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The Role of a Candidate microRNA in Mitochondrial Dysfunction and Inflammatory Responses *in vitro*: Relevance To Parkinson's Disease

Dissertação no âmbito do Mestrado em Genética Clínica Laboratorial orientada pela Doutora Ana Raquel Fernandes Esteves, coorientada pela Professora Doutora Sandra Isabel Morais de Almeida Costa Cardoso e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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A quem me acompanhou neste percurso e ajudou a construir quem sou.

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RESUMO

A Doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum e é caracterizada pela perda acentuada de neurónios dopaminérgicos na Substantia Nigra *pars compacta* (SNpc) e pela presença de inclusões proteicas intracitoplasmáticas denominadas de Corpos de Lewy. A DP é diagnosticada aquando do surgimento dos primeiros sintomas motores, nomeadamente bradicinesia, tremor em repouso, rigidez muscular e instabilidade postural. Atualmente, acredita-se que o início dos sintomas motores é precedido por uma fase prodrómica, durante a qual surgem sintomas não motores, como a disfunção gastrointestinal. Embora a neuropatologia da DP seja bem compreendida, a sua etiologia permanece um mistério. Foi proposto que a DP inicia no sistema nervoso entérico e se espalha, através do nervo vago, para o sistema nervoso central. Além disso, tanto a inflamação a nível cerebral como a nível intestinal têm sido associadas à DP, conduzindo à hipótese de que o sistema imunológico desempenha um papel ativo na patogénese da DP. Adicionalmente, vários estudos descreveram que a microbiota intestinal está alterada em doentes com DP, o que poderá comprometer a integridade da barreira intestinal e aumentar a sua permeabilidade. Além disso, vários estudos detetaram uma expressão alterada de microRNAs (miRNAs) em doentes com DP, bem como sua capacidade de modular e serem regulados pela microbiota intestinal do hospedeiro. Curiosamente, um estudo recente mostrou um enriquecimento do miRNA-486-5p em biópsias de cólon de doentes com DP. Alguns estudos sugerem que os miRNAs podem ser usados como biomarcadores para o estudo de malignidade intestinal em amostras de fezes e tecidos. Além disso, a identificação de perfis diferenciais de miRNAs, quer em amostras de intestino, quer em amostras fecais, entre doentes e controlos saudáveis, também sugere que os miRNAs poderão ser usados como potenciais biomarcadores para a deteção da DP na fase prodrómica. Trabalhos anteriores do nosso grupo mostraram que a desregulação da função mitocondrial é um dos principais contribuintes para o desenvolvimento da DP. Além disso, vários estudos têm descrito um papel crucial das mitocôndrias na regulação e ativação de respostas inflamatórias. Considerando estas linhas de evidência, a presente tese tem como objetivo compreender se a expressão do miR-486-5p poderá contribuir para a patogénese da DP, afetando a função mitocondrial e ativando respostas inflamatórias. Os efeitos *in vitro* foram estudados através da transfeção de células Caco-2 (para mimetizar células intestinais) e células SH-SY5Y (para mimetizar neurónios da DP) com o miRNA mencionado. Os

nossos resultados demonstraram um aumento dos níveis de marcadores inflamatórios em ambas as linhas celulares. Além disso, nas células Caco-2, os nossos resultados sugerem que a expressão de miR-486-5p pode levar ao comprometimento da barreira intestinal; nas células SH-SY5Y observamos a depleção de cardiolipina e consequente aumento de espécies reativas de oxigénio. No geral, podemos concluir que o miR-486-5p contribui para a inflamação intestinal e aumento da permeabilidade da barreira intestinal nas células Caco-2, enquanto nas células SH-SY5Y conduz à ativação de respostas inflamatórias devido à disfunção mitocondrial. Este estudo abre caminho para elucidar a contribuição de miRNAs específicos para a inflamação e disfunção mitocondrial no desenvolvimento da DP.

Palavras-chave: Doença de Parkinson; microRNAs, disfunção mitocondrial; inflamação; *in vitro*.

ABSTRACT

Parkinson's Disease (PD), the second most common neurodegenerative disorder, is characterized by the severe loss of dopaminergic neurons in the Substantia Nigra *pars compacta* (SNpc) and by the presence of intracytoplasmic proteinaceous inclusions called Lewy Bodies. PD is clinically diagnosed upon the onset of characteristic motor symptoms, such as bradykinesia, resting tremor, rigidity, and postural instability. It is currently accepted that the onset of motor symptoms is preceded by a prodromal phase, during which non-motor features emerge, such as gastrointestinal dysfunction. Although the neuropathology of PD is fairly well understood, its aetiology remains a mystery. It has been proposed that PD might start in the enteric nervous system and spread, via the vagus nerve, to the central nervous system. Moreover, both brain and intestinal inflammation have been associated with PD, leading to the hypothesis that the immune system plays an active role in PD pathogenesis. Furthermore, several studies have reported that gut microbiota is altered in PD patients, in a way that compromises the integrity of the intestinal barrier and increases its permeability. Additionally, several studies have detected an altered expression of microRNAs (miRNAs) in PD patients, as well as their ability to modulate and be regulated by the host's gut microbiota. Notably, a recent paper showed an enrichment of submucosal miRNA-486-5p in colon tissue from sporadic PD patients when compared to healthy age-matched controls. Interestingly, some studies suggested that miRNAs can be used as potential biomarkers for screening intestinal malignancy in stool and tissue samples. Moreover, in the future, identifying differential miRNA signatures either in intestine samples or in faecal samples between patients and healthy controls also suggest that miRNAs can be used as potential biomarkers for PD screening in a prodromal phase. Previous work from our group showed that dysregulation of mitochondrial function is a major contributor towards PD development. Moreover, several studies have now described a crucial role for mitochondria in the regulation and activation of inflammatory responses. Taking all these evidence into account, this thesis aims to investigate if miR-486-5p expression could contribute to PD pathogenesis by affecting mitochondrial function and activating inflammatory responses. *In vitro* effects were studied by transfecting Caco-2 cells (to mimic intestinal cells) and SH-SY5Y cells (to mimic PD neurons) with the mentioned miRNA. Our results demonstrated increased inflammatory markers levels in both Caco-2 and SH-SY5Y cells. Furthermore, our findings from Caco-2 cells propose that up-

regulation of miR-486-5p could ultimately lead to the impairment of the intestinal barrier; in SH-SY5Y cells, we observed the depletion of cardiolipin and consequent increase of ROS. Overall, we can conclude that miRNA-486-5p contributes to gut inflammation and loss of the gut barrier integrity in Caco-2 cells whereas in SH-SY5Y cells leads to the activation of inflammatory responses due to mitochondrial dysfunction. This study paves the way to clarify the contribution of specific miRNAs in inflammation and mitochondrial dysfunction in PD development.

Keywords: Parkinson's Disease; microRNAs; mitochondrial dysfunction; inflammation; *in vitro*.

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LIST OF ABBREVIATIONS

ALS, autophagy-lysosome system

APCs, antigen presenting cells

ASC, apoptosis-associated speck-like protein containing a CARD

BCR, B cell receptors

CCCP, carbonyl cyanide 3-chlorophenylhydrazone

CSF, cerebrospinal fluid

DAMPs, damage associated molecular patterns

ETC, electron transport chain

FBS, fetal bovine serum

FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

HLA, human leucocyte antigen

IFN-γ, interferon gamma

IL, interleukin

LBs, Lewy bodies

LP, Lewy pathology

LPS, lipopolysaccharide

miRNA, microRNA

MPP^+, 1-methyl-4-phenylpyridinium

MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA, messenger RNA

mtDNA, mitochondrial DNA

MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide

NAO, 10-N-Nonyl acridine orange

NLRP3, leucine-rich repeat pyrin domain containing 3

PAMPs, pathogen associated molecular patterns

PD, Parkinson's disease

PGC-1α, proliferator-activated receptor gamma coactivator1-alpha

PINK1, PTEN-induced putative kinase 1

RISC, RNA-induced silencing complex

ROS, reactive oxygen species

SNpc, substantia nigra *pars compacta*

TCR, T cell receptors

TMRM, tetramethylrhodamine, Methyl Ester dye

TNF-α, tumour necrosis factor alpha

UPS, ubiquitin-proteasome system

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CHAPTER I
INTRODUCTION

1.1. Parkinson's Disease

Parkinson's Disease (PD) was first described by James Parkinson more than two centuries ago, in "An Essay on the Shaking Palsy" in 1817. PD is the second most common progressive neurodegenerative disorder, after Alzheimer's Disease, with more than 10 million people affected worldwide in 2021. Its incidence and prevalence increase steadily with age (median age at onset at 60 years old), and men are 1.5 times more likely to suffer from the disease than women [1].

PD is clinically characterized by the development of numerous motor symptoms, including bradykinesia, resting tremor, rigidity, and postural instability [2]. These motor symptoms develop as a consequence of the severe loss of dopaminergic neurons in the Substantia Nigra *pars compacta* (SNpc), and only emerge when striatal dopamine levels are decreased by 60-70% as a result of the degeneration of 40-60% of neurons in the SNpc [3]. The presence of intracytoplasmic protein inclusions of α-synuclein, known as Lewy Bodies (LBs), is another important pathological hallmark of PD. Furthermore, it is believed that PD progression might also affect non-dopaminergic pathways, prior to the onset of nigral neurodegeneration, leading to the manifestation of several non-motor symptoms that appear at a prodromic stage, up to 15-20 years before the onset of motor symptoms (Fig. 1) [2]. Based on various population studies, a multitude of non-motor symptoms have been commonly associated with prodromal PD, namely: hyposmia, constipation, depression, dysautonomia, and rapid eye movement (REM) sleep behaviour disorder (RBD) (Fig. 1) [2, 3].

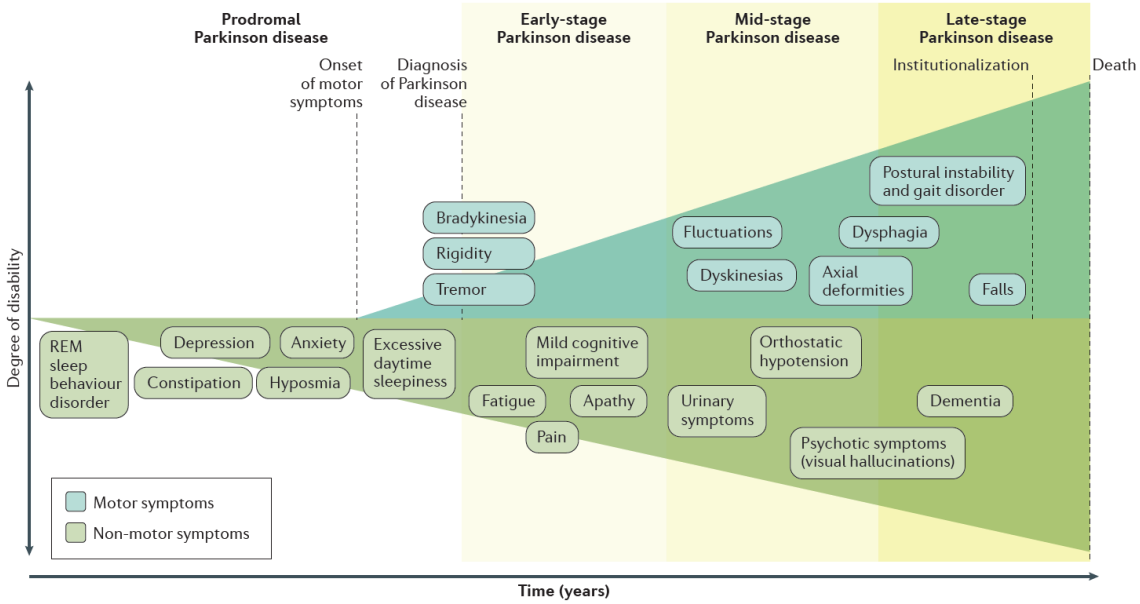

Figure 1. Clinical symptoms associated with Parkinson's Disease. Diagnosis of Parkinson's disease occurs with the onset of motor symptoms (early-stage Parkinson disease) but can be preceded by a prodromal phase of years or even decades, which is characterized by specific non-motor symptoms (prodromal Parkinson disease) (Adapted from Poewe *et al*., 2017).

Since the discovery of LBs, it was clear that a variety of other neuronal populations were affected by these proteinaceous inclusions, and not only the neurons in the SNpc [4]. In order to determine whether the pathology affects the nigral and extranigral structures simultaneously, Braak and colleagues conducted various studies which culminated in the formulation of a six-stage hypothesis for the progression of Lewy pathology (LP) in sporadic PD [5]. According to Braak's hypothesis, PD pathogenesis might initiate in non-dopaminergic areas, such as the olfactory bulb and the enteric nervous system (ENS), years and even decades before spreading, via the olfactory tract and the vagal nerve, respectively, towards the central nervous system (CNS), eventually reaching the SNpc and triggering the emergence of motor symptoms [6]. The earliest stages of LP (before SNpc involvement) have been linked to the prodromal non-motor symptoms of PD, like olfactory impairment and gastrointestinal dysfunctions [7], since constant evidence has detected the presence of α-synuclein aggregates in the gut during the prodromal phase, before the clinical diagnosis of PD [6, 8], and in the neurons of the olfactory tract [6]. Nonetheless, studies showed that some PD cases cannot be staged by the Braak's staging system, as some patients presented evident LB pathology first in the locus coeruleus, SNpc and the amygdala, and then in the dorsal motor nucleus of the vagus (DMV) and gut, representing a descending route of α-synuclein transmission from the CNS to the ENS [9]. These neuropathological evidences gave rise to two distinct hypotheses: body-first PD *vs.* brain-first PD (Fig. 2). In the body-first subtype of PD, LP originates in the enteric or autonomic nervous system and spreads to the CNS via the vagal nerve, which aligns with Braak's proposed staging [10]. These patients display more autonomic symptoms and a longer prodromal phase, with the development of non-motor symptoms like gastrointestinal dysfunction, RBD and hyposmia [9]. The opposite is true for the brain-first subtype of PD, where brain pathology presumably initiates in the amygdala or in closely connected areas such as the olfactory bulb [9]. These patients present fewer autonomic symptoms and a shorter prodromal phase, with a less frequent development of non-motor symptoms like RBD or hyposmia [9]. Existing evidence indicates most PD cases have been reported to follow the body-first approach [10].

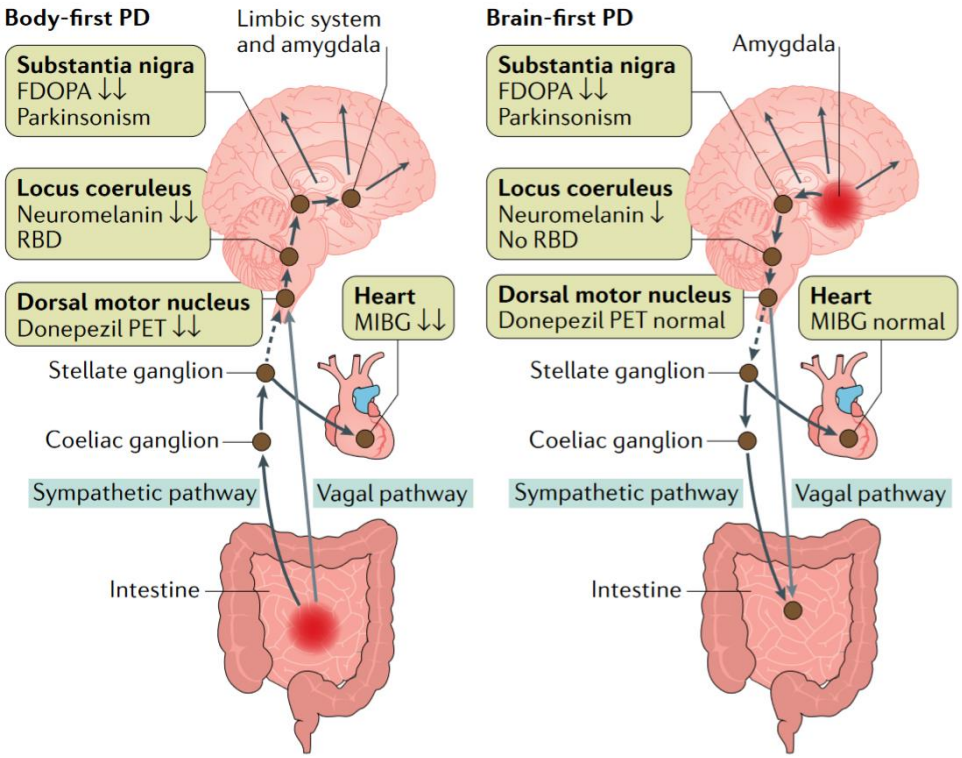

Figure 2. Characteristics of body-first *vs.* brain-first subtypes of Parkinson's Disease. Arrows indicate the direction of spread of α-synuclein pathology. In body-first PD (left), the first α-synuclein aggregation occurs in the gut and propagates through the autonomic nervous system to the spinal cord and brainstem. In brain-first PD (right), the initial α-synuclein pathology arises in vulnerable structures of the brain, such as the limbic system, or enters via an olfactory route. (Adapted from Berg *et al*., 2021).

