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ABSTRACT

Parkinson's Disease (PD) is a common and progressive neurodegenerative disorder characterised by motor impairments as well as non-motor symptoms. While dopamine-based therapies are effective in fighting the symptoms in the early stages of the disease, a lack of neuroprotective drugs means that the disease continues to progress. Along with the traditionally recognised pathological hallmarks of dopaminergic neuronal death and intracellular α-synuclein (α-syn) depositions, iron accumulation, elevated oxidative stress and lipid peroxidation damage are further conspicuous features of PD pathophysiology. However, the underlying mechanisms linking these pathological hallmarks with neurodegeneration still remain unclear. Ferroptosis, a regulated iron dependent cell death pathway involving a lethal accumulation of lipid peroxides, shares several features with PD pathophysiology. Interestingly, α-syn has been functionally linked with the metabolism of both iron and lipid, suggesting a possible interplay between dysregulated α-syn and other PD pathological hallmarks related to ferroptosis. This review will address the importance for understanding these disease mechanisms that could be targeted therapeutically. Anti-ferroptosis molecules are neuroprotective in PD animal models and the anti-ferroptotic iron chelator, deferiprone, slowed disease progression and improved motor function in two independent clinical trials for PD. An ongoing larger multi-centre phase 2 clinical trial will confirm the therapeutic potential of deferiprone and the relevance of ferroptosis in PD. This review addresses the known pathological features of PD in relation to the ferroptosis pathway with therapeutic implications of targeting this cell death pathway.

1. INTRODUCTION

Parkinson's Disease (PD) is the second most common neurodegenerative disorder after Alzheimer's Disease. Clinically, PD patients present with motor impairments such as bradykinesia and rigidity, as well as non-motor symptoms including anosmia, constipation, pain, anxiety, depression, psychosis and cognitive disorders that may progress to dementia [1]. The main pathological hallmarks of the sporadic and familial forms of the disease are a predominant and progressive degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNpc) associated with a systematic progressive iron accumulation, leading to a dopamine depletion in the striatum, disappearance of neuromelanin and appearance of intracellular Lewy Bodies with the major component consisting of aggregated α-synuclein. [2–5].

Current treatments **aim to increase dopamine neurotransmission, which offers symptomatic relief. The therapeutic arsenal includes the dopamine precursor Levodopa (L-DOPA), dopamine agonists and dopamine metabolism inhibitors [6]. L-DOPA remains the standard most effective treatment in combination with decarboxylase inhibitors[7,8]. Evidence from a randomized, double-blind, placebo-controlled trial suggested that L-DOPA slowed progression (as measured by UPDRS) between baseline and 42 weeks compared to the placebo group [9]. However, a more recent and larger study found no evidence of a disease-modifying effect of L-DOPA[10]. Disparity in the potential neuroprotective actions of this drug may arise from L-DOPA's interference with the striatal presynaptic dopamine transporter; standardly used as a reporter for nigro-striatal degeneration by DAT SPECT in clinical trials [9,11]. The powerful symptomatic action and short half-life of L-DOPA also weakens its clinical use with motor complications such as "wearing off" and dyskinesia occurring 4-6 years after chronic use, depending on disease severity [12–14]. To delay the onset of**

such complications, dopamine agonists, monoamine oxidase-B (MAO-B) inhibitors or catechol-O-methyltransferase (COMT) inhibitors are prescribed as concomitant therapies. The ADAGIO study clinically assessed the potential disease modifying effects of Rasagiline, an irreversible MAO-B inhibitor using a delayed-start protocol aimed at preventing the confounding symptomatic benefits of this drug. Although early treatment with rasagiline at a daily dose of 1mg slowed the rate of UPDRS deterioration in the early-start group, a similar response was not observed with the dose of 2mg [15,16]. Furthermore, a follow-up study failed to demonstrate long-term benefits for early-start rasagiline treatment [17].

