmunity Reviews*, *19*(1), 102429. https://doi.org/10.1016/j.autrev.2019.102429

Ryan, B. J., Hoek, S., Fon, E. A., & Wade-Martins, R. (2015). Mitochondrial dysfunction and mitophagy in Parkinson's: from familial to sporadic disease. *Trends in Biochemical Sciences*, *40*(4), 200–210. https://doi.org/10.1016/j.tibs.2015.02.003

Saikia, A., Bhattacharya, P., & Paul1, S. (2018). Importance of dopamine in parkinson's disease. *Advances in Tissue Engineering & Regenerative Medicine: Open Access*, *4*(3). https://doi.org/10.15406/atroa.2018.04.00077

Schaffrath, A., Schleyken, S., Seger, A., Jergas, H., Özdüzenciler, P., Pils, M., Blömeke, L., Cousin, A., Willbold, J., Bujnicki, T., Bannach, O., Fink, G. R., Willbold, D., Sommerauer, M., Barbe, M. T., & Tamgüney, G. (2023). Patients with isolated REM-sleep behavior disorder have elevated levels of alpha-synuclein aggregates in stool. *Npj Parkinson's Disease*, *9*(1), 1–11. https://doi.org/10.1038/s41531-023-00458-4

Schenck, C. H., Boeve, B. F., & Mahowald, M. W. (2013). Delayed emergence of a parkinsonian disorder or dementia in 81% of older men initially diagnosed with idiopathic rapid eye movement sleep behavior disorder: a 16-year update on a previously reported series. *Sleep Medicine*, *14*(8), 744–748. https://doi.org/10.1016/j.sleep.2012.10.009

Schirinzi, T., Salvatori, I., Zenuni, H., Grillo, P., Valle, C., Martella, G., Biagio Mercuri, N., & Ferri, A. (2022). Pattern of Mitochondrial Respiration in Peripheral Blood Cells of Patients with Parkinson's Disease. *International Journal of Molecular Sciences*, *23*(18), 10863–10863. https://doi.org/10.3390/ijms231810863

Schmidt, S., Stautner, C., Vu, D. T., Heinz, A., Regensburger, M., Karayel, O., Trümbach, D., Artati, A., Kaltenhäuser, S., Nassef, M. Z., Hembach, S., Steinert, L., Winner, B., Jürgen, W., Jastroch, M., Luecken, M. D., Theis, F. J., Westmeyer, G. G., Adamski, J., & Mann, M. (2023). A reversible state of hypometabolism in a human cellular model of sporadic Parkinson's disease. *Nature Communications*, *14*, 7674. https://doi.org/10.1038/s41467-023-42862-7

Schonhoff, A. M., Williams, G. P., Wallen, Z. D., Standaert, D. G., & Harms , A. S. (2020). Innate and adaptive immune responses in Parkinson's disease. *Progress in Brain Research*, *252*, 169–216. https://doi.org/10.1016/bs.pbr.2019.10.006

Schulte, C., & Gasser, T. (2011). Genetic basis of Parkinson's disease: inheritance, penetrance, and expression. *The Application of Clinical Genetics*, *4*, 67. https://doi.org/10.2147/tacg.s11639

Seger, A., Ophey, A., Christopher, Kickartz, J., Lindner, M.-S., Maximilian Hommelsen, Fink, G. R., & Sommerauer, M. (2023). Clinical subtypes in patients with isolated REM sleep behaviour disorder. *Npj Parkinson S Disease*, *9*(1). https://doi.org/10.1038/s41531-023-00598-7

Selvam, S., & Ayyavoo, V. (2024). Biomarkers in neurodegenerative diseases: a broad overview. *Exploration of Neuroprotective Therapy*, 119–147. https://doi.org/10.37349/ent.2024.00075

Shahnawaz, M., Tokuda, T., Waragai, M., Mendez, N., Ishii, R., Trenkwalder, C., Mollenhauer, B., & Soto, C. (2017). Development of a Biochemical Diagnosis of Parkinson Disease by Detection of α-Synuclein Misfolded Aggregates in Cerebrospinal Fluid. *JAMA Neurology*, *74*(2), 163. https://doi.org/10.1001/jamaneurol.2016.4547