Despite all the efforts and contributions of the scientific community, the primary cause of PD remains largely unknown; however, it has been hypothesised that the disease has a multifactorial aetiology, resulting from the interaction between environmental, genetic and age-associated factors [8]. Several environmental and lifestyle risk factors have been linked to PD, including environmental toxins, pesticides and heavy metals exposure, traumatic lesions and bacterial or viral infections [11]. Moreover, while most cases occur in a sporadic manner, approximately 5–10% of PD cases are of genetic origin [11]. The era of PD genetics started when several families seemed to exhibit a Mendelian inheritance pattern (dominant or recessive) of Parkinson's, suggesting a genetic cause of the disease [2]. In 1997, the first PD-associated mutation was discovered in the *SNCA* gene [12], originating a highly productive period of gene hunting that resulted in the identification of several PD-related genes, with either autosomal dominant (*SNCA*, *LRRK2*, *VPS35*) or autosomal recessive (*PRKN*, *PINK1*, *DJ1*) modes of inheritance [13]. Although genetic PD corresponds to a small fraction of all cases, focusing the research on genetic forms has proved to be valuable to understand the pathophysiology of PD, since some of the proteins encoded by PD-associated genes are involved in key

neuropathological mechanisms linked to the development and progression of both sporadic and familial PD (Fig. 3) [14], such as: α-synuclein aggregation and accumulation, mitochondrial dysfunction, proteasomal and autophagic impairment, oxidative stress and neuroinflammation [2]. α-synuclein, a small protein that is natively unfolded, adopts a β-sheet-rich amyloid-like structure in PD and becomes prone to aggregate [15]. Aggregates of α-synuclein as the core component of LBs and heritable forms of PD caused by mutations in the gene that encodes α-synuclein (*SNCA*) suggest that this protein plays an important role in the development and progression of PD. Although α-synuclein is implicated in several important cellular processes, such as intracellular trafficking, mitochondrial function, and synaptic vesicles dynamics [16], its misfolding and accumulation is toxic to dopaminergic neurons and leads to neuronal death [2]. Dopaminergic neurons are particularly vulnerable to oxidative stress because of their high energy demand [16]; several studies have shown increased oxidative stress in the brains of both genetic and sporadic PD cases, due to the accumulation of reactive oxygen species (ROS) that originate from the dopamine metabolism, mitochondrial dysfunction and neuroinflammatory processes [4]. A defective antioxidant response caused by mutations in genes that encode antioxidant proteins (such as *DJ1*) might also impair the removal of ROS, leading to their toxic build-up in the neurons [16]. Accumulation of protein aggregates and damaged organelles (mainly mitochondria) is characteristic of PD [4], and consistent evidence has shown the crucial role of impaired degradation of misfolded proteins or dysfunctional organelles in PD pathogenesis [4]. Ubiquitin-proteasome system (UPS) and autophagy-lysosome system (ALS) are the two main mechanisms responsible for the clearance of dysfunctional proteins and organelles, and their activity has been found diminished in the SNpc of PD brains, along with a reduced expression of their components [15]. Interestingly, mutated genes involved in UPS or ALS (*PRKN* and *PINK1*) have been described in familial PD, consequently impairing the removal and degradation of damaged proteins and organelles, such as misfolded α-synuclein and dysfunctional mitochondria [15]. Neuroinflammation has been considered a key feature of PD, as numerous studies have detected an increase of inflammatory markers in PD brains [4]. Initially, neuroinflammation was believed to arise only as a response to neuronal damage, however recent evidence revealed the existence of several genetic variations in the Human Leucocyte Antigen (HLA) coding region that are associated with a higher risk of developing late-onset PD [4, 8] , suggesting that neuroinflammatory responses might themselves contribute to disease pathogenesis [8,

15]. α-synuclein has also been implicated in neuroinflammation, as numerous studies propose that its aggregation is able to induce both innate and adaptive immunity; on the other hand neuroinflammation can also promote α-synuclein aggregation, proposing that the two processes participate in a self-aggravating vicious cycle [16].

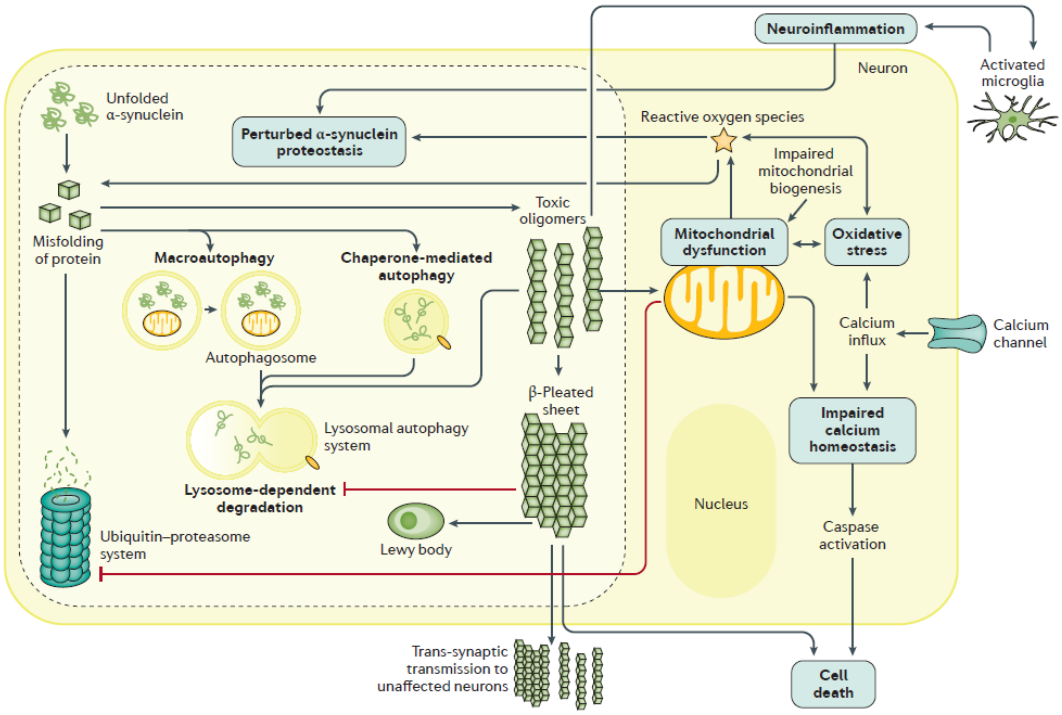

Figure 3. Molecular mechanisms involved in Parkinson's Disease. Schematic diagram depicting interactions between molecular pathways involved in the pathogenesis of the disease (Adapted from Poewe *et al*., 2017).

For several decades, numerous epidemiological studies have consistently shown that increased PD prevalence positively correlates with increasing age, highlighting aging as one of the most important contributing factors for PD. Indeed, the aging process is strongly associated with mitochondrial dysfunction, increased oxidative stress, neuroinflammation and impairment of protein clearance (which facilitates α-synuclein accumulation even in the normal ageing brain [16]), all of which have also been considered pathological markers of PD [4, 17].

James Parkinson optimistically stated that "*there appears to be sufficient reason for hoping that some remedial process may ere long be discovered, by which, at least, the progress of the disease may be stopped*" [18]. Over 200 years later, no cure or neuroprotective therapy has been discovered for PD, however, there has been great progress in understanding neurodegeneration in PD, hopefully bringing us closer to achieving truly disease-modifying therapies [8]. The currently available diagnostic

methods are not able to predict the onset of the disease early on, therefore the existing pharmacological and neurosurgical treatments are symptomatic, in order to ameliorate motor and non-motor symptoms and attempt to slow down the progression of the neurodegenerative process, but not being able to fully stop it [1].

1.2. Mitochondrial Dysfunction in Parkinson's Disease

Mitochondria are dynamic membrane-bound organelles known to play a critical role in multiple cellular functions, including energy production, intracellular Ca^{2+} homeostasis, ROS generation, and apoptosis [19]. The first evidence that mitochondrial dysfunction is a key player in PD pathogenesis dates back to 1983, when Langston and colleagues [20] reported several cases of a parkinsonian syndrome in humans caused by the use of a synthetic drug containing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These events rapidly received the attention of the scientific community, leading to the better understanding of how MPTP caused parkinsonism. The lipophilic nature of MPTP allows it to cross the blood-brain barrier and, once inside the brain, it is metabolized to 1-methyl-4-phenylpyridinium (MPP^+), which is taken up by dopaminergic neurons through the dopamine transporter (DAT), inhibiting complex I of the mitochondrial electron transport chain (ETC) and resulting in energy depletion and oxidative stress [19]. Mitochondrial dysfunction associated with PD pathogenesis can result from ETC dysfunction, impaired mitochondrial biogenesis, increased oxidative stress, defective mitophagy, altered mitochondrial dynamics, compromised trafficking, aberrant Ca^{2+} homeostasis, or combinations thereof [21]. The complex interplay between these compromised cellular functions leads to a cycle of progressive cellular dysfunction that ultimately results in dopaminergic neurodegeneration that underlies PD pathogenesis.

The hypothesis that PD can be triggered by mitochondrial dysfunction was corroborated when decreased activity of complex I was found in PD brain samples [22-25], platelets [26-29], lymphocytes [29], fibroblasts [30, 31], skeletal muscle [32-34] and in PD cytoplasmic hybrids (cybrids) [35]. Cybrids are generated through transferring of platelet mitochondria from either PD or control subjects to mitochondrial DNA (mtDNA)-depleted recipient cells (rho0 cells). The resulting cybrid lines express the nuclear genes of the recipient rho0 cell line and the mitochondrial genes of the platelet donor [36]. Besides MPP^+, other toxins and pesticides (like rotenone and paraquat) that impair mitochondrial complex I activity, also cause a Parkinsonian phenotype and dopaminergic

neuron loss in animals, and potentially in humans [37]. Mitochondrial dysfunction may be potentiated by impaired mitochondrial biogenesis, a highly regulated process essential for the maintenance of a healthy pool of mitochondria [19]. Mitochondrial respiration, mitochondrial antioxidant defence, as well as mitochondrial biogenesis are regulated by PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator-1alpha), that is significantly reduced in diverse PD models [38-41], therefore adversely affecting cellular bioenergetics. On the other hand, PGC-1α overexpression has been shown to mitigate α-synuclein oligomerization [38] and protect dopaminergic neurons [42].

The production of ROS through mitochondrial respiration is physiologic, however disruption of respiratory chain complexes causes excessive production of ROS and leads to oxidative stress, which is detrimental to cells [43]. Mitochondria are, simultaneously, the main source and the primary targets of ROS. Under normal conditions, antioxidant proteins like superoxide dismutase (SOD) and glutathione (GSH) prevent ROS levels from getting too high, but malfunction of these defence mechanisms leads to oxidative stress. Consequently, accumulated ROS damage the components of the respiratory chain, as well as mtDNA. PD brain biopsies revealed that complex I itself is oxidatively damaged, which prevents its proper assembly and function [24], leading to further inhibition and greater ROS production. Furthermore, post-mortem samples from PD patients exhibited increased lipid peroxidation while glutathione pathway was impaired [44]. ROS possess the ability to damage mtDNA by causing single and double-strand breaks that, when repaired inefficiently, originate mtDNA deletion mutations that affect the genes coding for essential proteins involved in the mitochondrial respiratory chain [43]. The mitochondrial genome is packaged in nucleoids by the mitochondrial transcription factor A (TFAM), in order to protect it from oxidative insults; however, TFAM deficiency has been observed in dopaminergic neurons of sporadic PD subjects [45, 46], suggesting an enhanced exposure of mtDNA to ROS damage in PD. Several studies have detected accumulation of deletions in the mtDNA of aged SNpc neurons of both healthy and PD individuals [45, 47-49]. Additionally, mtDNA copy number has been found to increase with age in healthy individuals [49], allowing the neurons to adapt to mitochondrial dysfunction via the maintenance of their mtDNA population. However, failure of such regulatory mechanisms of mtDNA copy number has been found in PD patients, leading to mtDNA copy number depletion [45, 47, 49, 50], despite accumulating mutations. Evidence has shown that oxidative stress is linked to dopamine metabolism,

justifying the selective neurodegeneration of dopaminergic neurons [51]. Auto-oxidation of dopamine generates free radicals and active quinones, which in turn interact with ROS scavengers, respiratory chain complexes and proteins involved in mitophagy [51], contributing to increased levels of ROS and defective mitophagy processes. Oxidative damage has also been implicated in impaired proteasomal ubiquitination and degradation of proteins, facilitating the accumulation of aggregated α-synuclein in the form of LBs [52]. α-synuclein accumulation has been noted to induce redox imbalance and mitochondrial fragmentation in *in vitro* and *in vivo* models of PD [53, 54]. Moreover, α-synuclein itself is known to interfere with mitochondrial function, by accumulating inside these organelles and damaging complex I of the ETC, resulting in mitochondrial dysfunction and increased levels of ROS [55-57].

Oxidative stress undoubtedly contributes to PD pathology, and it is now generally accepted that ROS are a by-product of mitochondrial dysfunction that contributes to exacerbate cell demise [58]. The interaction between these numerous mechanisms associated with mitochondrial dysfunction forms a positive feedback loop that drives uncontrolled pathogenesis conditions, resulting in the development and progression of PD.

1.3. Innate And Adaptive Immunity Activation in Parkinson's Disease

The immune system is the result of the complex interplay between innate and adaptive immunity. The innate immune system is ancient, highly conserved and operates through nonspecific mechanisms as a first line of defence (Fig. 4). It is comprised of tissue resident macrophages, dendritic cells, monocytes, granulocytes, neutrophils, and even non-immune cells that acquire immunological functions as necessary. In fact, besides immune cells, there is a plethora of non-immune cells capable of inducing innate immune responses, such as epithelial cells, epidermal keratinocytes, mesenchymal cells, stromal cells, and neurons [59]. A significant amount of evidence has observed the expression of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), on intestinal epithelial cells and neurons, suggesting that these non-immune cells possess the machinery to respond to pathogens through activation of innate immunity [60]. The expression of TLR4 has been found altered in inflammatory bowel diseases patients [61]. Colonic epithelial cells isolated from these patients were shown to secrete Interleukin (IL)-1β, produced through activation of the inflammasome, and IL-17 [60]. Likewise, it

has been demonstrated that neurons express TLR3 and TLR4 [62, 63] and also produce inflammatory cytokines, such as IL-6, Tumour Necrosis Factor Alpha (TNF-α) and interferon, that may mediate innate immunity in the absence of microglial cells [64]. Furthermore, evidence showed that neurons alone can activate NF-κB-dependent nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome under ischemic conditions [65]. Moreover, work from our group observed that both enriched mesencephalic and cortical neuronal cultures exposed to BMAA, a bacterial neurotoxin, activated the NLRP3 inflammasome accompanied by the release of IL-1β, indicating that neurons can initiate this activation without microglia contribution [62, 63]. Innate immunity's objective is to quickly resolve threats to the host, including clearance of invading pathogens, removal of dead/dying cells and wound repair. On the other hand, the adaptive immune system is highly specific and able to remember and effectively produce responses against previously encountered immunological threats, due to the ability of lymphocytes (T and B cells) to rearrange their genomes and create unique antigen specific receptors: T cell receptors (TCR) and B cell receptors (BCR) [66].

Macrophages and dendritic cells, also referred to as antigen presenting cells (APCs), are the main sensors of danger and initiators of innate immune responses. As the name suggests, APCs present antigens on their surface via major histocompatibility complex (MHC) molecules and mount effective immune responses by communicating and interacting with other immune cells when they encounter danger, such as monocytes, granulocytes, neutrophils, as well as adaptive immune system cells. Microglia, the main tissue resident macrophages and therefore the predominant APCs of the brain, are the largest population of innate immune cells in the CNS and function as the first responders to injury, modulators of homeostasis, and mediators of neuroinflammation. During CNS inflammation, microglia can upregulate MHCII, produce cytokines and chemokines and recruit peripheral myeloid cells, further contributing to the inflammatory process. APCs initiate the inflammatory processes upon activation of PRRs by pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) and through antigen presentation to T cells.