Over the last few years, several compounds have shown promising neuroprotective effects in *in vitro* and *in vivo* models but failed to translate to patient studies due to efficiency or safety concerns (reviewed in more detail in [18]). A huge unmet need remains for efficient neuroprotective or disease-modifying therapies. To overcome this demand, a better understanding of the mechanisms involved in producing the pathological hallmarks associated with the disease and their dynamic relationship to neuronal cell death is required. For many years, the pathological process involved in PD related neuronal death was considered to be apoptosis. This was mainly due to the fact that until recently, only a few types of programmed cell death were known, and these were identified predominantly by using oncogenic cell lines (i.e. neuroblastoma). Since then, multiple cell death mechanisms have been studied and implicated in PD pathogenesis (reviewed in [19]), including anoikis [20,21], autophagy [22,23], necroptosis [24,25], parthanatos [26,27] and pyroptosis [28].

More recently, ferroptosis has been established as a regulated necrosis that is morphologically and mechanistically distinct from apoptosis and other known cell death pathways [29]. Ferroptosis is characterised by iron dependent lipid peroxidation[30].

Interestingly, several PD pathological hallmarks are known key **features and/or triggers** in the ferroptotic cell death pathway. These include **iron overload** [4,31–34], **elevated lipid peroxidation** [35–37], **reduced GSH levels** [38–41], **XCT downregulation** [42], **DJ-1 depletion** [43,44] **and CoQ10 reduction** [45–47]**. Together, these well-established diseases features strongly** implicate this regulated cell death pathway in the neurodegeneration observed in PD.

In this review we propose that ferroptosis may represent the missing piece to the puzzle in explaining the vicious cycle between synucleinopathy, iron accumulation, oxidative stress and related cell death in PD. Establishing the implication of ferroptosis in neurodegenerative diseases such as PD will promote interest in generating a range of anti-ferroptosis based therapies that could delay disease onset and slow progression. The aim of this paper is to review the current understanding of ferroptosis and present the evidence for the involvement of this newly defined cell death in PD neuropathology.

2. FERROPTOSIS – AN IRON DEPENDENT FORM OF REGULATED CELL DEATH

The term ferroptosis was coined in 2012 to describe a novel regulated form of caspase-independent cell death resulting from iron induced lipid peroxides that accumulate to toxic levels [29]. Although the precise metabolic pathways are still currently being elucidated, the past few years of extensive research have deciphered several regulatory mechanisms as well as numerous specific inducer and inhibitor reagents (Figure 1). Keeping lipid hydroperoxides (LOOH) within physiological parameters is a key component in minimizing susceptibility to ferroptosis. Lipophilic antioxidants and lipid peroxide scavengers can block ferroptosis by preventing lipid peroxidation [48,49]. Under physiological conditions, the cell combats lipid peroxidation with glutathione peroxidase 4 (GPX4), an essential selenoprotein that reduces LOOH to lipid alcohols (LOH). Importantly, GPX4 is

the only member of the glutathione peroxidase family capable of reducing LOOH, supporting its central enzymatic role in the ferroptotic pathway [50,51]. Direct inactivation of GPX4 by RSL3 is one of the most common strategies to induce ferroptosis experimentally. Deletion of GPX4 in mice is embryonically lethal [52], whilst conditionally knocking out GPX4 in mice leads to acute renal failure, hippocampal and motor neuron neurodegeneration and early death of mice [48]. In addition, overexpression of GPX4 protects against cell death induced by RSL3 [53]. For the reduction of LOOH to LOH, GPX4 requires reduced glutathione (GSH) as an electron donor, releasing oxidised glutathione (GSSG). GSH is synthesised in the cell from glutamate and cysteine, the latter being the rate-limiting substrate. Cysteine can either be synthesised from methionine via the transsulfuration pathway or taken up in the form of an oxidised cystine dimer via the XcT antiporter before being reduced into the amino acid cystine. A heterodimer of the XcT unit (encoded by SLC7A11), that forms the Xc^- system, and the 4F2 unit (encoded by SLC3A2) that localizes the Xc^- system to the plasma membrane, are required for intracellular cystine transport [54]. An impairment of the XcT unit leads to a depletion in the intracellular cysteine pool, with consequential impairment of GSH biosynthesis and GPX4 activity. The subsequent lipid peroxide accumulation results in cell death by ferroptosis. Blocking GSH bioavailability through erastin-induced inhibition of the XcT antiporter or buthionine sulfoximine (BSO) induced inhibition of glutamate-cystein ligase (GCL); the rate-limiting enzyme in the first step of GHS synthesis, suffices to trigger ferroptosis (Figure 1). Elevating intracellular glutathione (GSH) levels with cysteine precursors such as n-acetylcysteine can protect against ferroptosis [53]. Recently in an attempt to uncover genes able to protect against ferroptosis through glutathione-independent pathways, the flavoprotein “apoptosis inducing factor mitochondria-associated 2” (AIFM2), was identified to rescue cell death caused by GPX4 deletion [47,55]. Renamed “ferroptosis suppressor-protein 1” (FSP1), it suppresses

ferroptosis by catalysing the regeneration of Coenzyme Q10 (CoQ10, also known as ubiquinone) to its reduced form CoQ10-H_2 or ubiquinol; a potent mitochondria and lipid peroxyl radical trapping antioxidant (Figure 1 and 2).