Signaevsky, M., Marami, B., Prastawa, M., Tabish, N., Iida, M. A., Zhang, X. F., Sawyer, M., Duran, I., Koenigsberg, D. G., Bryce, C. H., Chahine, L. M., Mollenhauer, B., Mosovsky, S., Riley, L., Dave, K. D., Eberling, J., Coffey, C. S., Adler, C. H., Serrano, G. E., & White, C. L. (2022). Antemortem detection of Parkinson's disease pathology in peripheral biopsies using artificial intelligence. *Acta Neuropathologica Communications*, *10*(1). https://doi.org/10.1186/s40478-022-01318-7

Singh, A., Zhi, L., & Zhang, H. (2019). LRRK2 and mitochondria: Recent advances and current views. *Brain Research*, *1702*, 96–104. https://doi.org/10.1016/j.brainres.2018.06.010

Singh, F., Prescott, A. R., Rosewell, P., Ball, G., Reith, A. D., & Ganley, I. G. (2021). Pharmacological rescue of impaired mitophagy in Parkinson's disease-related LRRK2 G2019S knock-in mice. *ELife*, *10*, e67604. https://doi.org/10.7554/eLife.67604

Singleton, A. B., Farrer, M. J., & Bonifati, V. (2013). The genetics of Parkinson's disease: Progress and therapeutic implications. *Movement Disorders*, *28*(1), 14–23. https://doi.org/10.1002/mds.25249

Slow, E. J., Postuma, R. B., & Lang, A. E. (2014). Implications of nocturnal symptoms towards the early diagnosis of Parkinson's disease. *Journal of Neural Transmission*, *121*(S1), 49–57. https://doi.org/10.1007/s00702-014-1168-4

Smith, A., Depp, C., Ryan, B. D., Johnston, G., Alegre-Abarrategui, J., Evetts, S., Rolinski, M., Baig, F., Ruffmann, C., Simon, A., Hu, M., & Wade-Martins, R. (2018). *Mitochondrial dysfunction and increased glycolysis in prodromal and early Parkinson's blood cells*. *33*(10), 1580–1590. https://doi.org/10.1002/mds.104

Smith, L., & Schapira, A. H. V. (2022). GBA Variants and Parkinson Disease: Mechanisms and Treatments. *Cells*, *11*(8), 1261. https://doi.org/10.3390/cells11081261

Sokhey, A. E., Depp, C., Ryan, B. D., Johnston, G., Alegre-Abarrategui, J., Evetts, S., Rolinski, M., Baig, F., Ruffmann, C., Simon, A., T.M. Hu, M., & Wade-Martins, R. (2018). *Mitochondrial dysfunction and increased glycolysis in prodromal and early Parkinson's blood cells*. *33*(10), 1580–1590. https://doi.org/10.1002/mds.104

Solmaz, V., Pekdaş Genç, E., Aksoy, D., Çevik, B., Kurt, S. G., & Benli, İ. (2018). Parkinson hastalarında nötrofil/lenfosit oranları, C reaktif protein ve sedimantasyon hızlarının değerlendirilmesi. *Cukurova Medical Journal*, *43*(2), 305–311. https://doi.org/10.17826/cumj.341649

Somerville, E. N., Krohn, L., Senkevich, K., Yu, E., Ahmad, J., Asayesh, F., Ruskey, J. A., Spiegelman, D., Fahn, S., Waters, C., Pablo Sardi, S., Alcalay, R. N., & Gan-Or, Z. (2024). Genome-wide association study of glucocerebrosidase activity modifiers. *BioRxiv (Cold Spring Harbor Laboratory)*. https://doi.org/10.1101/2024.03.27.586821