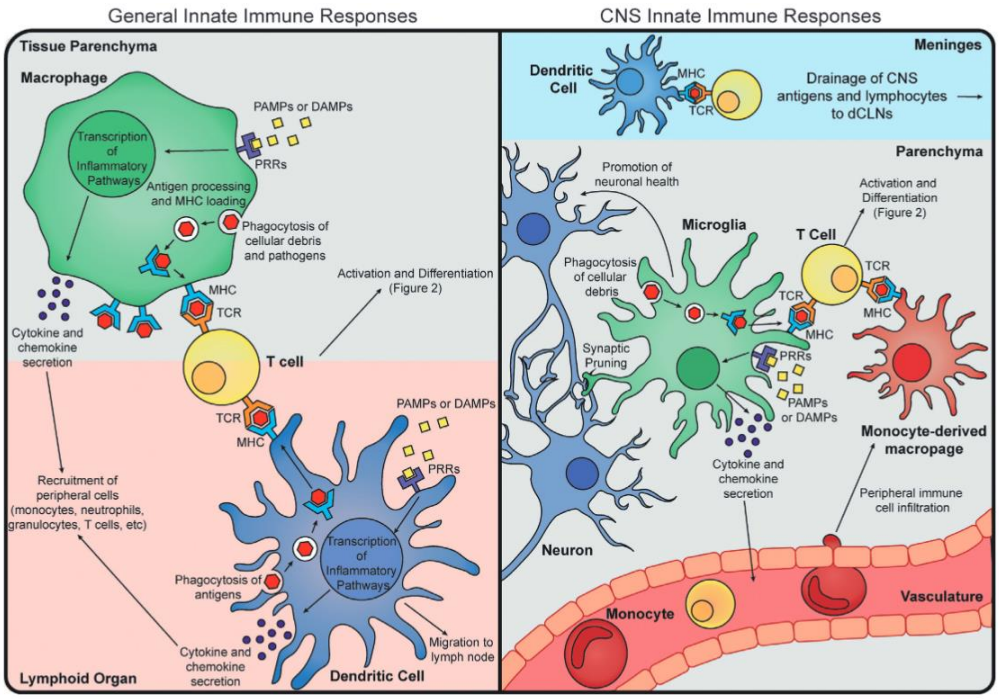

Figure 4. Overview of innate immune responses. General innate immune responses typically involve the recognition of pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) by a pattern recognition receptor (PRR) on the surface of antigen presenting cells (APCs). This process ultimately leads to the secretion of cytokines and chemokines to recruit more immune cells to the site. APCs can also phagocytose cellular debris and pathogens, process them, and load the antigenic peptides onto an MHC to be presented to T cells. In the CNS, the predominant APCs are microglia, but antigen uptake, pattern recognition, and cytokine secretion are thought to occur like general innate responses (Adapted from Schonhoff *et al*., 2020).

T cells use their TCR to detect antigens that have been loaded onto the MHC of other cells and are divided into two major subsets: CD8 T cells, referred to as cytotoxic T cells (Tc) and CD4 T cells (Fig. 5). CD8 T cells recognize antigens loaded onto MHCI (which can be displayed on the surface of any cell), proliferate and release cytokines and lytic molecules that promote the lysis of the infected cell. On the other hand, CD4 T cells recognize antigens loaded onto MHCII of APCs, namely innate immune cells and B cells. Upon TCR recognition of an MHC-bound antigen, CD4 T cells have the ability to differentiate into different T helper cell types (Th1, Th2, Th17 or Treg) to produce pathogen and tissue dependent cytokines that have different roles in inflammation. B cell activation can be T cell independent or T cell dependent, since B cells can either directly recognize pathogens via their BCR and initiate the production and secretion of antibodies targeted toward that particular pathogen or they can recognize a pathogen through their BCR, process it and load an antigenic peptide onto the MHCII, recruiting help from T cells that interact with the presented antigen and produce high-affinity antibodies to rapidly recognize and clear immunological threats (Fig. 5). The innate and adaptive immune systems function in harmony, producing effective and protective immune

responses. However, under pathological conditions, the immune system responds with excessive activation or misguided recognition of host antigens, leading to the damage of tissue [66].

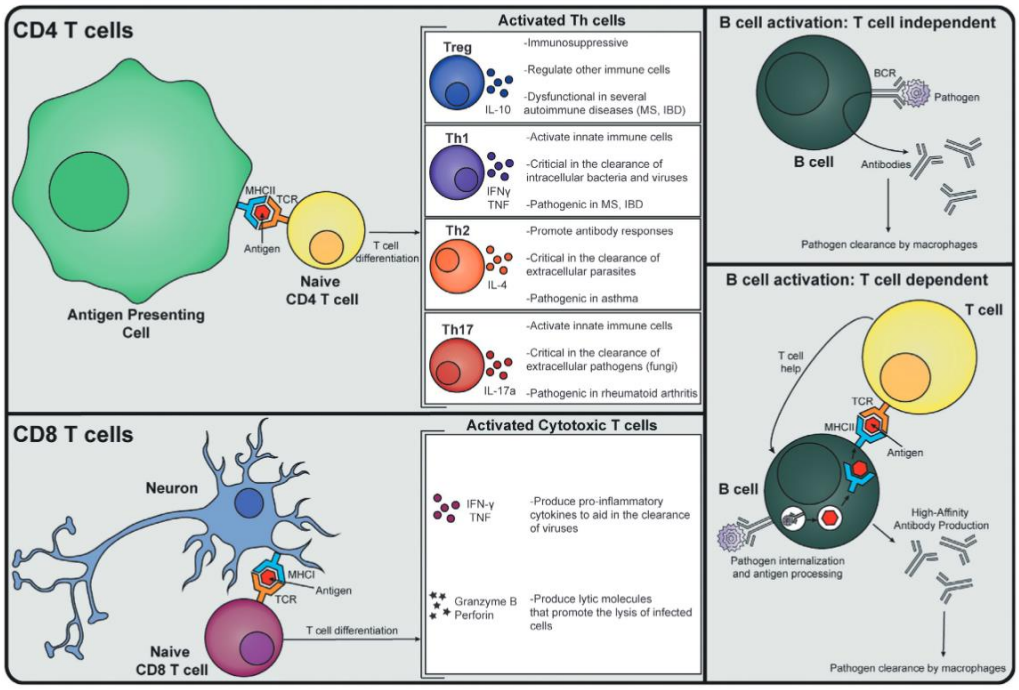

Figure 5. Overview of adaptive immune responses. CD4 T cells interact with antigen presenting cells (APCs) through their T cell receptor (TCR) via an antigen-loaded MHCII molecule. Upon antigen recognition a CD4 T cell can differentiate into a Treg, Th1, Th2, or Th17 cell type, each with different roles in inflammation and signature cytokines. A CD8 T cell interacts with antigen-loaded onto an MHCI molecule, which can be displayed on the surface of any cell, including neurons, recognizes the antigen and differentiates to produce inflammatory cytokines and lytic molecules. B cells can act with or without the help of T cells. The B cell receptor (BCR) can recognize extracellular pathogens and produce and secrete antibodies targeted towards that pathogen. A B cell can also recognize a pathogen via its BCR, process it, and load an antigenic peptide onto an MHCII molecule, which is then recognized by a CD4 T cell. These processes lead to the production of high affinity antibodies targeted towards the pathogen, resulting in its clearance. (Adapted from Schonhoff *et al*., 2020).

The involvement of the immune system in PD has been hypothesized since it was considered a multisystemic disorder, and evidence has grown substantially to support the idea that (neuro)inflammation drives the progression of PD and that it occurs long before symptomatology becomes apparent. One of the first observations that linked the immune system to the pathogenesis of PD arose in 1988, when McGeer and colleagues showed HLA-DR$^+$ (a component of the human MHCII) reactive microglia in the post-mortem SNpc of PD patients [67]. Similarly, accumulation of activated microglia around dopaminergic neurons was observed in post-mortem human brains with MPTP-induced parkinsonism [68]. Additionally, several groups provided further evidence of a pro-inflammatory phenotype or innate immunity activation of microglia through histological

studies [69-74], as well as through positron emission tomography (PET) imaging studies [75-79]. Extracellular α-synuclein can directly activate microglia early on, as shown in transgenic mice overexpressing wild-type or mutated α-synuclein [80, 81]. Additional studies demonstrated that α-synuclein directly promotes microglia activation [82-85], inducing production and release of pro-inflammatory cytokines [82, 83] and increasing expression of antioxidant enzymes [83]. Moreover, besides microglial activation, intranigral injection of α-synuclein also resulted in the upregulation of mRNA expression of major pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and the expression of endothelial markers of inflammation [85]. The same group that identified HLA-DR^+ reactive microglia in the SNpc of PD patients also reported the presence of CD8 T cells in the brain of patients with PD [86], which was further corroborated by the detection of increased levels of CD4 and CD8 T cells in both the SNpc of PD patients and in the MPTP mouse model of PD [87]. Moreover, higher levels of CXCR4, a chemokine receptor expressed by T cells, and its reciprocal ligand CXCL12, were observed in the post-mortem brains of PD and MPTP mouse model of PD [88]. The accumulation of α-synuclein aggregates has been demonstrated to induce MHCII expression by microglia in *in vivo* models, while knockout of MHCII prevents α-synuclein-induced microglial activation, antigen presentation, and the degeneration of dopaminergic neurons; *in vitro* aggregated α-synuclein drives the activation of antigen processing and presentation to $CD4^+$ T cells, sufficient to initiate cytokine production [82]. Mitochondrial toxins, such as 6-OHDA, MPTP and rotenone, have the ability to trigger an immune reaction in the striatum and SNpc, implying that a primary damage to the ETC represents by itself a trigger for microglial activation and neuroinflammatory processes [89-92]. Rotenone was shown to cause microglial activation in rodent models [93] and in human microglial cell lines [94]. A similar phenotype of microglial activation was observed in the brains of 6-OHDA rats [95-97], as well as $CD3^+$, $CD4^+$ and $CD8^+$ T cells that migrated from blood vessels to the SNpc [98].

Innate and adaptive immune systems cause upregulation of inflammatory cytokines that are crucial to the recruitment, stimulation, and regulation of inflammatory responses. There is considerable evidence that these molecules are secreted in both the brain and cerebrospinal fluid (CSF) of PD subjects, as shown by increased levels of TNF-α in both the caudate and putamen, as well as the CSF of PD patients [99]. Several other studies performed in the CSF of PD patients revealed higher levels of IL-1β [100-103], IL-2 [101,

104], IL-4 [101], IL-6 [100-102, 104], and TNF-α [99, 103, 104]. In addition to the evidence of an activated innate and adaptive immune response in the CNS of PD patients, there is data supporting the occurrence of similar inflammatory responses throughout the body, validating the hypothesis that PD is a multisystemic disorder. The major pathway for immune cell trafficking throughout the body is the bloodstream. Indeed, higher levels of inflammatory cytokines are also found in the blood of PD patients, including IL-1β [105-109], IL-2 [105, 107], IL-6 [105, 107, 110], TNF-α [107, 111-113] and IFN-γ [112-114].

As previously referred, α-synuclein pathology has also been found in the colon, in neurons of the ENS, and in the vagus nerve itself. Chronic constipation, which is linked to peripheral inflammation [58], is one of the most frequent non-motor symptoms of PD. Enteric inflammation occurs in PD and it has been demonstrated by increased levels of inflammation markers in the gastrointestinal tract, such as glial fibrillary acidic protein (GFAP) and pro-inflammatory cytokines including IL-1β, IL-6, TNF-α and IFN-γ, further reinforcing the role of peripheral inflammation in the initiation and/or progression of PD [115]. Interestingly, these higher cytokines levels in the gut occur earlier during disease progression and decrease with time, possibly indicating that gut inflammation could be an early event in the pathogenesis of PD [66]. Observations of both gastrointestinal dysfunction and inflammation in individuals with PD led to the investigation of variations in the gut microbiome of these individuals, due to its importance in overall gut function and its role in shaping, as well as being shaped by the host's immune system [66]. Several studies have reported alterations in the microbiome of PD patients from multiple geographical populations [112, 116-121]. *Roseburia* spp. has been shown to promote anti-inflammatory processes through (1) enhancement of intestinal barrier health via increased expression of tight junction proteins [122], (2) modulation of immune responses promoting downregulation of pro-inflammatory cytokines (e.g., IL-17) and differentiation of Treg cells [123] and (3) stimulation of anti-inflammatory cytokines (e.g., IL-10, TGFβ) [124]. Additionally, *Faecalibacterium* has been shown to reduce inflammation through multiple potential mechanisms including regulation of Th17 and Treg cell differentiation [125], downregulation of pro-inflammatory pathways and cytokine production [126], secretion of anti-inflammatory molecules [127], and promotion of intestinal barrier health [128]. Although certain bacteria, including *Lachnospiraceae* and some of its genera *Roseburia*, *Blautia*, and *Faecalibacterium*, are

associated with an anti-inflammatory environment, they have consistently been found to be reduced in PD stool. With reduced abundance of these species, it is possible that the gut becomes more susceptible to inflammation. Overall, the composition of the gut microbiota is evidently altered in PD, and these findings are consistent with the hypothesis that gut dysbiosis is linked to an inflammatory environment that may contribute to the initiation and/or progression of PD pathology. The data presented suggest a crucial role of the immune system in both the pathogenesis and progression of PD, as multiple observations of abnormal innate and adaptive immune responses in the CNS, blood and gut of PD patients link the immune system to PD risk. Additionally, PD patients display signature inflammation profiles with elevated blood and CSF cytokine levels, infiltration of peripheral immune cells into the CNS, hyperreactive circulating immune cells, and a dysregulated gut microbiome.

1.4. The Interplay Between Mitochondria and Innate Immunity

Upon microbial infection, the innate immune system is triggered through the recognition of PAMPs by PRRs expressed on the cells' surface. Invading microorganisms and infected cells are then removed following an orchestrated pro-inflammatory immune response [58]. Nevertheless, PRRs have the ability to initiate innate immune responses independently of infection – a process known as sterile inflammation –, through recognition of DAMPs, which are endogenous signals released upon injury or stress [129]. Mitochondria has been recognized as an important source of DAMPs; the reason behind it is the fact that mitochondria share a common origin with bacteria, and both display some similarities: circular DNA with CpG motifs, double-membrane structure with abundance of cardiolipin in the inner membrane, secretion of N-formylated proteins and reproduction by binary fission [129]. Upon injury or stress, the mitochondrial release of DAMPs activates the innate immune system, much like bacterial PAMPs, triggering sterile inflammation that mimic the response to infection [58, 129].

Numerous studies have attributed a crucial role for mitochondria in the regulation and activation of the NLRP3 inflammasome [130], an intracellular immune sentinel activated upon changes in cellular homeostasis, which activates pro-inflammatory cytokines (IL-1β and IL-18) to trigger pyroptotic cell death (Fig. 6) [131, 132]. While initial studies showed that mitochondrial dysfunction and ROS production are required for NLRP3

inflammasome activation [133, 134], further evidence has also revealed an active role of mtDNA translocation to the cytosol, where it can directly bind to and activate the NLRP3 inflammasome [134, 135]. Moreover, cardiolipin, a phospholipid located exclusively in mitochondrial inner and bacterial membranes [136, 137], can also act as an activator of NLRP3 inflammasome by directly binding to NLRP3, downstream of mitochondrial dysfunction [138]. Mitochondria, along with mitochondrial DAMPs (mtDNA, ROS and cardiolipin), are crucial for the activation and regulation of NLRP3 inflammasome (Fig. 6), which in turn incorporates mitochondrial dysfunction in a pro-inflammatory signalling response, thus elucidating the association of mitochondrial damage with inflammation [129]. The potential for mitochondrial DAMPs to trigger and/or exacerbate neuroinflammation has only being investigated recently. Neuronal and microglial cell lines exposed to mitochondrial lysates displayed increased markers of inflammation, with mtDNA being identified as the candidate DAMP responsible for the inflammatory changes [139]. Extracellular recombinant TFAM treatment of different models of human microglia, in combination with IFN-γ, was shown to induce the expression of pro-inflammatory cytokines, such as IL-1β, IL-6 and IL-18, thus supporting the hypothesis that TFAM may also act as a pro-inflammatory intercellular signalling molecule recognized by microglia [140]. Moreover, mice injected with isolated mitochondria into the brain also revealed increased markers of inflammation, such as increased TNF-α, NF-κB phosphorylation, GFAP protein and decreased *Trem2* mRNA [141]. Despite the described role for extracellular mitochondrial DAMPs as pro-inflammatory signalling molecules in the brain, the mechanisms by which mitochondria act as a transcellular signalling platform in the CNS remain unknown. Some studies have revealed that neurons and astrocytes can exchange mitochondria as a potential mode of cell-to-cell signalling [142, 143]. However, during neurodegeneration, increased disposal of damaged mitochondria by compromised neurons (due to impaired mitochondrial quality control mechanisms), or its inefficient uptake by the recipient astrocytes (due to the presence of extracellular protein aggregates), might result in extracellular accumulation of mitochondrial DAMPs and, consequently, exacerbation of neuroinflammation [58].