As the name implies, iron plays a central role in ferroptosis. Co-treatment with several sources of iron sensitizes cells to ferroptosis triggered by erastin or RSL3 [29,56] and depletion of iron using drugs such as deferiprone (DFP) or genetically silencing transferrin receptor 1 (TfR1) to prevent cellular iron import protects cells against ferroptosis [56,57]. Although the exact mechanisms through which iron promotes ferroptosis remain unclear, it has been suggested that i. ferrous iron directly induces lipid peroxidation[58], ii. iron loads the iron-dependent 12/15 lipoxygenase which enzymatically induces lipid peroxidation [41,59,60], and iii. iron loading of the hypoxia inducible factor prolyl-hydroxylase 1 induces ATF4-dependent pro-death gene transcription [61]. In biofluids such as cerebrospinal fluid (CSF), iron is predominantly bound to transferrin (Tf). Iron-loaded Tf (holo-Tf) incorporates two ferric (Fe^{3+}) atoms and is internalized into a cell through clathrin-mediated TfR1-dependent endocytosis (Belaidi and Bush 2016). Once in the endosome, iron is released from Tf due to its acidic environment and the metalloreductase STEAP3 reduces the bound insoluble Fe^{3+} to its soluble ferrous (Fe^{2+}) form [63]. Iron is then released into the cytosol through the divalent metal transporter 1 (DMT1), with Tf and TfR1 being recycled back to the membrane for further use. Neuronal uptake of iron can also occur directly through the DMT1 channel on the plasma membrane, allowing for a less regulated import pathway [62] (Figure 1). The free cytosolic Fe^{2+} that constitutes the labile iron pool (LIP) participates in the Fenton reaction producing highly reactive hydroxyl radicals (·OH) from hydrogen peroxide (H_2O_2)[58]. It is thus essential that appropriate levels of free iron in the LIP are tightly maintained to avoid the excess generation of free ·OH and other radicals (see section 3.2. for further details). The

function of ferritin is in part to safely store excess iron in the cytosol but neuromelanin also serves as a key iron storage protein specifically in dopaminergic neurons. The only known export pathway for iron is through the transmembrane channel ferroportin (FPN) in which iron, oxidized to Fe^{3+} by a ferroxidase such as ceruloplasmin, can exit the cell[64]. In select cells (including neurons) β-amyloid precursor protein (APP) is required to facilitate iron efflux by stabilising FPN on the plasma membrane [65–68] (Figure 1).

In addition to the Fenton reaction, iron can mediate the generation of lipid peroxides by serving as a cofactor to the family of Lipoxygenase enzymes (LOX). Of particular interest to ferroptosis, 15-LOX can enzymatically generate additional LOOH on long-chain polyunsaturated fatty acids (PUFA); mainly phosphatidylethanolamines containing arachidonic acid (AA) or adrenic acid (AdA) present on the plasma membrane (Shintoku et al. 2017; Shah, Shchepinov, and Pratt 2018). The lipid composition of the plasma membrane can therefore determine cellular susceptibility to ferroptosis whereby long chain PUFAs containing AA increase the risk for lipid peroxidation whilst monounsaturated fatty acids (MUFAs) appear to decrease such risk [71]. For PUFAs to be incorporated into the phospholipids (PL) of the plasma membrane, they first need to be conjugated to Coenzyme-A (CoA) by the enzyme acyl-CoA synthetase long-chain family member 4 (ACSL4). PUFA-CoAs can then be incorporated into the plasma membrane by lysophosphatidylcholine acyltransferase 3 (LPCAT3) where 15-LOX specifically oxidise the PLs rendering the plasma membrane more permeable and fragile. ACSL4, but not other members of the ACSL family, is a key player in the ferroptosis pathway as it enriches cellular membranes with long chain ω6 fatty acids. Inhibiting or genetically depleting ACSL4 prevents ferroptotic lipid peroxidation and subsequent associated cell death [72]. The selectivity of ACSL4 over the other ACSLs in regulating the ferroptosis pathway is likely to be due to its substrate