Song, Y., Zhou, Y., & Zhou, X. (2020). The role of mitophagy in innate immune responses triggered by mitochondrial stress. *Cell Communication and Signaling*, *18*(1). https://doi.org/10.1186/s12964-020-00659-x

Springer, M. Z., & Macleod, K. F. (2016). In Brief: Mitophagy: mechanisms and role in human disease. *The Journal of Pathology*, *240*(3), 253–255. https://doi.org/10.1002/path.4774

Spurlock, B., Tullet, J., Hartman, J. L., & Mitra, K. (2020). Interplay of mitochondrial fission-fusion with cell cycle regulation: Possible impacts on stem cell and organismal aging. *Experimental Gerontology*, *135*, 110919. https://doi.org/10.1016/j.exger.2020.110919

Stoker, T. B., Torsney, K. M., & Barker, R. A. (2018). Pathological Mechanisms and Clinical Aspects of GBA1 Mutation-Associated Parkinson's Disease. *Exon Publications*, 45–64. https://doi.org/10.15586/codonpublications.parkinsonsdisease.2018.ch3

Subramaniam, S. R., & Chesselet, M.-F. (2013). Mitochondrial dysfunction and oxidative stress in Parkinson's disease. *Progress in Neurobiology*, *106-107*, 17–32. https://doi.org/10.1016/j.pneurobio.2013.04.004

Sumi, Y., Masuda, F., Kadotani, H., & Ozeki, Y. (2022). The prevalence of depression in isolated/idiopathic rapid eye movement sleep behavior disorder: A systematic review

and meta-analysis. *Sleep Medicine Reviews*, *65*, 101684. https://doi.org/10.1016/j.smrv.2022.101684

Sun, X., Jiang, Q., Zhang, Y., Su, J., Liu, W., Lv, J., Yang, F., & Shu, W. (2024). Advances in fluorescent probe development for bioimaging of potential Parkinson's biomarkers. *European Journal of Medicinal Chemistry*, *267*, 116195–116195. https://doi.org/10.1016/j.ejmech.2024.116195

Tait, S. W. G., & Green, D. R. (2012). Mitochondria and cell signalling. *Journal of Cell Science*, *125*(4), 807–815. https://doi.org/10.1242/jcs.099234

Tait, S. W. G., & Green, D. R. (2013). Mitochondrial Regulation of Cell Death. *Cold Spring Harbor Perspectives in Biology*, *5*(9). https://doi.org/10.1101/cshperspect.a008706

Tan, E.-K. ., Kwok, H.-K. ., Tan, L. C., Zhao, W.-T. ., Prakash, K. M., Au, W.-L. ., Pavanni, R., Ng, Y.-Y. ., Satake, W., Zhao, Y., Toda, T., & Liu, J.-J. . (2010). Analysis of GWAS-linked loci in Parkinson disease reaffirms PARK16 as a susceptibility locus. *Neurology*, *75*(6), 508–512. https://doi.org/10.1212/WNL.0b013e3181eccfcd

Tanaka, K. (2020). The PINK1–Parkin axis: An Overview. *Neuroscience Research*, *159*, 9–15. https://doi.org/10.1016/j.neures.2020.01.006

Tansey, M. G., Wallings, R. L., Houser, M. C., Herrick, M. K., Keating, C. E., & Joers, V. (2022). Inflammation and immune dysfunction in Parkinson disease. *Nature Reviews Immunology*, *22*, 1–17. https://doi.org/10.1038/s41577-022-00684-6

Taylor, J. M., Main, B. S., & Crack, P. J. (2013). Neuroinflammation and oxidative stress: Co-conspirators in the pathology of Parkinson's disease. *Neurochemistry International*, *62*(5), 803–819. https://doi.org/10.1016/j.neuint.2012.12.016

Taymans, J.-M., Fell, M., Greenamyre, T., Hirst, W. D., Mamais, A., Padmanabhan, S., Peter, I., Rideout, H., & Thaler, A. (2023). Perspective on the current state of the LRRK2 field. *Npj Parkinson's Disease*, *9*(1), 1–9. https://doi.org/10.1038/s41531-023-00544-7