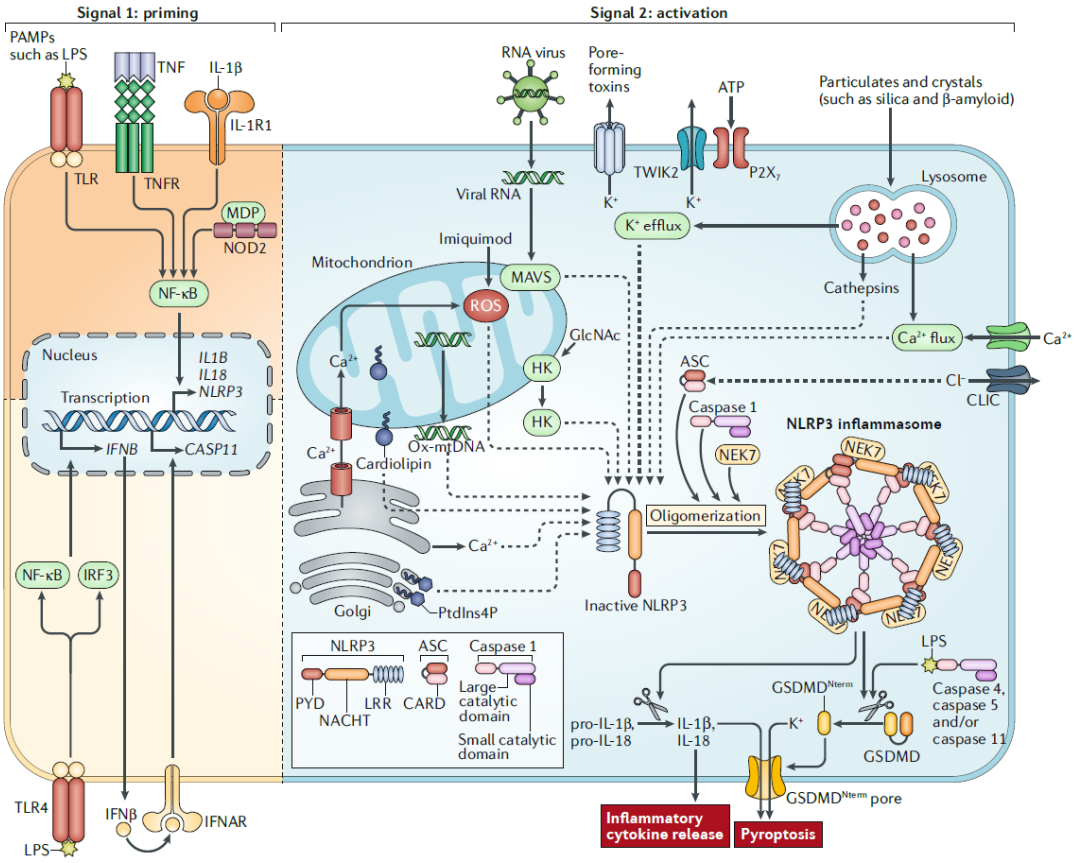

Figure 6. NLRP3 inflammasome priming and activation. Priming is provided by the activation of cytokines or PAMPs, leading to the transcriptional upregulation of NLRP3 inflammasome components. Activation is provided by any of numerous PAMPs or DAMPs, such as mitochondrial reactive oxygen species (mtROS) production, the relocalization of cardiolipin to the outer mitochondrial membrane and the release of oxidized mtDNA (Ox- mtDNA). Formation of the inflammasome activates caspase 1, which in turn cleaves pro- IL-1β into IL-1β (Adapted from Swanson *et al*., 2019).

The cause of the neuronal loss observed in PD brains is still poorly understood. It has been hypothesized that mitochondrial dysfunction, gut bacteria or even their metabolites targeting the mitochondria, could activate NLRP3 inflammasome in dopaminergic neurons, through the exposure of DAMPs, contributing to low-grade inflammation [129]. It was shown in PD cellular and animal models that mitochondrial network is highly fragmented [144]. Mitochondrial fission is required for the selective targeting of dysfunctional mitochondria for degradation by the lysosome [144, 145], however, it was recently proven that mitochondrial fission leads to the exposure of cardiolipin, which serves an important defensive function for the elimination of damaged mitochondria [146]. Since cardiolipin is only found in mitochondrial and bacterial membranes it is considered a mitochondrial-derived DAMP that is detected by the NLRP3 [147]. These results seem to point to a positive feedback loop whereas mitochondrial dysfunction

increases cardiolipin exposure, which in turn activates NLRP3 inflammasome, an innate immune sentinel involved in neuronal innate immunity.

1.5. miRNAs Involvement in Parkinson's Disease

1.5.1. miRNAs Basics

MicroRNAs (miRNAs) are small (~ 20–26 nucleotides), non-coding RNAs generated from genomic DNA [148]. miRNAs exert negative post-transcriptional regulation, including translational repression, mRNA destabilization, and/or mRNA degradation, by binding to target messenger RNAs (mRNAs) [148]. In general, each miRNA has the potential to target many mRNAs due to an imperfect match mode of action, and multiple miRNAs can regulate a single gene/mRNA [148]. Hence, dysregulating even one miRNA may disrupt the diligent balance of many cellular systems or pathways, triggering the development of diseases.

miRNA biogenesis is a closely controlled process and can be divided into various steps: miRNA transcription, processing by Drosha, transportation, processing by Dicer and RNA-induced silencing complex (RISC) loading (Fig. 7) [149]. Two main pathways of miRNA biogenesis were identified as canonical and non-canonical ones. In the canonical biogenesis pathway (Fig. 7), miRNA genes are transcribed by RNA polymerase II as a long primary transcript of approximately 70 nucleotides (pri-miRNA) with a typical hairpin loop structure. pri-miRNA is then processed by the microprocessor complex, comprised of Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8), which cleaves the pri-miRNA at the base of the stem-loop structure to produce the precursor miRNA (pre-miRNA). The pre-miRNA hairpin is exported by exportin 5 (XPO5) from the nucleus to the cytoplasm, where it is cleaved by Dicer near the loop, yielding a small asymmetrical double-stranded RNA. The mature miRNA associates with Argonaute (Ago), that cleaves the double-stranded RNA, leaving a single-stranded miRNA to be loaded into the assembled RNA-induced silencing complex (RISC), which mediates the recognition of the targeted mRNA. In the noncanonical pathway of biogenesis (mirtron), pri-miRNA is processed to pre-miRNA through splicing and debranching by the spliceosome machinery, therefore bypassing the Drosha-DGCR8 step. The resulting pre-miRNA is exported to the cytoplasm by exportin 5 to continue with the canonical pathway. Both pathways ultimately lead to the assembly of a functional RISC complex

loaded with the miRNA (miRISC), that is then directed to the target mRNA to inhibit multiple steps of protein synthesis affecting mRNA target stability and/or translation [149].

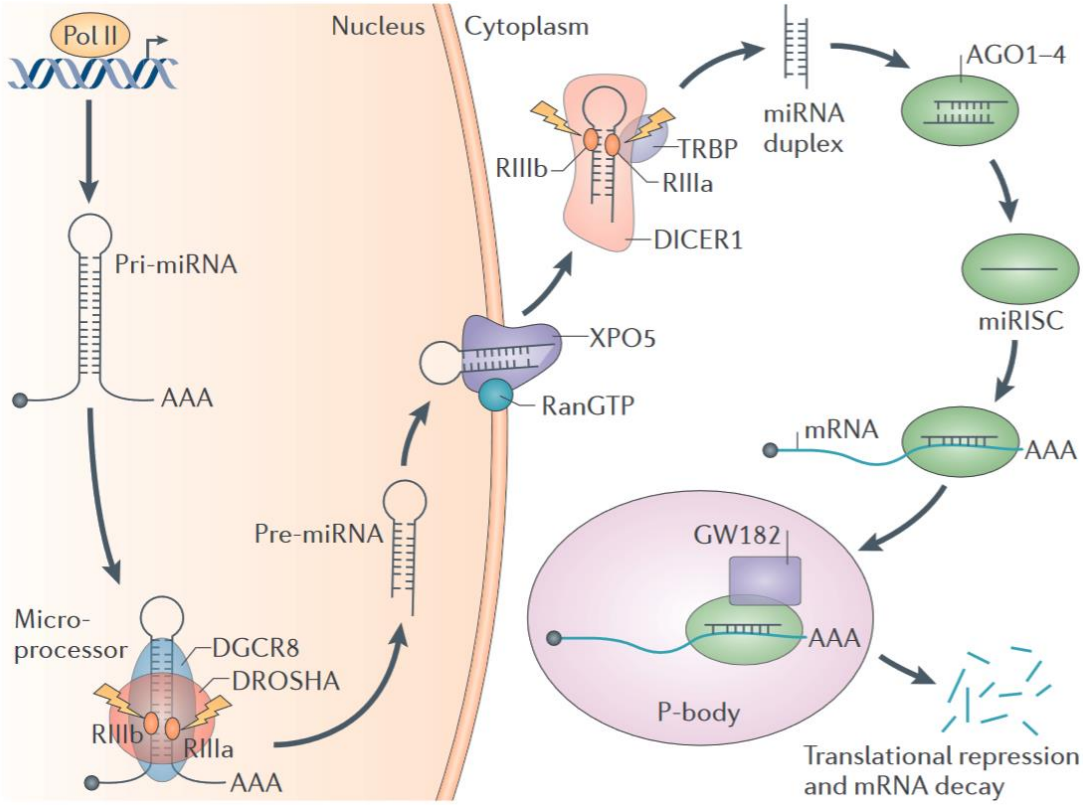

Figure 7. Schematic illustration of miRNA biogenesis. During miRNA biogenesis, an RNA transcript forms stem-loop primary-miRNAs (pri-miRNAs) that are processed into hairpin premature-miRNAs (pre-miRNAs) by Drosha and transported to the cytoplasm by exportin 5. Pre-miRNAs are further cleaved into mature miRNAs by Dicer. Associated with AGO2, the premature miRNAs are loaded into RISC and negatively regulate the expression of mRNAs (Adapted from Lin *et al*., 2015).

Cells, either within the same tissue or in different tissues/organs, can communicate in long distance by sending information from one cell to another to coordinate their behaviours in order to grow, develop and survive [148]. Recent studies proposed that miRNAs contribute to cell-to-cell communication, by being secreted and transported to other cells via circulation to affect recipient cells [148]. This hypothesis has been validated by the detection of extracellular/circulating miRNAs in a multitude of biological fluids, such as blood, CSF, saliva, breast milk, urine, and others [150]. miRNAs can be found circulating in vesicles (exosomes, microvesicles, apoptotic bodies) or associated with proteins (AGO2), and, contrary to cellular RNA, extracellular miRNAs present high stability, providing a desirable characteristic for long distance cellular communication [151]. It is now accepted that extracellular/circulating miRNAs can not

only serve as biomarkers or therapeutic approaches for a variety of diseases, but also play important roles in intercellular communication.

1.5.2. Human and Animal Studies

In the past few years, miRNA dysregulation has been implicated in several neurodegenerative diseases, including PD, where it contributes to neurodegeneration and disease progression [151]. The prominent role of miRNAs on the integrity of the CNS has been exemplified by experiments inducing a selective depletion of Dicer in midbrain dopaminergic neurons in mice, which impairs miRNA biogenesis and results in neurodegeneration and locomotor symptoms mimicking PD [152]. Furthermore, a multitude of screening studies have reported differentially expressed miRNAs on the brain [153-158], CSF [159-162] and blood [163-172] of PD individuals. In PD, some miRNAs have been associated with neuroinflammation and dopaminergic neurons demise, thereby worsening disease pathogenesis. It has been observed that overexpression of miR-494 significantly decreased the levels of DJ-1 both *in vitro* in 3T3-L1 and Neuro-2a cell lines, rendering cells more susceptible to oxidative stress, and *in vivo*, in a MPTP mouse model, exacerbating MPTP-induced neurodegeneration [173]. A report from 2018 showed that miR-494-3p negatively regulates sirtuin 3 (SIRT3) expression in both MPP^+-treated SH-SY5Y cells and in MPTP-induced PD mouse model, worsening motor impairment of these mice [174]. On the other hand, miR-7 was shown to exert a protective role by: (1) repressing expression of α-synuclein, (2) accelerating the clearance of α-synuclein and its aggregates through autophagy in differentiated ReNcell VM cells and (3) facilitating the degradation of pre-formed fibrils of α-synuclein transported from outside the cells [175]. Moreover, Zeng and colleagues highlighted the protective role of miR-135b since it inhibits pyroptosis by targeting FoxO1 in MPP^+-treated SH-SY5Y and PC-12 cells [176]. Additionally, a recent study found that miR-421 targets Pink1. miR-421 expression was upregulated in mice treated with MPTP, as well as in SH-SY5Y cells treated with MPP^+, and inhibition of miR-421 alleviated neurodegeneration in MPTP-treated mice and promoted mitophagy in MPP^+-treated SH-SY5Y cells, which revealed that miR-421 regulates mitophagy through the Pink1/Parkin pathway [177]. Another study revealed that miR-486-3p targets sirtuin 2 (SIRT2) and reduces its expression levels. However, the authors reported a risk-conferring polymorphism in the *SIRT2* gene and showed that a single nucleotide polymorphism

(SNP) in this gene alters the binding efficiency of miR-486-3p to *SIRT2*, thereby increasing the expression level of SIRT2, which could increase α-synuclein aggregation and toxicity and ultimately contribute to the progression and risk of PD [178].

Given the important involvement of gut dysbiosis and inflammation in PD, and the potential of miRNAs to serve as diagnostic biomarkers, Kurz and colleagues [179] investigated the expression of miRNAs in routine colonic biopsies from PD patients and detected several differentially expressed miRNAs; amongst all, miR-486–5p upregulation showed the highest specificity for PD and correlated with age and disease severity in PD. In a follow-up analysis, 301 target genes of miR-486–5p were identified, as well as the biological processes affected by the mentioned miRNA, with brain development and post-synapse organization processes having the strongest functional association with miR-486-5p target gene network [179].

1.5.3. Gut Microbiota and microRNAs

The gut microbiota comprises tens of trillions of microorganisms, including bacteria, viruses, fungi, and protozoans. It contributes to both the maintenance and breakdown of gut homeostasis through interactions with the intestinal epithelial barrier (IEB) and immune system [180]. Enteric bacteria directly interact with intestinal epithelial cells, contributing directly to preserve the integrity of the IEB by regulating epithelial cell growth and differentiation, tight junction protein expression and mucosal permeability [180]. Variations in gut microbiota composition could lead to the impairment of IEB and alterations of mucosal permeability, with consequent infiltration of enteric bacteria and their products into the lamina propria, triggering immune/inflammatory responses (Fig. 8) [180]. Specific bacterial products (such as short chain fatty acids (SCFAs), vitamins or neurotransmitters) can translocate into the blood stream and spread upwards to the brain, where they can influence the regulation of CNS immune/inflammatory cell activities, including the microglia [180]. In addition, bacterial products can directly activate circulating immune cells, that then migrate to the CNS and modulate the brain physiology [180]. Particularly, the immune-bacterial interplay represents one of the main networks involved in gut-brain communication. As previously stated, it has been shown that gut microbiota is significantly altered in PD patients compared with healthy controls, becoming evident that it might play an important role in the pathogenesis of PD.

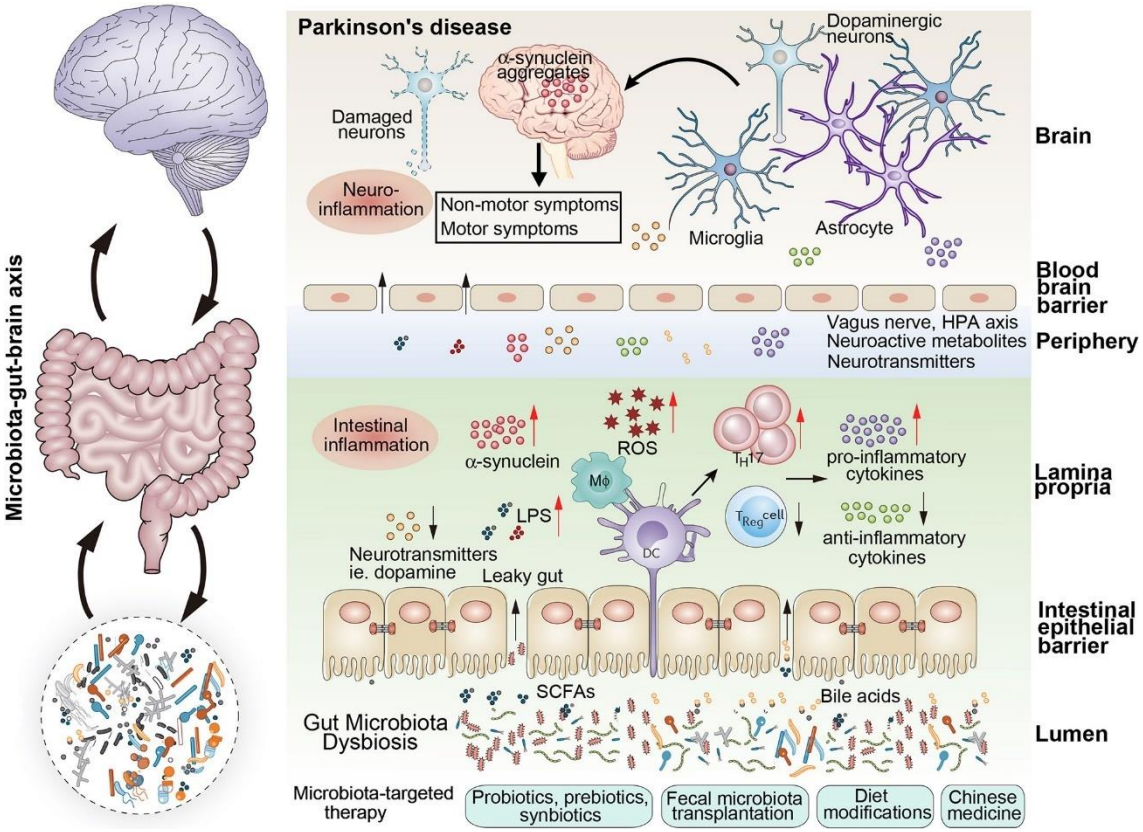

Figure 8. Schematic representation of the gut microbiota dysbiosis associated with Parkinson's Disease. Microbiota dysbiosis plays an important role in the occurrence and development of PD, and it has been associated with increased intestinal permeability, aggravated neuroinflammation, abnormal aggregation of α-synuclein, oxidative stress, and decreased neurotransmitter production, all of which have vital roles in the pathogenesis of PD (Adapted from Zhu *et al*., 2022).