preference for AA, the main fatty acid implicated in ferroptosis [73]. The importance of LPCAT3 in the ferroptosis pathway was initially pointed out by Dixon *et al* [74] but later confirmed by Doll *et al* [72] where LPCAT3 deletion mildly protected fibroblast against ferroptosis (Figure 1).

The initial discovery of ferroptosis occurred within the context of certain oncogenic Ras-dependent cancer types. However, thanks to extensive ongoing research since its emergence, this type of cell death has now also been implicated in an array of other pathological conditions. These include ischemia-reperfusion injury (IRI) of the liver, kidney, brain and heart [75–82], **stroke** [83–87] and multiple neurodegenerative disorders including PD [57], Alzheimer's disease [88] and amyotrophic lateral sclerosis [89].

3. FERROPTOSIS IN PARKINSON'S DISEASE

Ferroptosis has been shown to be a prevalent type of cell death in *in vitro*, *ex vivo* and *in vivo* models of PD [57]. Dopaminergic neurons within a differentiated cellular model (Lund human mesencephalic cells; LUHMES) or *ex vivo* organotypic slice cultures are sensitive to erastin induced ferroptosis which can be rescued by the ferroptosis specific inhibitors ferostatin-1 (Fer-1) and liproxstatin-1 (Lpx-1) as well as the iron chelator DFP. These specific inhibitors are also protective against neurotoxin induced cell death associated with sporadic PD (e.g. rotenone, paraquat and MPP+). *In vivo*, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) associated neuropathology has been identified to have a ferroptosis component as inhibition by Fer-1 and Lpx-1 prevents dopaminergic neuronal loss in the SN and striatum as well as behavioural and motor impairments [57]. **These results further** provide promise that inhibition of ferroptosis may alleviate and/or prevent PD associated neuropathology.

3.1 The role of iron in PD pathology

Iron serves as a cofactor in a myriad of metabolic processes throughout the body, and is particularly essential for brain health as it is involved in neurotransmitter synthesis, mitochondrial respiration, myelin synthesis and sulfur-cluster protein synthesis amongst other processes [90,91]. Due to the high metabolic activity and reliance on iron for many of these processes it should not be a surprise that this metal accumulates in the brain through age. However, this accumulation is even greater in regions of the brain that happen to be associated with neurodegenerative disorders [62,92]. In PD, iron elevation is especially observed in glia and dopaminergic neurons of the SNpc, where levels correlate with disease severity[4,32,33,93]. In patients, this has been measured by iron-sensitive high-field MRI [33,94] and quantitative susceptibility mapping (QSM) analyses [95] as well as post-mortem tissue and is strongly supported in many parkinsonian animal models [34,96–98]. In PD, abnormal iron accumulation is most likely due to an imbalance in the iron homeostatic pathway caused by alterations in iron regulatory proteins [99,100]. Patients carrying mutations that cause iron-related proteins to be dysfunctional (e.g. transferrin) have an increased a risk of developing PD [101,102]. Increased levels of DMT1, reported in the SNpc of PD patients as well as several mouse models of PD, are likely to contribute to an increased cellular iron import [103,104]. Ferritin levels are also decreased in the SN of post-mortem brains of PD patients [105], and FPN is decreased in several models of PD including MPTP and 6-hydroxydopamine (6-OHDA)[106]. Of relevance to impaired neuronal efflux through ferroportin, rare variants of APP with loss of membrane function predispose humans to develop PD [107]. In addition, APP expression is decreased in the SN of PD patients, leading to a similar iron-associated phenotype as APP knockout mice [66,68]. Furthermore, the ferroxidase activity of CP required to facilitate iron efflux through FPN is decreased in both patients and animal models of PD[108,109]. Further support for iron elevation as a cause of parkinsonian pathology comes from the genetic disorder aceruloplasminemia, in which CP is mutated, often leading to a

parkinsonian phenotype including gait difficulties, ataxia, involuntary movements and cognitive decline that correlate with brain iron deposition [110,111]. It is likely that an unregulated modulation of iron import and efflux contribute towards the elevation of intracellular iron required for increased vulnerability to free radical formation and ferroptosis.