Terkelsen, M. H., Klaestrup, I. H., Hvingelby, V., Lauritsen, J., Pavese, N., & Romero-Ramos, M. (2022). Neuroinflammation and Immune Changes in Prodromal Parkinson's Disease and Other Synucleinopathies. *Journal of Parkinson's Disease*, *12*(s1), S149–S163. https://doi.org/10.3233/jpd-223245

Thorne, N. J., & Tumbarello, D. A. (2022). The relationship of alpha-synuclein to mitochondrial dynamics and quality control. *Frontiers in Molecular Neuroscience*, *15*. https://doi.org/10.3389/fnmol.2022.947191

Tolosa, E., Garrido, A., Scholz, S. W., & Poewe, W. (2021). Challenges in the diagnosis of Parkinson's disease. *The Lancet Neurology*, *20*(5), 385–397. https://doi.org/10.1016/S1474-4422(21)00030-2

Tönges, L., Buhmann, C., Klebe, S., Klucken, J., Kwon, E. H., Müller, T., Pedrosa, D. J., Schröter, N., Riederer, P., & Lingor, P. (2022). Blood-based biomarker in Parkinson's disease: potential for future applications in clinical research and practice. *Journal of Neural Transmission*, *129*(9), 1201–1217. https://doi.org/10.1007/s00702-022-02498-1

Toomey, C. E., Heywood, W., Evans, J. R., Lachica, J. I., Pressey, S. N., Foti, S. C., Al Shahrani, M., D'Sa, K., Hargreaves, I. P., Heales, S., Orford, M., Troakes, C., Attems, J., Gelpí, E., Palkovits, M., Lashley, T., Gentleman, S., Révész, T., Mills, K., & Gandhi, S. (2022). Mitochondrial dysfunction is a key pathological driver of early stage Parkinson's. *Acta Neuropathologica Communications*, *10*(1). https://doi.org/10.1186/s40478-022-01424-6

Tran, J., Anastacio, H., & Bardy, C. (2020). Genetic predispositions of Parkinson's disease revealed in patient-derived brain cells. *Npj Parkinson's Disease*, *6*(1), 1–18. https://doi.org/10.1038/s41531-020-0110-8

Triarhou, L. C. (2013). Dopamine and Parkinson's Disease. In *www.ncbi.nlm.nih.gov*. Landes Bioscience. https://www.ncbi.nlm.nih.gov/books/NBK6271/#:~:text=Neurons%20of%20the%20substantia%20nigra

Trist, B. G., Hare, D. J., & Double, K. L. (2019). Oxidative stress in the aging substantia nigra and the etiology of Parkinson's disease. *Aging Cell*, *18*(6). https://doi.org/10.1111/acel.13031

Tysnes, O.-B., & Storstein, A. (2017). Epidemiology of Parkinson's Disease. *Journal of Neural Transmission*, *124*(8), 901–905. https://doi.org/10.1007/s00702-017-1686-y

Ullas, S., & Sinclair, C. (2024). Applications of Flow Cytometry in Drug Discovery and Translational Research. *International Journal of Molecular Sciences*, *25*(7), 3851–3851. https://doi.org/10.3390/ijms25073851

Valente, E. M., Salvi, S., Ialongo, T., Marongiu, R., Elia, A. E., Caputo, V., Romito, L., Albanese, A., Dallapiccola, B., & Bentivoglio, A. R. (2004). PINK1 mutations are associated with sporadic early-onset parkinsonism. *Annals of Neurology*, *56*(3), 336–341. https://doi.org/10.1002/ana.20256

Van Laar, A. D., & Jain, S. (2004). Non-motor Symptoms of Parkinson Disease: Update on the Diagnosis and Treatment. *The Neurologist*, *10*(4), 185–194. https://doi.org/10.1097/01.nrl.0000131146.08278.a5