In addition, recent evidence indicates that miRNAs are involved in PD pathophysiology, hinting at novel disease-related mechanisms that are now beginning to be explored, and positing miRNAs as potential biomarkers for PD screening. Interestingly, miRNAs were found in human faecal samples. Furthermore, it was recently demonstrated that secreted miRNAs are able to enter bacteria and regulate bacterial gene transcripts and affect their growth [181], giving rise to the possibility of the host's miRNAs to affect and shape their own gut microbiome in PD (Fig. 9). This hypothesis was proposed after *Fusobacterium nucleatum* cultured with human miR-515-5p (a miRNA present in human faeces) showed an increased ratio of 16S rRNA/23S rRNA transcripts and altered growth [181]. In order to better understand the potential interactions between miRNAs and the gut metagenome, Hewel and co-workers [182] performed an *in silico* target screen for binding sites of PD-associated miRNAs on human gut metagenome sequences, from which resulted a massive number of interactions. They found numerous miRNAs that may be key regulators in bacterial pathways relevant to PD, such as the bacterial secretion system,

lipopolysaccharide (LPS) biosynthesis and biofilm formation [182]. On the other hand, gut microbiota may affect the host by producing miRNAs and modulating human gene expression (Fig. 9), primarily through gut microbiota metabolites. In fact, Peck *et al*. have confirmed that the microbiota regulates miRNA expression in intestinal epithelium cell subtypes, and this regulation may alter intestinal homeostasis [183].

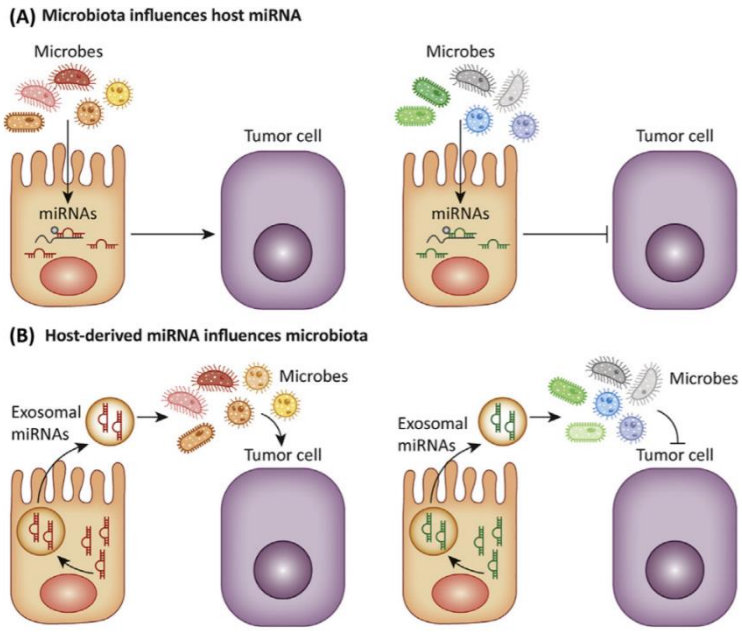

Figure 9. Reciprocal regulation of miRNAs and gut microbiota. (A) Microbiota can influence host miRNA expression. (B) Host cells release miRNAs via extracellular vesicles to control intestinal homeostasis and gut microbiota (Adapted from Dong *et al*., 2019).

Based on these findings, miRNAs could potentially be diagnostic biomarkers and a therapeutic option, used to revert the microbiome of PD patients to a healthy state. This could even be applied years before the disease breaks out, to keep the pathological changes beyond a threshold level and prevent the outbreak of the disease altogether.

1.6. Objectives

As previously mentioned, PD, which has been traditionally characterized by motor impairment, is now considered a multisystemic disorder displaying a plethora of non-motor symptoms, such as gastrointestinal complaints, including constipation, that appear years before PD clinical diagnosis. Interestingly, many studies have shown a direct correlation between gut microbiota dysbiosis and disease progression in PD patients. In fact, several groups showed that PD patients have a different composition of faecal and mucosa-associated gut microbiota compared to healthy subjects. Remarkably, recent

evidence indicates a crosstalk between gut microbiota and miRNAs hinting its involvement in PD pathophysiology. Most importantly, miRNA-486-5p was found to be upregulated in colonic tissue from PD patients comparatively to healthy aged-matched individuals [179].

With the present study we, particularly, aim to disclose whether miR-486-5p can modulate mitochondrial dysfunction and inflammatory responses in both Caco-2 and SH-SY5Y cell lines and understand how variations induced by the selected miRNA in these pathways can relate to PD pathogenesis. Our results provide new insights for the future development of efficient diagnostic biomarkers and therapies for prodromal PD, allowing an early diagnosis and a better quality of life for PD patients.

CHAPTER II
MATERIALS AND METHODS

2.1. Chemicals, Antibodies and Kits

Table 1. List of chemicals.

Chemicals	Manufacturer
10-N-Nonyl Acridine Orange (NAO)	Enzo Lifesciences Cat. No. 08091739
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma Chemical Co Cat. No. M2128
Alamar Blue	Sigma Chemical Co R7017
Amplex Red	Molecular Probes Cat. No. A22188
Caspase-1 Substrate	Sigma Chemical Co Cat. No. SCP0066
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Sigma Chemical Co Cat. No. C2759
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)	Sigma Chemical Co Cat. No. C2920
Hoechst	Invitrogen (Cat. No. H1399)
Lipofectamine RNAiMAX	Invitrogen Cat. No. 13778075
Lipopolysaccharides (LPS)	Sigma Chemical Co Cat. No. L2654
miRNA mimic	Invitrogen Cat. No. 4464066
Negative Control	Invitrogen Cat. No. 4464058
Oligomycin	Alfa Aesar Cat. No. J60211
Tetramethylrhodamine, Methyl Ester dye (TMRM)	Molecular Probes Cat. No. T668

Table 2. List of antibodies.

Antibodies	Manufacturer
Mouse anti-IL23R	abcam (Cat. No. ab228426)
Mouse anti-phospho-STAT3	Santa Cruz Biotechnology (Cat. No. sc-8059)
Mouse anti-STAT3	Santa Cruz Biotechnology (Cat. No. sc-293151)
Mouse anti-TLR4	Santa Cruz Biotechnology (Cat. No. sc-293072)
Mouse anti-ASC	Santa Cruz Biotechnology (Cat. No. sc-271054)
Rabbit anti-NLRP3	Cell Signaling Technology (Cat. No. 13158)
Rabbit anti-ZO-1	abcam (Cat. No. ab96587)
Rabbit anti-Occludin	Invitrogen (Cat. No. 40-4700)
Mouse anti-α-Tubulin	Sigma Chemical Co (Cat. No. T6199)
Mouse anti-vinculin	Millipore (Cat. No. MAB3574)
Goat anti-rabbit Alexa Fluor 488	Molecular Probes (Cat. No. A11008)

Table 3. List of ELISA kits.

Kits	Manufacturer
Mouse IL-1β Quantikine ELISA	R&D Systems (Cat. No. MLB00C)
Mouse IL-6 Quantikine ELISA	R&D Systems (Cat. No.M6000D)
Mouse IL-17 Quantikine ELISA	R&D Systems (Cat. No. DY317-05)
NFκB p65 Total SimpleStep ELISA Kit	Abcam (Cat. No. ab176648)

2.2. Cell Lines Culture and Treatments

The human colorectal adenocarcinoma Caco-2 cell line was kept in culture under a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Chemical Co.) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% sodium pyruvate (Gibco) and 1% penicillin/streptomycin solution (Gibco). Cells were seeded at a density of $1.7x10^5$ cells/mL. Cells were grown on coverslips in 12-well plates for immunocytochemistry analysis; in 6-well plates for western blot analysis and inflammatory markers assessment and in 24-well plates for qRT-PCR, MTT and Alamar Blue cell viability assays, analysis of Mitochondrial Membrane Potential and Reactive Oxygen Species. SH-SY5Y human neuroblastoma cells were kept in culture under a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin solution (Gibco). Cells were seeded at a density of $1x10^5$ cells/mL. Cells were seeded in 6-well plates for western blot analysis, inflammatory markers and Caspase-1 assessment; in 24-well plates for qRT-PCR, MTT and Alamar Blue cell viability assays, analysis of Mitochondrial Membrane Potential and Reactive Oxygen Species and in μ-slide 8-well plates from ibidi for determination of Cardiolipin fluorescence intensity. Caco-2 cells were incubated for 24 h with carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), while SH-SY5Y cells were incubated for 2 h with carbonyl cyanide 3-chlorophenylhydrazone (CCCP), with a final concentration of 2.5 μM and 5 μM, respectively. Both Caco-2 and SH-SY5Y cell lines were incubated for 24 h with 1μg/mL LPS. FCCP, CCCP and LPS were added to the culture medium at the mentioned final concentrations and used as positive controls of the experiments.

2.3. Transfection

SH-SY5Y and Caco-2 cells were cultured to reach 80% confluence prior to transfection. Cells were transfected with miR-486-5p mimic or negative control (Invitrogen) using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. As the negative control, we used a random sequence miRNA molecule that had been tested in human cell lines and tissues and validated to not produce identifiable effects on known miRNA function. Briefly, Lipofectamine RNAiMAX combined with miR-486-5p mimic or negative control at a concentration of 10nM were incubated in Opti-MEM serum-free medium for 5 min prior to transfection. The mixture was then added into the cells for 4 h in serum-free medium. After that, the serum-free medium was replaced by fresh culture medium for 20 h. Then the cells were harvested for the following experiments.

2.4. cDNA Synthesis and qRT-PCR

Total RNA, including miRNAs, was extracted from cells using miRNeasy Tissue/Cells Advanced Micro Kit from Qiagen (Cat. No. 217684) according to the manufacturer's protocol. RNA concentration was determined using a NanoDrop 2000c/2000 UV-Vis Spectrophotomer. For quantitative PCR purposes, previously isolated RNA, including miRNAs, was converted into cDNA using miRCURY® LNA® RT Kit (Qiagen), following the manufacturer's instructions. cDNA templates were diluted in RNAse-free water and PCR was performed by adding diluted cDNA to master mix containing SYBR® Green, primer (miRCURY® LNA® miRNA PCR Assays), ROX Reference Dye and RFW. All reactions were performed in duplicate (two cDNA reactions per RNA sample). Samples were analysed on StepOnePlus™ thermocycler (Applied Biosystems™), and cycling conditions were used following the manufacturer's instructions. Negative controls such as NRT (no reverse transcriptase control) and NT (no template control) were used, in order to monitor eventual sample and/or reagent contaminations. The amplification rate for each target was evaluated from the cycle threshold (Ct) numbers obtained with cDNA dilutions. The qPCR analysis for hsa-miR-486-5p LNA (YP00204001, Qiagen) was normalized with endogenous control U6 snRNA (v2) LNA (YP02119464, Qiagen) and differences between control and experimental samples were calculated using the 2-ΔΔCt method.

2.5. Prediction of Putative miRNA targets

Putative miRNA targets were predicted using 2 different databases: Target Scan (http://www.targetscan.org/vert_61/) [184] and miRDB (http://mirdb.org/miRDB/) [185].

2.6. MTT Cell Viability Assay

Cellular viability was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In viable cells, NAD(P)H-dependent oxidoreductase enzymes reduce the MTT reagent to formazan, an insoluble crystalline product with a purple colour that absorbs light at 570 nm. Following cellular treatment with different concentrations of LPS or FCCP for 24h, or CCCP for 2h, 0.5 mL of MTT (0.5 mg/ml dissolved in sodium medium) was added to each well. Cells were then incubated at 37 °C for 2 to 3 h. At the end of the incubation period, the formazan precipitates were solubilized with 0.5 ml of acidic isopropanol (0.04M HCl/Isopropanol). The absorbance was measured at 570 nm using a Spectramax Plus 384 spectrophotometer.

2.7. Alamar Blue

The Alamar Blue assay uses the reducing power of living cells to quantitatively measure cell viability *in vitro*. Resazurin, the active ingredient of Alamar Blue reagent, is a non-toxic, cell-permeable compound that is blue in colour and virtually non-fluorescent. Upon entering living cells, resazurin is reduced to resorufin, a compound that is red in colour and highly fluorescent. The continued growth of viable cells maintains a reducing environment (fluorescent, red) and inhibition of growth maintains an oxidized environment (non-fluorescent, blue). Changes in viability can be easily detected by reading absorbance at 570 and 600 nm. After treatments, cells were washed with PBS (1×) and incubated with Alamar Blue (0.1 mg of resazurin/1 mL of PBS (1×)) 10% (v/v) in culture medium for 2 h at 37 ºC. After the incubation period, absorbance was read at 570 nm and 600 nm using a Spectramax Plus 384 spectrophotometer. Cell viability was determined as follows:

$$\text{Cell Viability (\% control)} = \frac{(A_{570} - A_{600})\ \text{treated cells} \times 100}{(A_{570} - A_{600})\ \text{control cells}}$$

2.8. Analysis of Mitochondrial Membrane Potential (Δψm) with TMRM Probe

To monitor changes in mitochondrial membrane potential tetramethylrhodamine methyl ester dye (TMRM) (Molecular Probes) was used [62]. TMRM is a cell permeable fluorescent indicator that accumulates in the highly negatively charged interior of mitochondria. The accumulation in functional mitochondria takes place as a consequence of TMRM positive charge; thereby, a decrease in TMRM cellular retention is associated with a decrease in Δψm. After treatments cells were washed with PBS (1×) and subsequently loaded in the dark with 300 nM TMRM in HBSS (5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $NaH_2PO_4.2H_2O$, 5 mM Glucose, 5.36 mM Sodium Pyruvate, 5.36 mM HEPES, pH 7.4) and 1.8 mM $CaCl_2$. Basal fluorescence was recorded for 5 min at 37 °C (λex = 540 nm and λem = 590 nm). Afterward, 1 μM FCCP (protonophore) and 2 μg/mL oligomycin (an inhibitor of H^+ transporting ATP synthase and an inhibitor of Na^+/K^+ transporting ATPase) were added to each well in order to achieve maximal mitochondrial depolarization and to prevent ATP synthase reversal, respectively. FCCP and oligomycin were purchased from Sigma Chemical Co. Measurements were recorded for another 3 min at 37 °C. TMRM retention ability was calculated by the difference between the total fluorescence (after depolarization) and the initial value of fluorescence (basal fluorescence). Results are expressed as a percentage of the dye retained within the untreated cells. Measurements were performed using a SpectraMax Gemini EM fluorimeter.

2.9. Analysis of ROS with Amplex Red Probe

ROS levels were measured by using the Amplex Red reagent, a highly sensitive and stable probe for H_2O_2, and that acts as a fluorogenic substrate for peroxidase. In the presence of horseradish peroxidase (HRP), Amplex Red probe reacts in a 1:1 stoichiometry with H_2O_2 to produce highly fluorescent resorufin. After treatments, cells were washed with PBS (1×) at 37 °C, and then incubated with 10 μM Amplex Red probe in HBSS medium and 0.5 U/mL HRP. The plate was read on a SpectraMax Gemini EM fluorimeter, with settings on kinetic mode during 30 min and using excitation and emission wavelengths corresponding to 550 and 580 nm, respectively, with auto cut-off and high sensitivity.

2.10. Preparation of Whole Cellular Extracts

For western blot analysis, for the analysis of immunity markers with Elisa kits and for Caspase-1 determination whole cellular extracts were prepared as followed. Cell lines were washed in ice-cold PBS (1×) and lysed in a hypotonic lysis buffer (25 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA, pH 7.5) supplemented with 10% Triton X-100, 2 mM DTT, 0.1 mM PMSF, protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail from Roche) and phosphatase inhibitors (commercial phosphatase inhibitor cocktail, PhosSTOP™, Roche). Cell suspensions were frozen three times in liquid nitrogen and centrifuged at 20,000×g for 10 min at 4 ºC. The resulting supernatants were removed and stored at −80 °C. Protein content was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions.

2.11. Western Blot Analysis

Samples were suspended in 6× sample buffer (4× Tris.HCl/SDS, pH 6.8, 30% glycerol, 10% SDS, 0.6M DTT, 0.012% bromophenol blue) and boiled for 5 min at 95 °C. Depending on the protein molecular weight of interest, samples containing 25 μg of protein were loaded onto 7% or 15% SDS-PAGE gels. After transfer to PVDF membranes (Millipore), nonspecific binding was blocked by gently agitating the membranes in Tris-Buffered Solution (TBS) (20mM Tris, 150mM NaCl, pH 7.6) containing 0.1% Tween (TBS-T) and 3% BSA for 1 h at room temperature. The blots were subsequently incubated with the respective primary antibodies overnight at 4 °C with gentle agitation: 1:500 anti-IL23R from abcam (Cat. No. ab228426); 1:200 anti-phospho-STAT3 from Santa Cruz Biotechnology (Cat. No. sc-8059); 1:200 anti-STAT3 from Santa Cruz Biotechnology (Cat. No. sc-293151); 1:200 anti-TLR4 from Santa Cruz Biotechnology (Cat. No. sc-293072); 1:200 anti-ASC from Santa Cruz Biotechnology (Cat. No. sc-271054); 1:1000 anti-NLRP3 from Cell Signaling Technology (Cat. No. 13158); 1:500 anti-ZO-1 from abcam (Cat. No. ab96587). Finally, 1:10000 anti-α-Tubulin from Sigma Chemical Co (Cat. No. T6199) and anti-vinculin from Millipore (Cat. No. MAB3574) were used for loading control. Membranes were washed in TBS-T three times (each time for 5 min) and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at RT with gentle agitation. After three washes, specific bands of interest were detected by developing with an alkaline phosphatase enhanced chemical

fluorescence reagent (ECF from Sigma Chemical Co. Cat. No. GERP N3685). Fluorescence signals were detected using a Bio-Rad Versa-Doc Imager. Analysis of Western blot band densities were determined using Quantity One Software (Bio-Rad). Regions of interest were drawn around bands of interest and the background was automatically subtracted. Relative densities were calculated and normalized to housekeeping proteins (α-Tubulin, vinculin).