Based on the extensive evidence supporting the impact of iron on PD pathology, iron chelation has been investigated as a possible therapeutic strategy[100,112,113]. Iron chelation in the MPTP mouse model restores iron to physiological levels in correlation with preventing cell toxicity and behavioural deficits [97,98]. DFP is currently used clinically for systemic iron overload disorders such as beta thalassemia and was recently investigated in a double-blind, randomized, placebo-controlled clinical trial of early-stage PD [98]. Over 12 months, patients receiving daily doses of DFP showed a promising decrease in motor handicap progression as well as reduced iron deposition in the SN [98]. The potential of DFP as a disease modifying treatment for PD is now being assessed in a phase 3 multicentre clinical trial with outcomes expected within the near future (FAIRPARK II – NCT01539837). Importantly, similar results have been observed in an independent phase 2 randomised double-blind placebo controlled clinical trial [114].

3.2 The role of oxidative stress in PD Pathology

Oxidative stress (OS) is considered a major contributor to the pathophysiology underlying PD and is well reported in patients as well as all PD animal models [40,41,115,116]. Indeed, a number of parkinsonian models induce an OS response that results in a phenotype similar to PD. Cellular damage caused by OS comprises of protein oxidation, leading to protein dysfunction and structural changes, DNA oxidation and cell membrane disruption due to lipid peroxidation. OS is induced by an imbalance in the redox state caused not only

by an excessive reactive oxygen species (ROS) production but also by an insufficient antioxidant system response to reduce these reactive species. ROS are defined as highly reactive molecules derived from oxygen and include free radicals such as superoxide (O_2^-) and ·OH as well as non-radical molecules like H_2O_2 [116]. ·OH are considered one of the most volatile ROS responsible for the cytotoxicity effect underlying oxidative stress and are predominantly generated from H_2O_2 and free cytosolic Fe^{2+} through the Fenton Reaction. Mitochondria are one of the main sites of H_2O_2 and ROS production, particularly O_2^-, via the respiratory chain complexes used to transport electrons [117,118]. In the brain, the majority of the O_2^- is produced by Complex I, and it is not a coincidence that this is the primary location from which ROS is generated in various neurodegenerative diseases [119]. Indeed, in PD patients a dysfunctional Complex I is present in the SN and frontal cortex as well as fibroblast and platelets from these patients; all of which may lead to increased superoxide production [118,119]. The deficiency in complex I is related to a CoQ10 deficit, further contributing to ROS production in mitochondria and lipid peroxidation in membranes. Interestingly, CoQ10 was recently reported to play an important anti-ferroptosis role on an FSP1-NAD(P)H-CoQ10 axis [47,55].

Cells are equipped with an antioxidant system to maintain a balanced redox state, which, if compromised, can result in excessive oxidative stress and subsequent cell death. The maintenance of glutathione GSH is one such antioxidant system heavily used by the brain to remove ROS by directly interacting and removing the highly reactive O_2^- and ·OH radicals [120,121]. Oxidative stress in PD may in part be due to a reduction in GSH levels that appear to be particularly evident in the SN of PD [38–40]. **The** Sian *et al* **study measured GSH levels in several port-mortem brain regions from PD, progressive supranuclear palsy, multiple-system atrophy and Huntington's disease patients, and the only significant change observed was a specific 40% reduction in the SN from PD patients. The fact that no**

changes were reported in the other diseases suggests that the alterations in GSH levels were not a general consequence of neurodegeneration [39]. Interestingly, a recent analysis of DNA methylation in 1132 PD cases and 999 controls associated hypermethylation in the promoter region of the SLC7A11 gene (encoding the cysteine-glutamate antiporter XcT-) with risk of PD. This hypermethylation of SLC7A11 results in a downregulation of system XcT- which could contribute to the decreased intracellular GSH levels observed in PD and increase a susceptibility to ferroptosis[42]. A similar reduction in GSH level has been shown to trigger the activation of neuronal 12-lipoxygenase (12-LOX) and subsequent accumulation of LOOH [41]. These observations further support the concept that the decrease of GSH in the SN from PD patients is not simply a consequence of neuronal death but a direct indication of oxidative stress. Moreover, as GSH is a natural ligand for Fe^{2+} [122], its reduction in the SN of PD patients would not only impair the antioxidant capacity of dopaminergic neurons but also increase the LIP, further contributing to the generation of ·OH and other ROS. The aforesaid intracellular environment would render the dopaminergic neurons particularly vulnerable to ferroptotic cell death.