Vásquez, K. A., Valverde, E. M., Aguilar, D. V., & Gabarain, H.-J. H. (2019). Montreal Cognitive Assessment scale in patients with Parkinson Disease with normal scores in the Mini-Mental State Examination. *Dementia & Neuropsychologia*, *13*(1), 78–81. https://doi.org/10.1590/1980-57642018dn13-010008

Vilariño-Güell, C., Rajput, A., Milnerwood, A. J., Shah, B., Szu-Tu, C., Trinh, J., Yu, I. S., Mary Joy Encarnacion, Munsie, L. N., Tapia, L., Gustavsson, E. K., Chou, P., Igor Tatarnikov, Evans, D. M., Pishotta, F. T., Volta, M., Dayne Beccano-Kelly, Thompson, C., Lin, M. K., & Sherman, H. E. (2013). *DNAJC13 mutations in Parkinson disease*. *23*(7), 1794–1801. https://doi.org/10.1093/hmg/ddt570

Vissers, M., Troyer, M. D., Thijssen, E., Heuberger, J., Geert Jan Groeneveld, & Huntwork-Rodriguez, S. (2023). A leucine-rich repeat kinase 2 (LRRK2) pathway biomarker characterization study in patients with Parkinson's disease with and without LRRK2 mutations and healthy controls. *Clinical and Translational Science*, *16*(8), 1408–1420. https://doi.org/10.1111/cts.13541

Wallings, R. L., & Tansey, M. G. (2019). LRRK2 regulation of immune-pathways and inflammatory disease. *Biochemical Society Transactions*, *47*(6). https://doi.org/10.1042/bst20180463

Wang, C., Chen, L., Yang, Y., Zhang, M., & Wong, G. (2019). Identification of potential blood biomarkers for Parkinson's disease by gene expression and DNA methylation data integration analysis. *Clinical Epigenetics*, *11*(1). https://doi.org/10.1186/s13148-019-0621-5

Weintraub, D., Caspell-Garcia, C., Simuni, T., Hyunkeun Ryan Cho, Coffey, C. S., Dag Aarsland, Alcalay, R. N., Barrett, M. J., Chahine, L. M., Eberling, J. L., Espay, A. J., Hamilton, J. L., Hawkins, K. A., Leverenz, J. B., Litvan, I., Richard, I. H., Rosenthal, L. S., Siderowf, A., York, M. K., & Parkinson's Progression Markers Initiative. (2020). Neuropsychiatric symptoms and cognitive abilities over the initial quinquennium of Parkinson disease. *Annals of Clinical and Translational Neurology*, *7*(4), 449–461. https://doi.org/10.1002/acn3.51022

Wider, C., Skipper, L., Solida, A., Brown, L., Farrer, M., Dickson, D., Wszolek, Z. K., & Vingerhoets, F. J. G. (2008). Autosomal dominant dopa-responsive parkinsonism in a

multigenerational Swiss family. *Parkinsonism & Related Disorders*, *14*(6), 465–470. https://doi.org/10.1016/j.parkreldis.2007.11.013

Wijeyekoon, R. S., Kronenberg-Versteeg, D., Scott, K. M., Hayat, S., Jones, J. L., Clatworthy, M. R., Floto, R. A., Barker, R. A., & Williams-Gray, C. H. (2018). Monocyte Function in Parkinson's Disease and the Impact of Autologous Serum on Phagocytosis. *Frontiers in Neurology*, *9*. https://doi.org/10.3389/fneur.2018.00870

Williams, G. P., Schonhoff, A. M., Sette, A., & Lindestam Arlehamn, C. S. (2022). Central and Peripheral Inflammation: Connecting the Immune Responses of Parkinson's Disease. *Journal of Parkinson's Disease*, *12*(Suppl 1), S129–S136. https://doi.org/10.3233/JPD-223241