2.12. Immunocytochemistry and Confocal Microscopy Analysis

Cells were grown on glass coverslips (16 mm diameter) in 12-well plates. Following treatment, cells were washed twice with PBS (1×) and fixed with 4% paraformaldehyde for 20 min at RT. The fixed cells were washed again with PBS (1×), permeabilized with 0.25% Triton X-100 for 10 min and incubated with 5% BSA, to prevent non-specific binding, for 1 h at RT. Then, cells were incubated with the primary antibody 1:250 Occludin for 3 h at RT. Cells were then incubated with the appropriate secondary antibody (1:250 Alexa Fluor 488 from Molecular Probes). Subsequently, cells were incubated with Hoescth (15 μg/μL) for 5 min at RT and protected from light. After a final wash, the coverslips were immobilized on a glass slide with mounting medium Dako Cytomation (Dako). Negative controls omitting the primary antibody were performed in each case, and no staining was seen. Images were acquired using a Plan-Apochromat/1.4NA 63× lens on a confocal microscope LSM710 (Zeiss Microscopy). The images of cells stained with Occludin were extracted to grayscale, inverted to show Occludin-specific fluorescence as black pixels and thresholded to optimally resolve Occludin staining. Background fluorescence and specific Occludin fluorescence were determined. The final value for fluorescence intensity resulted from the subtraction of background fluorescence from specific fluorescence and the result was further divided by the number of cells from each acquired image. At least 20 cells were examined for each condition.

2.13. Determination of Cardiolipin Fluorescence Intensity

10-N-Nonyl acridine orange (NAO) binds to negatively charged phospholipids cardiolipin, phosphatidylinositol, and phosphatidylserine, but with higher affinity to cardiolipin, and is largely independent of mitochondrial membrane potential. Cardiolipin distribution and fluorescence was measured using the NAO probe. After treatments, cells

were washed with HBSS (5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $NaH_2PO_4.H_2O$, 5 mM Glucose, 5.36 mM Sodium Pyruvate, 5.36 mM HEPES, pH 7.2) and then loaded in the dark with 100 nM Cardiolipin in HBSS for 1 h. After a gentle wash, cells were kept in HBSS during image acquisition. Images were obtained using a Plan-Apochromat/1.4NA 63× lens on a Spinning Disk Cell Observer microscope (Zeiss Microscopy). The images of cells stained with NAO were extracted to grayscale, inverted to show NAO-specific fluorescence as black pixels and thresholded to optimally resolve NAO staining. Background fluorescence and specific NAO fluorescence were determined. The final value for fluorescence intensity resulted from the subtraction of background fluorescence from specific fluorescence and the result was further divided by the number of cells from each acquired image.

2.14. Inflammatory Markers Assessment by ELISA

Inflammation markers were evaluated in 25 μg cell lysates by using the NF-κB p65, IL-1β, IL-17 and IL-6 ELISA kits. Absorbance was registered at 450 nm in a SpectraMax Plus 384 spectrophotometer.

2.15. Caspase 1-like Activity Assay

Caspase 1 activation was measured using a colorimetric substrate in which the substrate cleavage was monitored at 405 nm. Cell lysates containing 40 μg protein were incubated at 37 °C for 2 h in 25mM HEPES, pH 7.5 containing 0.1% CHAPS, 10% sucrose, 2mM DTT, and 40 μM of Ac-VAD-4-methoxy-2-naphtylamide (Sigma Chemical Co.) to determine caspase 1 activation. Detection was evaluated at 405 nm using a SpectraMax Plus 384 spectrophotometer.

2.16. Statistical Analysis

All data are expressed as the means ± SEM. Statistical significance was analysed by unpaired Student's *t*-test or one-way ANOVA followed by Tukey or Bonferroni post-hoc test. A p value of less than 0.05 was considered statistically significant.

CHAPTER III
RESULTS

3.1. Cell viability assays and miR-486-5p expression levels

LPS, an outer membrane component of gram-negative bacteria, is commonly used to induce inflammatory responses *in vitro*. FCCP and CCCP are mitochondrial oxidative phosphorylation uncouplers that disrupt the mitochondrial proton gradient and cause a severe loss of mitochondrial membrane potential. Therefore, LPS, FCCP and CCCP were used as positive controls on different experiments.

Cell viability assays were performed in order to determine which concentration of these compounds represented a minor damage to both Caco-2 and SH-SY5Y cell lines. Through MTT assays, we observed that 1 μg/mL LPS and 2.5 μM FCCP for Caco-2 cells and 1 μg/mL LPS and 5 μM CCCP for SH-SY5Y cells were the highest non-toxic concentrations (Fig. 10 A). Caco-2 cells treated with 2.5 μM FCCP presented significant reduction in cell viability when compared with untreated cells. After transfecting both cell lines with miR-486-5p, we conducted Alamar Blue assays to determine cell viability (Fig. 10 B) followed by qRT-PCR analysis to assess miR-486-5p expression levels (Fig. 10 C). No significant alterations were identified in cell viability of transfected cells and a significant increase of miR-486-5p expression levels was detected in both cell lines transfected with the mentioned miRNA.

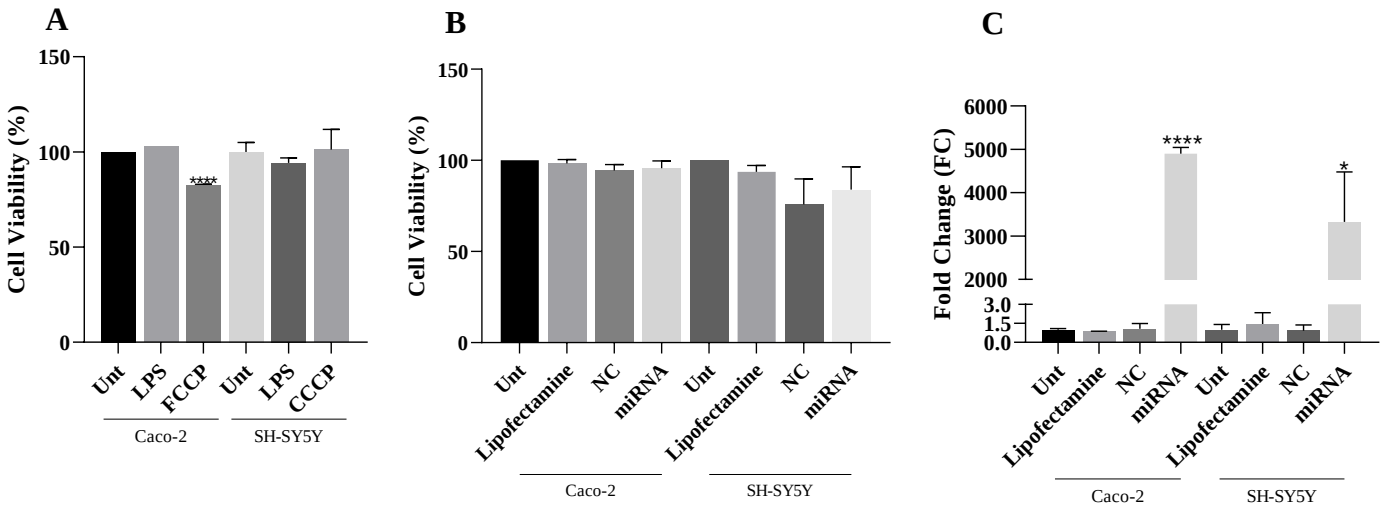

Figure 10. Cell viability and expression levels of miR-486-5p in both Caco-2 and SH-SY5Y cell lines. Both cell lines were exposed to 1 μg/mL LPS for 24 h. Caco-2 cells were incubated with 2.5 μM FCCP for 24 h, while SH-SY5Y cells were incubated with 5 μM CCCP for 2 h. Cell viability was determined through MTT cell viability assay (n=2-5) **(A)** and Alamar Blue (n=3-5) **(B)**, as described in Materials and Methods. **(C)** Expression levels of miR-486-5p were measured after transfection by qRT-PCR analysis (n=3). Data represent the mean ± SEM and are expressed in comparison with untreated cells. * $p < 0.05$ and **** < 0.0001, significantly different relative to untreated cells. Statistical differences were analysed using one-way ANOVA followed by Tukey's test (Fig. A-B) or Bonferroni's test (Fig. C). NC: negative control.

3.2. Caco-2

3.2.1. miR-486-5p failed to induce mitochondrial defects in Caco-2 cells

As previously mentioned, several lines of evidence link PD to mitochondrial dysfunction. In fact, mitochondrial dysfunction in PD tissues and models is characterized by a decrease in the mitochondrial membrane potential (Δψmit) [36] and increased levels of ROS. We evaluated mitochondrial function of transfected Caco-2 cells by measuring Δψmit using the TMRM probe (Fig. 11 A) and determined ROS levels with Amplex Red probe (Fig. 11 B). However, no significant alterations were detected, except for the positive control FCCP.

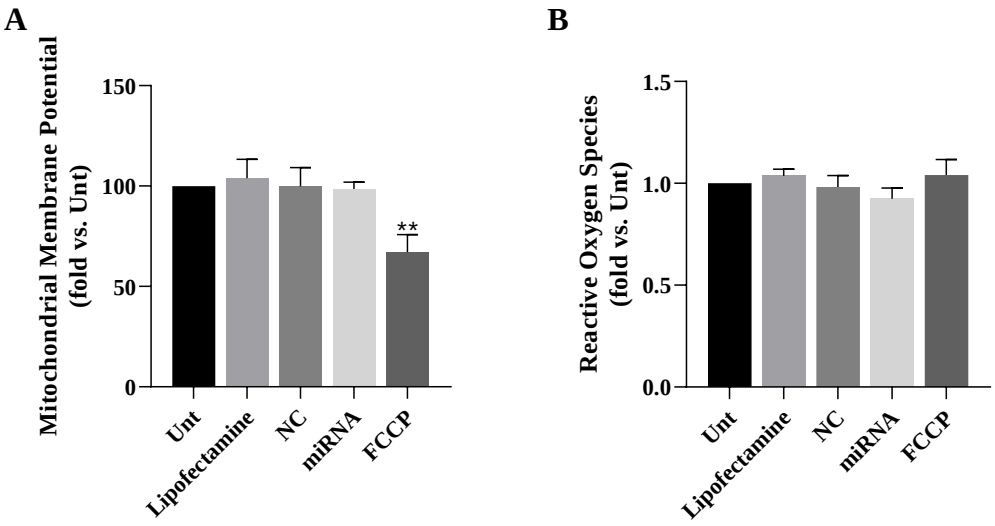

Figure 11. Mitochondrial Membrane Potential (Δψmit) and Reactive Oxygen Species in Caco-2 cells transfected with miR-486-5p. **(A)** Effect of miR-486-5p overexpression on Δψmit of Caco-2 cells. Mitochondrial membrane potential was estimated using TMRM probe (n=4). **(B)** Effect of miR-486-5p overexpression on ROS levels of Caco-2 cells. ROS levels were measured using Amplex Red probe (n=4). Data represent the mean ± SEM and are expressed in comparison with untreated cells. ** $p < 0.01$, significantly different from the untreated cells. Statistical differences were analysed using one-way ANOVA followed by Tukey's test.

3.2.2. miR-486-5p increases inflammatory markers in Caco-2 cells

Evidence has suggested that mitochondria is involved in signalling pathways responsible for the activation of innate immune responses. This activation converges in the formation of NLRP3 inflammasome that promotes the processing of precursor cytokines, such as pro-IL-1β, through Caspase 1 activation (Fig. 6). Although no alterations were found in mitochondrial function after transfection with miR-486-5p in Caco-2 cells we evaluated NLRP3 inflammasome activation. To do so, we evaluated the levels of TLR4, NLRP3 and ASC (Fig. 12 A, B, C, D) along with pro-inflammatory cytokines (IL-1β and IL-6) (Fig. 12 E, F). Western Blot analysis of TLR4, NLRP3 and its adaptor ASC showed no

significant increase of these proteins in transfected Caco-2 cells. Nevertheless, the significantly increased levels of IL-1β and the tendential higher levels of IL-6 in transfected Caco-2 cells suggest the activation of inflammatory responses. In addition, we observed that LPS, as expected, induces an increase in IL-1β and IL-6 levels. Importantly, no alterations were found after transfection with the negative control, indicating the specificity of miR-486-5p transfection.

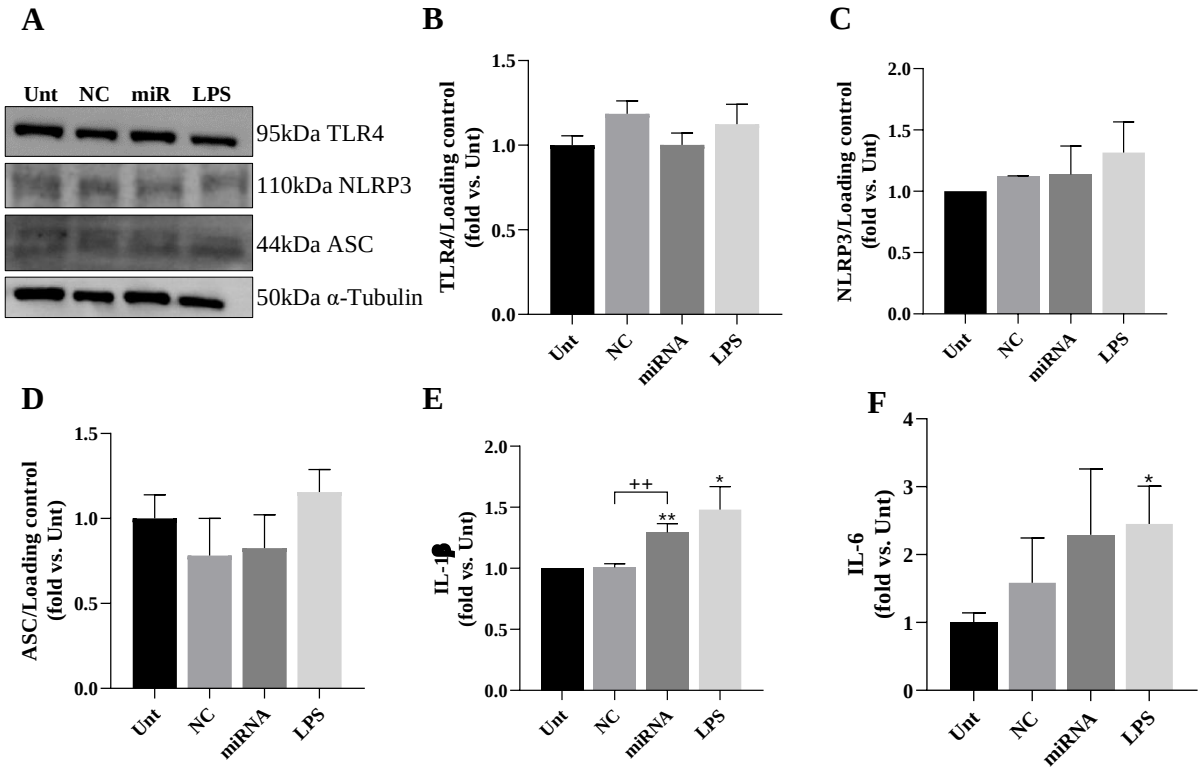

Figure 12. TLR4, NLRP3, ASC, IL-1β and IL-6 levels after transfection of Caco-2 cells with miR-486-5p. **(A)** Western Blot analysis of TLR4, NLRP3 and ASC levels. **(B)** Densitometric analysis of TLR4 corrected with α-tubulin (n=7). **(C)** Densitometric analysis of NLRP3 corrected with α-tubulin (n=2). **(D)** Densitometric analysis of ASC corrected with α-tubulin (n=6). The blots were re-probed for α-tubulin or vinculin to confirm equal protein loading. **(E)** IL-1β was measured using an ELISA kit (n=4). **(F)** IL-6 was measured using an ELISA kit (n=4). Data represent the mean ± SEM and are expressed in comparison with untreated cells. * $p < 0.05$ and ** $p < 0.01$, significantly different from the untreated cells. ++ $p < 0.01$, significantly different from the NC. Statistical differences were analysed using unpaired Student's t-test (Fig. F) or using one-way ANOVA followed by Tukey's test (Fig. E).