DJ-1 is another cellular antioxidant enzyme known to play a key role in regulating oxidative stress, ROS formation and mitochondrial function. Loss of function mutations in the DJ-1 gene (PARK7) have been linked to autosomal-recessive early onset PD with increased mitochondrial oxidant stress, a drop in basal cellular respiration and oxidized dopamine accumulation[123]. Recently, Cao *et al* have shown that DJ-1 acts as a ferroptosis inhibitor by preserving the transsulfuration pathway, and thereby the biosynthesis of cysteine and GSH. DJ-1 depletion leads to lipid ROS accumulation and a heightened sensitivity to ferroptosis cell death [44]. The discovery of DJ-1 as a ferroptosis suppressor further supports that ferroptosis is implicated in PD pathology.

One would anticipate that such changes to GSH levels and the increase in LOOH would impact upon the expression and activity of key antioxidant enzymes such as GPX4. In post-mortem tissue from PD patients' brains, protein levels of GPX4 are increased compared to control subjects [124]. More recently, GPX4 levels were confirmed to be increased relative to cell density of surviving neurons, despite the apparent reduction when measuring against total tissue [125]**. This implies that it is only the remaining neurons in the SN of PD patients that were able to counter an oxidative and pro-ferroptotic environment through an appropriate GPX4 response to prevent cell death** [125]**Interestingly, this phenomenon has also been reported in *in vitro* and *in vivo* models of stroke** [83,84].

Recent advances in imaging technology are increasing our capability to measure oxidative stress in living PD patients using positron emission tomography (PET). Copper(II)-diacetyl-bis(4-methylthiosemicarbazonato) (Cu-ATSM) is a PET tracer initially developed for hypoxia imaging but has recently shown potential in reflecting the redox state within the body [126]. Interestingly, this brain blood barrier penetrant imaging agent was identified as being neuroprotective in multiple animal models of Parkinson's disease [127] and prevent lipid peroxidation without altering the oxidation state of iron. Similar to Fer-1 and Lpx-1, Cu-ATSM may block ferroptosis by preventing the propagation of lipid radicals rather than preventing iron oxidation [128] and could offer an opportunity to monitor disease progression as well as efficacy in PD patients during treatment. While functional imaging of oxidative stress is currently considered to mainly detect mitochondrial dysfunction, it still appears sensitive enough to show an elevation of striatal oxidative stress in PD patients when compared to controls [129].

Despite the strong evidence implicating oxidative stress in PD pathology and the neuroprotective properties of antioxidants reported in models of PD, outcomes from clinical trials have remained inconsistent (reviewed in [116]). Clinical studies with exogenous

antioxidant therapies are mixed with some reporting efficacy [130–132] whilst others fail to demonstrate a significant effect [133]. A major component of this disparity could lie with many clinical trials not having the appropriate pharmacokinetic measurements taken, a lack of confirmation in target engagement in the brain, inappropriate treatment duration and/or uncontrolled variances in endogenous antioxidant potential within the patient.

3.2.1 The role of lipid peroxidation in PD pathology

Lipid metabolism and cellular lipid composition can determine cellular sensitivity to ferroptosis. Lipidomic analyses revealed that polyunsaturated-fatty-acid-containing phospholipids (PUFA-PLs), and in particular phosphatidylethanolamines (PE) containing arachidonic acid (AA) or adrenic acid (AdA) in the plasma membrane, are the lipids most susceptible to ferroptosis-related peroxidation [134,135]. The ability of cells to undergo ferroptosis is therefore determined by the abundance and cellular localisation of PUFA, as confirmed by the fact that human cells enriched with AA are sensitised to ferroptosis [59]. As previously mentioned, free PUFAs undergo esterification by ACSL4 before incorporation into the plasma membrane phospholipids by LPCAT3 [74]. The bis-allylic carbons, adjacent to two carbon atoms with double bonds, are the key positions within lipids that drive ferroptosis as they increase susceptibility to attack from reactive radicals, lipoxygenases and surrounding lipid peroxides [59]. Thus, the peroxidation potential of a PUFA is linearly dependent on their number of double bonds [136]. The process of lipid peroxidation takes place via three steps: initiation, propagation and termination. Firstly, reactive radicals abstract a hydrogen atom from a bis-allylic carbon to form the carbon-centred lipid radical (PL·). Such radicals rapidly react with oxygen forming lipid peroxyl radicals (PLOO·), which can subsequently abstract further hydrogens from neighbouring lipids to propagate the generation of new PLOO· and lipid hydroperoxides (PLOOH) [137].The lipid peroxidation