Williamson, M., Madureira, M., McGuinness, W., Heon-Roberts, R., Mock, E. D., Naidoo, K., Cramb, K. M. L., Caiazza, M. C., Malpartida, A. B., Lavelle, M., Savory, K., Humble, S. W., Patterson, R. W., Davis, J. B., Connor-Robson, N., Ryan, B. J., & Wade-Martins, R. (2023). Mitochondrial dysfunction and mitophagy defects in *LRRK2-R1441C* Parkinson's disease models. *Human Molecular Genetics*, *32*(18), 2808–2821. https://doi.org/10.1093/hmg/ddad102

World Health Organisation (1993). *International programme on chemical safety biomarkers and risk assessment: concepts and principles,* www.inchem.org/documents/ehc/ehc/ehc155.htm

World Health Organisation. (2022, June 14). *Launch of WHO's Parkinson Disease Technical Brief*. Www.who.int. https://www.who.int/news/item/14-06-2022-launch-of-who-s-parkinson-disease-technical-brief

Wu, C.-C., Islam, Md. M., Lee, A.-J., Su, C.-H., Weng, Y.-C., Yeh, C.-Y., Lee, H.-H., & Lin, M.-C. (2022). Association between Statin Use and Risk of Parkinson's Disease: Evidence from 18 Observational Studies Comprising 3.7 Million Individuals. *Journal of Personalized Medicine*, *12*(5), 825. https://doi.org/10.3390/jpm12050825

Wu, Z., Ho, W. S., & Lu, R. (2021). Targeting Mitochondrial Oxidative Phosphorylation in Glioblastoma Therapy. *NeuroMolecular Medicine*. https://doi.org/10.1007/s12017-021-08678-8

Wu, Z., Wu, S., Liang, T., & Wang, L. (2021). Lipoprotein-Associated Phospholipase A2 Is a Risk Factor for Patients With Parkinson's Disease. *Frontiers in Neuroscience*, *15*. https://doi.org/10.3389/fnins.2021.633022

Xiang, W., Schlachetzki, J. C. M., Helling, S., Bussmann, J. C., Berlinghof, M., Schäffer, T. E., Marcus, K., Winkler, J., Klucken, J., & Becker, C.-M. (2013). Oxidative stress-induced posttranslational modifications of alpha-synuclein: Specific modification of alpha-synuclein by 4-hydroxy-2-nonenal increases dopaminergic toxicity. *Molecular and Cellular Neuroscience*, *54*, 71–83. https://doi.org/10.1016/j.mcn.2013.01.004

Xiromerisiou, G., Marogianni, C., Lampropoulos, I. C., Dardiotis, E., Speletas, M., Ntavaroukas, P., Androutsopoulou, A., Kalala, F., Grigoriadis, N., & Papoutsopoulou, S. (2022). Peripheral Inflammatory Markers TNF-α and CCL2 Revisited: Association with Parkinson's Disease Severity. *International Journal of Molecular Sciences*, *24*(1), 264–264. https://doi.org/10.3390/ijms24010264

Xu, J., He, X., Xu, Y., Chen, X., Li, M., Zhang, L., Fu, X., Pan, M., Wang, Q., & Hu, X. (2022). Characteristics of systemic inflammation and brain iron deposition in Parkinson's disease patients. *Annals of Clinical and Translational Neurology*, *9*(3), 276–285. https://doi.org/10.1002/acn3.51512

Yakhine-Diop, S. M. S., Niso-Santano, M., Rodríguez-Arribas, M., Gómez-Sánchez, R., Martínez-Chacón, G., Uribe-Carretero, E., Navarro-García, J. A., Ruiz-Hurtado, G., Aiastui, A., Cooper, J. M., López, A., Pedro, J. M. B.-S., González-Polo, R. A., & Fuentes, J. M. (2018). Impaired Mitophagy and Protein Acetylation Levels in Fibroblasts from Parkinson's Disease Patients. *Molecular Neurobiology*, *56*(4), 2466–2481. https://doi.org/10.1007/s12035-018-1206-6

Yamashita, K. Y., Sweta Bhoopatiraju, Silverglate, B., & Grossberg, G. T. (2023). Biomarkers in Parkinson's Disease: A State of the Art Review. *Biomarkers in Neuropsychiatry*, *9*, 100074–100074. https://doi.org/10.1016/j.bionps.2023.100074