3.2.3. miR-486-5p modulates the intestinal barrier integrity in Caco-2 cells

Using Target Scan and miRDB, we found a putative miR-486-5p target that has been highly associated with inflammatory diseases: IL-23 receptor (IL23R). IL-23 is a pro-inflammatory cytokine that has been implicated in the development of chronic inflammatory diseases, such as psoriasis, inflammatory bowel diseases, multiple sclerosis, and rheumatoid arthritis in humans [186]. IL-23 is produced by dendritic cells and activated macrophages, and stimulates the production of inflammatory mediators,

such as IL-17, by binding to IL-23R (Fig. 13) present on the surface of target populations, mainly Th17 and IL-17-secreting TCRγδ cells (Tγδ17) [186]. Dysregulated IL-17 production can result in excessive pro-inflammatory cytokine expression and chronic inflammation, which lead to tissue damage and contribute to the pathogenesis of inflammatory diseases. On the other hand, IL-17 can also mediate protective innate immunity [187]. Indeed, IL-17 released from homeostatic Th17 gut cells can signal the production of tight junction proteins having a key role in the maintenance of barrier properties of epithelial tissues. The protective role of IL-17 has been linked to the regulation of the microbiota, maintenance of intestinal epithelial cell tight junctions and barrier integrity, and to the stimulation of repair upon intestinal epithelial damage [186]. Due to the pivotal role of IL-23 and IL-17 in inflammatory diseases, and the association that has emerged between (gut) inflammation and PD, we investigated the IL-23/IL-17 axis. Therefore, we evaluated the levels of IL23R, STAT3 and phospho-STAT3 in Caco-2 cells (Fig 14 A, B, C, D), as well as IL-17 levels (Fig. 14 F). Western blot analysis revealed a statistically significant reduction of IL23R levels and a tendential decrease of STAT3 levels of transfected Caco-2 cells, which correlates with the significantly reduced levels of IL-17 in transfected Caco-2 cells. Phospho-STAT3 levels remained unaltered. Additionally, we evaluated intestinal barrier integrity by measuring the levels of two tight junctions present in the intestinal barrier: Zonula occludens-1 (ZO-1) (Fig. 14 A, E) and occludin (Fig. 14 G, H). Up-regulation of miR-486-5p caused a statistically significant decrease of both ZO-1 and occludin levels in Caco-2 cells, further supporting the role of IL-17 in the maintenance of the intestinal barrier. Moreover, LPS tends to decrease ZO-1 levels and significantly decreases occludin levels. Once again, no alterations were found after transfection with the negative control.

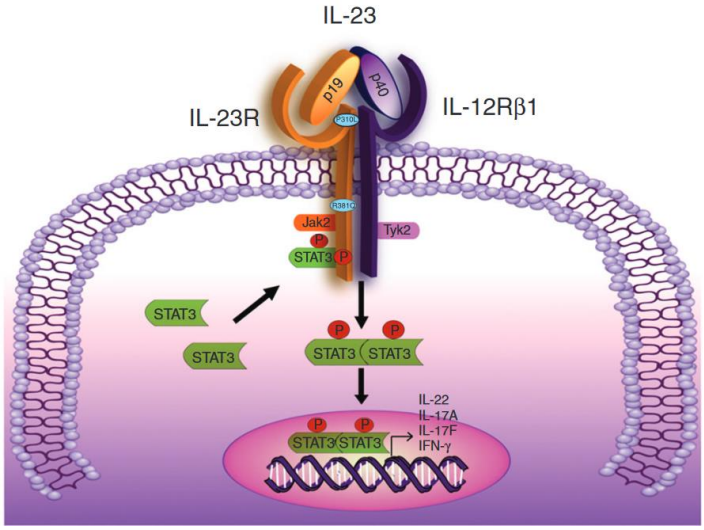

Figure 13. IL-23 signalling pathway. IL-23 binds to its IL-23 receptor, and results in Jak2-mediated phosphorylation of tyrosine residues located in the intracellular domain of the IL-23R subunit. Phosphorylated tyrosine residues serve as a docking site for STAT3 molecules, which in turn get phosphorylated. Phospho-STAT3 proteins homodimerize and translocate into the nucleus, inducing transcription of cytokines, such as IL-17A, IL-17F, IL-22 and IFN-γ (Adapted from Di Cesare *et al*., 2009).

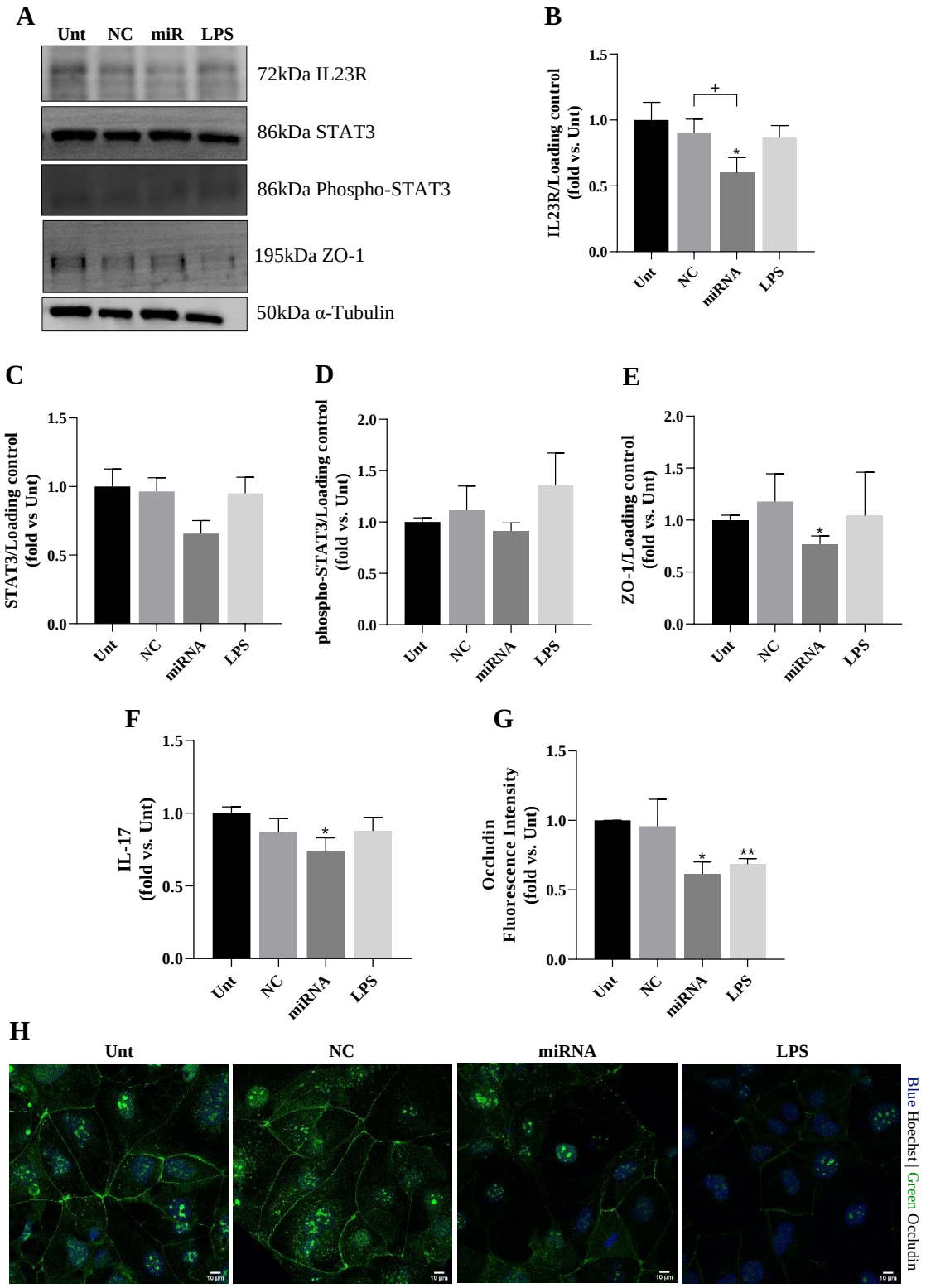

Figure 14. IL23R, STAT3, phospho-STAT3, ZO-1, IL-17 and occludin levels of Caco-2 cells transfected with miR-486-5p. **(A)** Western Blot analysis of IL23R, STAT3, phospho-STAT3 and ZO-1. **(B)** Densitometric analysis of IL23R corrected with α-tubulin (n=6). **(C)** Densitometric analysis of STAT3 corrected with α-tubulin (n=5). **(D)** Densitometric analysis of phospho-STAT3 corrected with α-tubulin (n=9). **(E)** Densitometric analysis of ZO-1 corrected with α-tubulin (n=5). The blots were re-probed for α-tubulin or vinculin to confirm equal protein loading. **(F)** IL-17 levels were measured using an ELISA kit (n=6). **(G)(H)** Immunostaining of Caco-2 cells with anti-occludin antibody (green) after transfection with miR-486-5p (n=3). Data represent the mean ± SEM and are expressed in comparison with untreated cells. * $p < 0.05$ and ** $p < 0.01$, significantly different from the untreated cells. + $p < 0.05$, significantly different from the NC. Statistical differences were analysed using unpaired Student's t-test (Fig. G) or using one-way ANOVA followed by Tukey's test (Fig. B-F). Hoechst 33342 stained nuclei are in blue. Scale bars: 10 µm.

3.3. SH-SY5Y

3.3.1. miR-486-5p induces mitochondrial defects in SH-SY5Y cells

As previously mentioned, mitochondrial dysfunction is characterized by the loss of mitochondrial membrane potential and increased levels of ROS. While miR-486-5p did not alter mitochondrial membrane potential on both cell lines (Fig. 15 A), it did cause a significant increase in ROS levels of transfected SH-SY5Y cells (Fig. 15 B).

Cardiolipin is a phospholipid localized exclusively in the inner mitochondrial membrane. Upon mitochondrial dysfunction, cardiolipin is externalized and functions as DAMPs to initiate and/or exacerbate innate immunity responses through NLRP3 activation [138]. Using Target Scan and miRDB, we found that cardiolipin synthase is also a putative miR-486-5p target. Cardiolipin, which is synthetized by cardiolipin synthase, is critically involved in a multitude of mitochondrial and cellular processes, in addition to being required for the optimal activity of complex I, complex III, and complex IV of the ETC [188]. Cardiolipin exposure was evaluated using 10-N-Nonyl acridine orange (NAO) through live cell imaging (Fig. 15 C, D). We detected a decrease in cardiolipin fluorescence intensity in SH-SY5Y cells transfected with miR-486-5p when compared with untreated cells, indirectly indicating that cardiolipin synthase is being down-regulated by miR-486-5p. This decrease in cardiolipin levels can therefore be responsible for the increase in ROS production. Furthermore, and as expected, CCCP triggered the loss of mitochondrial membrane potential and increased ROS production.

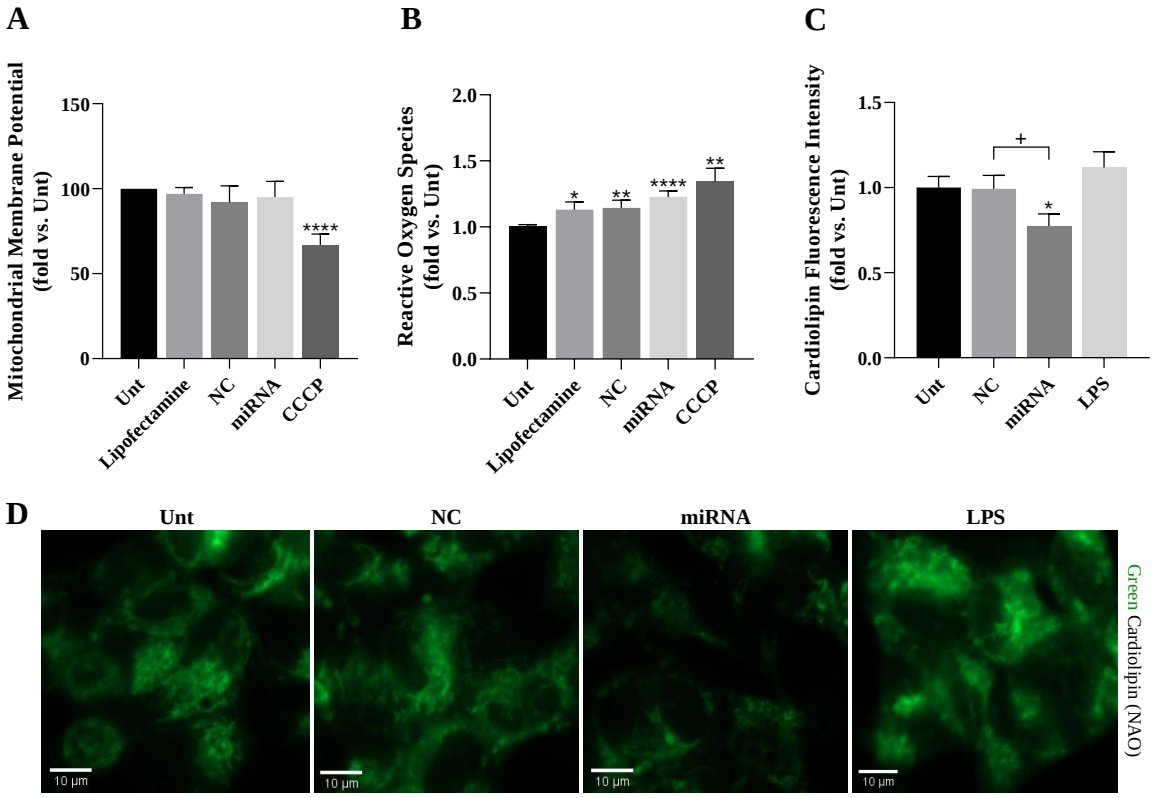

Figure 15. Mitochondrial Membrane Potential (Δψmit), ROS levels and Cardiolipin levels in SH-SY5Y cells transfected with miR-486-5p. **(A)** Effect of miR-486-5p overexpression on Δψmit of SH-SY5Y cells. Mitochondrial membrane potential was estimated using TMRM probe (n=5). **(B)** Effect of miR-486-5p overexpression on ROS levels of SH-SY5Y cells. ROS levels were measured using Amplex Red probe (n=5). **(C)** Staining of living SH-SY5Y cells with 10-N-Nonyl acridine orange (NAO) probe after transfection with miR-486-5p. **(D)** Quantification of cardiolipin fluorescence intensity (n=3). Data represent the mean ± SEM and are expressed in comparison with untreated cells. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$, significantly different from the untreated cells. + $p < 0.05$, significantly different from the NC. Statistical differences were analysed using unpaired Student's t-test (Fig. B) or using one-way ANOVA followed by Tukey's test (Fig. A-C). Scale bars: 10 µm.

3.3.2. miR-486-5p increases inflammatory markers in SH-SY5Y cells

Innate immunity and NLRP3 inflammasome activation in SH-SY5Y cells were evaluated in a similar manner to Caco-2 cells. The levels of TLR4, NLRP3 and ASC (Fig. 16 A, B, C, D) were determined, along with Caspase 1 activity and NF-κB levels (Fig. 16 E, F), as well as pro-inflammatory cytokines (IL-1β and IL-6) levels (Fig. 16 G, H). TLR4, NLRP3 and ASC levels were not significantly altered in transfected SH-SY5Y cells, although there seems to be a tendential increase of TLR4 and ASC levels. On the other hand, inflammatory markers like IL-1β, Caspase 1 and NF-κB were significantly increased in transfected cells. Additionally, SH-SY5Y cells exposed to LPS tend to increase TLR4 and ASC levels and significantly increase IL-1β, IL-6 and NF-κB levels and Caspase 1 activation.