reaction can be inhibited by the FSP1-CoQ10H_2 system or when antioxidant enzymes, such as GPX4, donate electrons and reduce the pLOOH to PLOH (Figure 2). In ferroptosis, an insufficient GPX4 activity leads to an overwhelming accumulation of LOOH.

Noteworthy, the brain has the second highest concentration of lipids, after adipose tissue. The proportion of these lipids are largely AA and docosahexaenoic acid (DHA) which contain four and six double bonds respectively [138]. Furthermore, the high oxygen consumption of the brain makes it particularly sensitive to lipid peroxidation. Dexter *et al*, were the first to demonstrate the involvement of lipid peroxidation in PD as a cause of nigral cell death [35]. Brain post-mortem analyses revealed a reduction in PUFAs but not MUFAs in the SN of PD patients compared to controls (D. Dexter et al. 1986). Conversely, levels of malondialdehyde (MDA), a toxic by-product of lipid peroxidation, were elevated in the same tissue [36] and LOOH increased in plasma [139]. MDA levels were increased both in early and late PD patients whilst LOOH levels were only significantly increased in later stages of the disease. This suggests that while MDA could be a useful biomarker for PD, changes in LOOH levels may correlate better with disease progression [37]. Further studies have revealed a correlative increase in another lipid peroxidation metabolite, 4-hydroxy-2-nonenal (HNE), as well as HNE-protein adducts, with pathology from Lewy bodies in the SN of PD patients and brainstem of DLB [140]. Interestingly, in the CSF of PD patients, elevated HNE correlates with an accumulation of iron in the SN [140,141].

The mechanism by which lipid peroxidation is involved with PD pathology was expanded upon by Shchepinov *et al* whereby MPTP treated mice were protected against nigrostriatal injury upon supplementation with deuterated-PUFAs (D-PUFAs) [142]. D-PUFAs have deuterium in the place of the bis-allylic hydrogens, which slows radical generation compared to Hydrogenate-PUFAs [142]. More recently, *Yang et al* confirmed that pre-treating cells with D-PUFA prevents PUFA oxidation and ferroptosis[59].

The 85-kDa group VI calcium-independent phospholipase A2 beta (PLA2G6) is another key enzyme with possible implications to ferroptosis. This enzyme hydrolyses the sn-2 acyl chain of glycerophospholipids to release free fatty acids and lysophospholipids. PLA2G6 localizes to the mitochondria and has proposed roles in the remodeling of membrane phospholipids, signal transduction, calcium signaling, cell proliferation and cell death[143]. Patients with homozygous PLA2G6 mutations present a form of neurodegeneration with brain iron accumulation (NBIA) that has neuropathological similarities to both Parkinson's and Alzheimer's disease: as well as iron accumulation, these include widespread Lewy bodies, dystrophic neurites and cortical neuronal neurofibrillary tangles[144]. In a recent study, Kinghorn *et al*, showed that knocking-out the *drosophila* homologue of the PLA2G6 gene, iPLA2-VIA, resulted in reduced survival, locomotor deficits, organismal hypersensitivity to oxidative stress (in particular the mitochondria) and a strong association with increased lipid peroxidation levels [145]. D-PUFA has also shown to rescue the aged-associated locomotor abnormalities and restore mitochondrial membrane potential in this model.

An elevation of iron in combination with high levels of PUFAs within dopaminergic neurons creates an environment particularly sensitive to lipid peroxidation meaning that a slight imbalance in iron, dopamine or lipid homeostasis could sensitise dopaminergic neurons to ferroptosis. Characterising the distinct and regulated pathways of lipid peroxidation sheds light in deciphering the neuropathology involved in nigral cell death in PD and encourages the pursuit of therapeutic strategies that will inhibit ferroptosis.