Yang, W., Chang, Z., Que, R., Weng, G., Deng, B., Wang, T., Huang, Z., Xie, F., Wei, X., Yang, Q., Li, M., Ma, K., Zhou, F., Tang, B., Mok, V. C. T., Zhu, S., & Wang, Q. (2020). Contra-Directional Expression of Plasma Superoxide Dismutase with Lipoprotein Cholesterol and High-Sensitivity C-reactive Protein as Important Markers of Parkinson's Disease Severity. *Frontiers in Aging Neuroscience*, *12*. https://doi.org/10.3389/fnagi.2020.00053

Yuvraj Anandrao Jagtap, Kumar, P., Kinger, S., Dubey, A. R., Choudhary, A., Gutti, R. K., Singh, S., Jha, H. C., Mohan Poluri, K., & Mishra, A. K. (2023). Disturb mitochondrial associated proteostasis: Neurodegeneration and imperfect ageing. *Frontiers in Cell and Developmental Biology*, *11*. https://doi.org/10.3389/fcell.2023.1146564

Zaltieri, M., Longhena, F., Pizzi, M., Missale, C., Spano, P., & Bellucci, A. (2015). Mitochondrial Dysfunction andα-Synuclein Synaptic Pathology in Parkinson's Disease: Who's on First? *Parkinson's Disease*, *2015*, 1–10. https://doi.org/10.1155/2015/108029

Zeiser, R. (2018). Immune modulatory effects of statins. *Immunology*, *154*(1), 69–75. https://doi.org/10.1111/imm.12902

Zhang, H., Gu, Z., Yao, C., Cai, Y., Li, Y., Mao, W., Xu, E., Postuma, R. B., & Chan, P. (2020). Risk factors for possible REM sleep behavior disorders. *Neurology*, *95*(16), e2214–e2224. https://doi.org/10.1212/wnl.0000000000010610

Zhao, Y., Zhang, Y., Zhang, J., & Yang, G. (2021). Plasma proteome profiling using tandem mass tag labeling technology reveals potential biomarkers for Parkinson's disease: a preliminary study. *PROTEOMICS – Clinical Applications*, *16*(2), 2100010. https://doi.org/10.1002/prca.202100010

Zheng, W., & Fan, D. (2022). Glucocerebrosidase Mutations Cause Mitochondrial and Lysosomal Dysfunction in Parkinson's Disease: Pathogenesis and Therapeutic Implications. *Frontiers in Aging Neuroscience*, *14*. https://doi.org/10.3389/fnagi.2022.851135

Zhou, Z., Yi, L., Wang, Q., Tit Meng Lim, & Eng King Tan. (2023). Role of dopamine in the pathophysiology of Parkinson's disease. *Translational Neurodegeneration*, *12*(1). https://doi.org/10.1186/s40035-023-00378-6

Zhuang, W., Camacho, L., Silva, C. S., & Hong, H. (2020). Reproducibility challenges for biomarker detection with uncertain but informative experimental data. *Biomarkers in Medicine*, *14*(13), 1255–1263. https://doi.org/10.2217/bmm-2019-0599

Zimmermann, M., & Brockmann, K. (2022). Blood and Cerebrospinal Fluid Biomarkers of Inflammation in Parkinson's Disease. *Journal of Parkinson's Disease*, 1–18. https://doi.org/10.3233/jpd-223277

Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., Hulihan, M., Uitti, R. J., Calne, D. B., Stoessl, A. Jon., Pfeiffer, R. F., Patenge, N., Carbajal, I. C., Vieregge, P., Asmus, F., Müller-Myhsok, B., Dickson, D. W., Meitinger, T., & Strom, T. M. (2004). Mutations in LRRK2 Cause Autosomal-Dominant Parkinsonism with Pleomorphic Pathology. *Neuron*, *44*(4), 601–607. https://doi.org/10.1016/j.neuron.2004.11.005