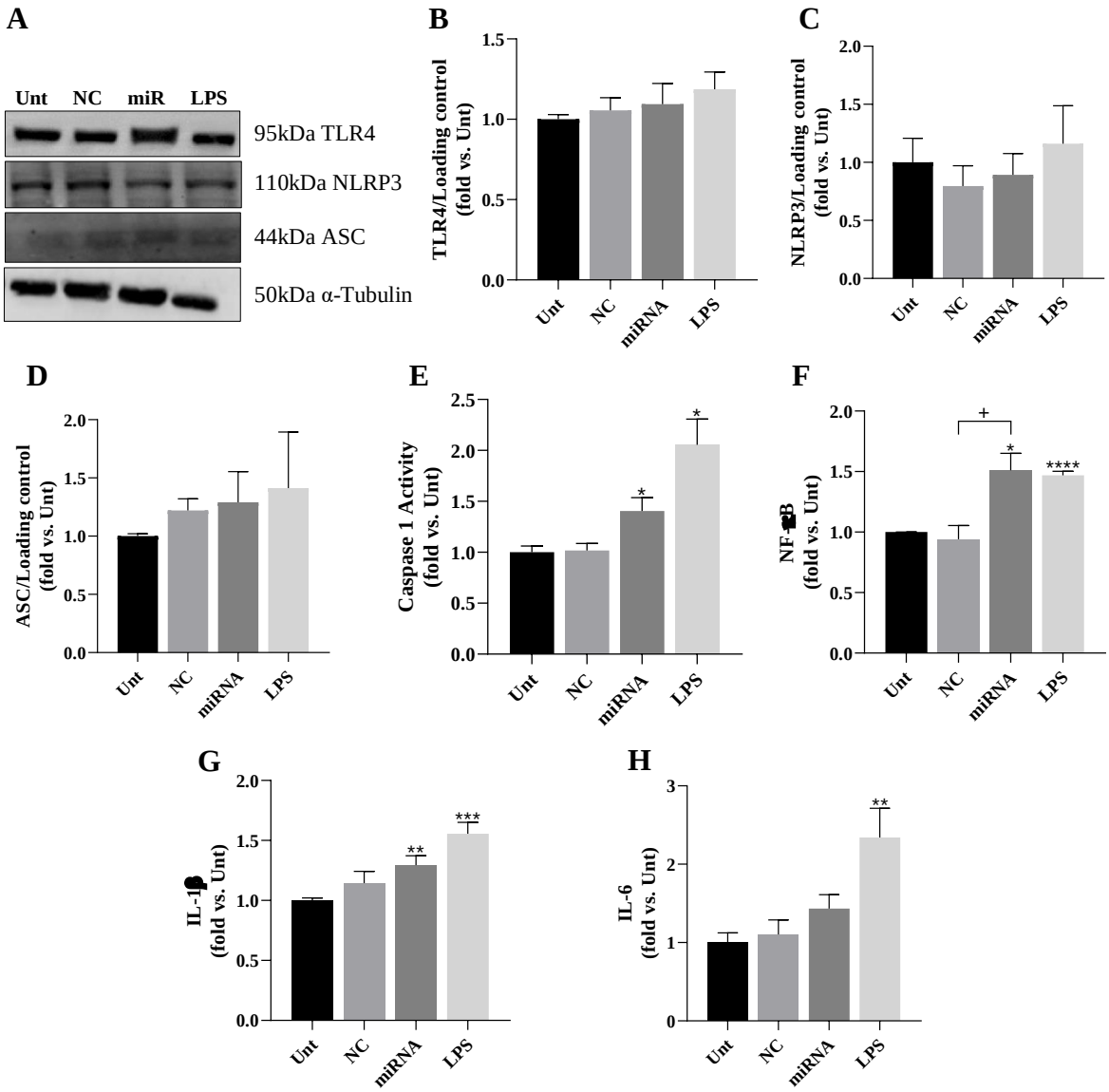

Figure 16. TLR4, NLRP3, ASC, NF-κB, IL-1β, IL-6 levels and Caspase 1 activity after transfection of SH-SY5Y cells with miR-486-5p. **(A)** Western Blot analysis of TLR4, NLRP3 and ASC. **(B)** Densitometric analysis of TLR4 corrected with α-tubulin (n=9). **(C)** Densitometric analysis of NLRP3 corrected with α-tubulin (n=4). **(D)** Densitometric analysis of ASC corrected with α-tubulin (n=6). The blots were re-probed for α-tubulin or vinculin to confirm equal protein loading. **(E)** Caspase 1 activity was measured spectrophotometrically at 405 nm, as described in Materials and Methods (n=3). **(F)** NF-κB was measured using an ELISA kit (n=4). **(G)** IL-1β was measured using an ELISA kit (n=6). **(H)** IL-6 was measured using an ELISA kit (n=5). Data represent the mean ± SEM and are expressed in comparison with untreated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, significantly different from the untreated cells. + $p < 0.05$, significantly different from the NC. Statistical differences were analysed using one-way ANOVA followed by Tukey's test.

3.3.3. miR-486-5p does not influence IL-23/IL-17 pathway in SH-SY5Y cells

Similar to Caco-2 cells, the IL-23/IL-17 pathway mediated by IL23R was studied in SH-SY5Y cells. IL23R, STAT3 and phospho-STAT3 levels were measured (Fig. 17 A, B, C, D), in addition to IL-17 levels (Fig. 17 E). However, no significant alterations were detected.

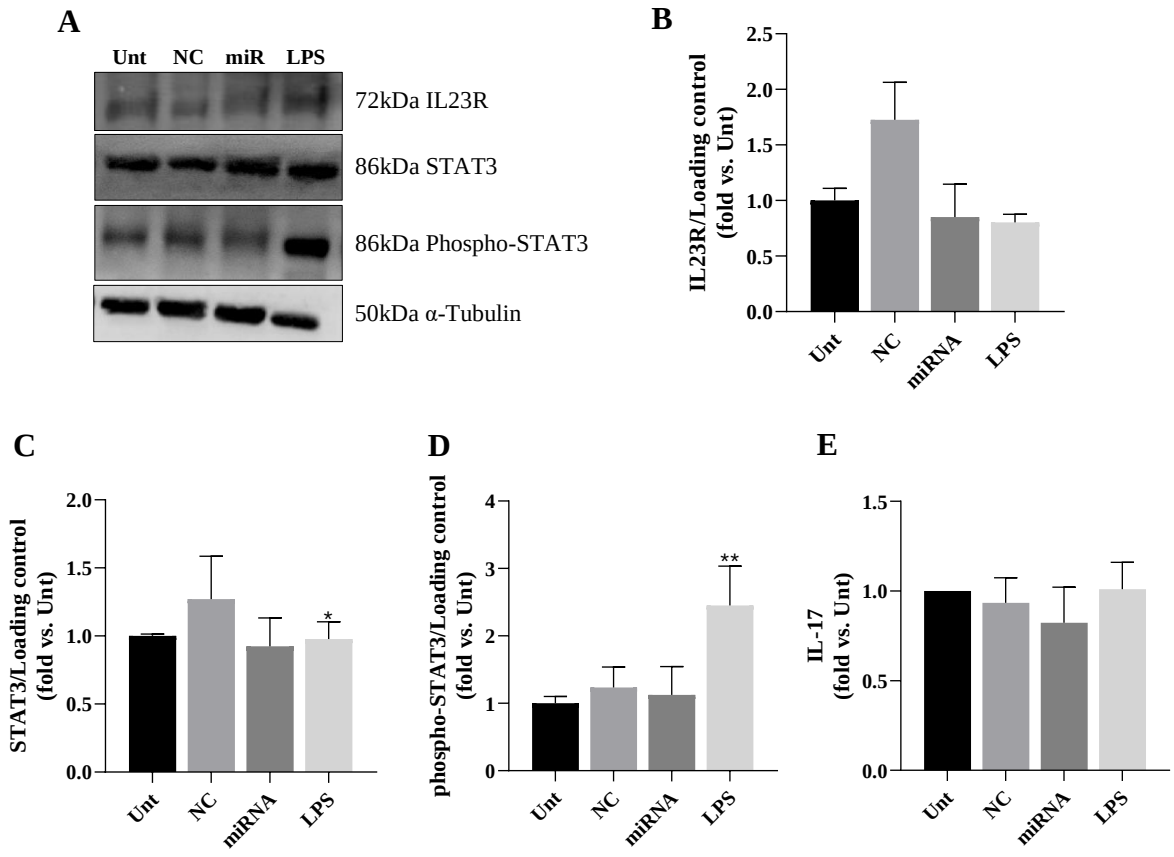

Figure 17. IL23R, STAT3, phospho-STAT3 and IL-17 levels of SH-SY5Y cells transfected with miR-486-5p. **(A)** Western Blot analysis of IL23R, STAT3 and phospho-STAT3. **(B)** Densitometric analysis of IL23R corrected with α-tubulin (n=6). **(C)** Densitometric analysis of STAT3 corrected with α-tubulin (n=5). **(D)** Densitometric analysis of phospho-STAT3 corrected with α-tubulin (n=6). **(E)** IL-17 levels were measured using an ELISA kit (n=4). Data represent the mean ± SEM and are expressed in comparison with untreated cells. * $p < 0.05$ and ** $p < 0.01$, significantly different from the untreated cells. Statistical differences were analysed using Student's t-test (Fig. C) or using one-way ANOVA followed by Tukey's test (Fig. D).

CHAPTER IV
DISCUSSION

PD is a neurodegenerative disease hallmarked by severe loss of dopaminergic neurons of the SNpc, and α-synuclein-containing inclusion bodies in the surviving neurons, resulting in characteristic motor impairment [189]. It is currently accepted that the onset of motor symptoms is preceded by a prodromal phase, during which non-motor features emerge, such as gastrointestinal dysfunction with a prevalence as high as 77% - 81% [189]. Although the neuropathology of PD is fairly well understood, its aetiology remains elusive. It has been proposed that PD might start in the ENS before spreading, via the vagus nerve, to the CNS. Indeed, accumulation of α-synuclein aggregates, a major PD pathological hallmark, was found in the gut of PD patients early on in the prodromal phase. In addition, work from our group and others have consistently proved that defects in mitochondrial function and oxidative stress play a major role in sporadic PD [145, 190]. Furthermore, both neuroinflammation and intestinal inflammation have been linked to PD, suggesting that the immune system plays a crucial role in PD pathogenesis. Moreover, several studies have suggested that the gut microbiota alterations reported in PD patients compromises the integrity of the intestinal barrier and increases its permeability [10]. It has also been suggested that gut inflammation can potentiate blood-brain barrier permeability and neuroinflammation, culminating in nigrostriatal neuronal damage and PD-like motor dysfunction [62]. Additionally, numerous studies have reported an altered expression of miRNAs in PD patients, as well as the ability of miRNAs to shape host's gut microbiota [181] and be regulated by host's gut microbiota [183]. In fact, the selected miRNA was found to be increased in colon tissue from PD patients and this increase is correlated with age and disease severity in PD [179]. Taking all these evidences into account, in this thesis we investigated if miR-486-5p expression could contribute to PD pathogenesis by affecting mitochondrial function and activating inflammatory responses. *In vitro* effects were studied by transfecting Caco-2 cells (to mimic intestinal cells) and SH-SY5Y cells (to mimic PD neurons) with the mentioned miRNA.

We provide evidence that miR-486-5p significantly increases the levels of pro-inflammatory markers, such as IL-1β and IL-6, in both cell lines. Additionally, transfected SH-SY5Y cells also presented significantly increased levels of NF-κB, an inducible transcription factor that targets genes involved in inflammation, and an increase in Caspase 1 activity, the prototypical human inflammatory caspase. Other miRNAs have been reported to induce inflammation, namely miR-155-5p, that has been found to be up-

regulated in blood samples of PD patients [171]. Other studies have suggested the central role of miR-155-5p in inflammation, since deletion of this miRNA reduced pro-inflammatory responses in a mouse model of PD [191]. Several studies have implicated mitochondrial dysfunction in PD pathogenesis elucidating its ability to activate innate immune responses [62]; therefore, we evaluated mitochondrial function by measuring mitochondrial membrane potential and ROS levels. Mitochondrial membrane potential remained unaltered in both cell lines (Fig. 11 and Fig. 15), and only SH-SY5Y cells showed increased ROS levels (Fig. 15). This suggests that mitochondrial dysfunction was not the origin of the elevated levels of pro-inflammatory markers in Caco-2 cells, which was corroborated by the absence of alterations in the levels of proteins involved in mitochondrial-mediated innate immunity (TLR4, NLRP3, ASC). Using Target Scan and miRDB, we selected a miR-486-5p target of interest for the present study: IL23R. IL-23 is a pro-inflammatory cytokine that has been implicated in the development of chronic inflammatory diseases, such as inflammatory bowel diseases that have been associated with a higher PD risk [186]. IL23R is expressed on the surface of Th17 and IL-17-secreting TCRγδ cells (Tγδ17) [186], however we were able to detect the presence of IL23R on Caco-2 cells through Western Blot analysis. Apart from the mainstream understanding of the immune system, we thereby propose that other cells can express IL23R and trigger adaptive immune responses. IL-23 binds to IL23R and triggers the production and secretion of IL-17 (Fig. 13). We investigated the proteins involved in this pathway (IL23R, STAT3, phospho-STAT3) and detected a statistically significant decrease of IL23R and a tendential decrease of STAT3 levels in transfected Caco-2 cells (Fig. 14 B, C), implying the silencing of IL23R by miR-486-5p. The diminished levels of these proteins could justify the significantly reduced levels of IL-17 detected in Caco-2 cells transfected with miR-486-5p (Fig. 14 F). IL-17 has been shown to have a key role in the maintenance of barrier properties of epithelial tissues, and has been linked to the regulation of the microbiota, maintenance of intestinal epithelial cell tight junctions and barrier integrity, and to the stimulation of repair upon intestinal epithelial damage [186]. Our results corroborate this hypothesis, because up-regulation of miR-486-5p resulted in the decrease of occludin levels, demonstrated by immunocytochemistry (Fig. 14 G, H), and in the decrease of ZO-1 levels by western blot analysis (Fig. 14 A, E), two tight junctions of the intestinal barrier. Based on our findings, we propose that miR-486-5p up-regulation ultimately leads to the impairment of the intestinal barrier by silencing IL23R in Caco-2 cells. It has been hypothesized that the increase of intestinal permeability could

be sufficient to expose enteric neurons to bacterial derived pro-inflammatory products, such as LPS, inducing local inflammation and oxidative stress, which in turn leads to neuronal pathological α-synuclein aggregation and accumulation. Experimental findings have showed that local (intestinal) or systemic administration of LPS in mice is associated with higher α-synuclein expression [192, 193] or aggregation [194]. The bloodstream is another mechanism by which gut hyperpermeability may influence the brain in PD. Systemic inflammation has been shown in PD patients [195] and evidence from animal models supports a role for systemic inflammation in the exacerbation of neurodegeneration [196].

Although the mitochondrial membrane potential of SH-SY5Y cells remained unaltered, ROS levels were significantly increased upon up-regulation of miR-486-5p (Fig. 15 B), suggesting an impaired mitochondrial function. Since cardiolipin synthase is a target of the studied miRNA, and cardiolipin is highly related to mitochondrial function, we evaluated cardiolipin levels in these cells. We detected a significant reduction of cardiolipin levels in SH-SY5Y cells transfected with miR-486-5p (Fig. 15 C, D), probably resulting from the silencing of cardiolipin synthase by miR-486-5p, which correlates with the increased levels of ROS. Cardiolipin is a phospholipid of the inner mitochondrial membrane and is required for the optimal activity of complexes I, III and IV of the ETC [188]. Decreased levels of cardiolipin due to silencing of cardiolipin synthase will most likely impair the ETC and lead to the production of ROS [197], which in turn might activate immune responses by triggering the activation of NLRP3 inflammasome [198]. In fact, we observed an increase in ROS production, NF-κB levels, Caspase 1 activity and IL-1β levels (Fig. 15 B, Fig. 16 E-G) in SH-SY5Y cells transfected with miR-486-5p. The NLRP3 inflammasome is a cytosolic protein complex that is required for caspase 1 activation, and its assembly involves NLRP3, procaspase-1 and ASC proteins. Upon inflammasome activation, caspase 1 is activated and triggers the processing of pro-IL-18 and pro-IL-1β into their active forms (IL-18 and IL-1β) [130]. Because Caspase 1 activity was significantly increased, as well as NF-κB and IL-1β levels, we expected to observe an increase of the levels of proteins involved in mitochondrial-mediated innate immunity and NLRP3 inflammasome assembly (TLR4, NLRP3, ASC), however only ASC and TLR4 levels showed a tendential increase in transfected SH-SY5Y cells. The NLRP3 inflammasome activation pathway could be at its final stage at the time cellular extracts were obtained, activating Caspase 1 and inducing IL-1β production, but already being

degraded, thus explaining why significant differences were seen only in Caspase 1 activity and IL-1β levels but not in TLR4, NLRP3 and ASC levels. ROS levels, however, remain increased throughout the whole pathway since inflammation promotes pyroptosis, releasing contents from the damaged cell and leading to the further production of ROS [199]. Moreover, miR-486-5p up-regulation does not seem to affect the IL-23/IL-17 axis in SH-SY5Y cells, since no alterations were found in the levels of IL23R, STAT3, phospho-STAT3 and IL-17.

Based on our findings, we suggest that up-regulation of miR-486-5p ultimately leads to the impairment of the intestinal barrier, increasing its permeability, in Caco-2 cells. This results from the silencing of IL23R by the studied miRNA. Impairment of this pathway leads to reduced levels of IL-17, which is strictly connected to the maintenance of the intestinal barrier integrity. We showed that transfection of Caco-2 cells with miR-486-5p causes a significant decrease of IL23R and IL-17 levels, as well as a significant reduction of the levels of two tight junction proteins, ZO-1 and occludin. Since an impaired intestinal barrier has been associated with PD [189], our findings thereby suggest that the studied miRNA could also be responsible for this PD feature. Our results also indicate that miR-486-5p affects SH-SY5Y cells by silencing cardiolipin synthase, thus reducing cardiolipin levels and increasing ROS production which acts as a DAMP and activates inflammatory responses by triggering NLRP3 inflammasome activation. Inflammation has been widely linked to PD, which indicates that miR-486-5p could also be one of the numerous factors triggering inflammation in PD.

In conclusion, these results pave the way for future studies regarding miRNAs involvement in PD ultimately leading to significant breakthroughs into the development of innovative therapies to address some of the disorder's most pressing unmet requirements. Although some current treatments may have the capacity to slow down the disease progression, we still do not possess the ability to arrest PD or even to reach an early diagnosis. The recent focus on the role of miRNAs in PD might speed the identification of robust, efficient, non-invasive, and cost-effective diagnostic biomarkers of pathogenesis and disease progression, allowing an early diagnosis of PD. Furthermore, miRNAs could also function as therapeutic targets, granting the possibility for individually tailored treatment options.

CHAPTER V
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