3.3 The role of other ferroptosis regulators in PD pathology

Nuclear factor erythroid-2-related factor 2 (Nrf2), a master regulator of the antioxidant **response, was recently shown to protect against ferroptosis** [146,147]**.** Under

oxidative stress, Nrf2 is translocated to the nucleus to induce the expression of endogenous antioxidant **proteins responsible for preventing lipid peroxidation. Of relevance to ferroptosis, continuous exposure to erastin results in Nrf2-dependent upregulation of cystathionine β-synthase (CBS), the enzyme responsible for the biosynthesis of cysteine, in an attempt to counter cell death** [147]**. Nrf2 also controls the expression of NAD(P)H:quinone oxidoreductase 1** [148]**, several iron metabolism proteins (e.g. ferritin and ferroportin), GPX4** [149] **and other key ferroptosis proteins involved in GSH biosynthesis (e.g. XcT, glutamate-cystein ligase and gluthathione synthetase)** [147,149,150] **(reviewed by** [151]**).** Nrf2 has been extensively studied in the context of PD pathology where an age-related decline in activity leads to reduced GSH levels [152,153]**. In PD patients, Nrf2 and downstream effectors are highly transcribed in blood leukocytes compared to controls** [154]**. Interestingly, these Nrf2 transcripts correlate with PD duration, suggesting that Nrf2 plays a role in fighting the intrinsic oxidative stress observed during disease pathology. Furthermore, the cellular localisation of Nrf2 appears to be predominantly nuclei in the SN of PD patients, in contrast to the cytoplasmic location in affected brain regions from other neurodegenerative diseases such as AD or Lewy body variant of AD** [155]**. Such nuclear translocation in PD indicates a cellular and/or disease dependent recruitment of Nrf2 caused by an intrinsic vulnerability of dopaminergic neurons to oxidative stress.** *In vitro*, activation and nucleus translocation of Nrf2 is also protective against MPP+ insult [156], **and sufficient in preventing locomotor impairment as well as neuronal loss in a *drosophila* model of PD** [157]**. It is worth noting that Nrf2 response to oxidative stress is not unique to ferroptosis as Nrf2 inhibition has also been implicated in apoptosis cell death** [158]**. Therefore, the role of Nrf2 in PD may be associated with several forms of cell death.**

NADPH is an intracellular reductant involved in the elimination of LOOH. Intracellular NADPH levels are considered a biomarker for ferroptosis sensitivity, but accurate measurement of NADPH levels is difficult in patients. Several studies with biofluids from PD patients have demonstrated an altered level of NADPH oxidase; an enzymatic complex which oxidises NADPH to generate oxygen species. The NADPH oxidase subunits NADPH oxidase 1 (NOX1) and 4 (NOX4) are both increased in the SN in several PD mammalian models [159,160] as well as PD patients [161].

CoQ10 and its reduced form CoQ10-H_2 are potent mitochondria and lipid ROS antioxidants also considered as endogenous ferroptosis inhibitors [30]. The role of **CoQ10 as a ferroptosis inhibitor** has been further established by the two recent studies that show FSP1 as being instrumental in regenerating CoQ10 from NADPH and having an ability to suppress both phospholipid peroxidation and ferroptosis independent of GPX4 [47,55]. Interestingly, CoQ10 is reduced in patients and animal models of PD [45, 46], resulting in increased ROS production. CoQ10 supplementation can decrease lipid peroxidation markers in the plasma, liver and brain of PD mouse models [162] as well as protect against MPTP induced dopaminergic neurodegeneration and α-syn aggregation [163]. The lack of a beneficial outcome of CoQ10 in a randomized early PD clinical trial [133] may have arisen from challenges around its **biodistribution in the central nervous system. Duration of treatment is another factor to take into consideration as the CARE-HD (Coenzyme 10 and Remacemide Evaluation in Huntington's Disease) study only indicated a benefit after two years of treatment [164]. A related alternative treatment rationale could be** CoQ10-H_2 , as a **3-fold higher plasma concentration can be achieved compared to oxidised CoQ10** [165], or the introduction of a combinatorial therapy with currently used drugs and/or **ferroptosis inhibitors.**

Selenium (Se) is considered a key element in the cellular antioxidant machinery as it is crucial for selenocysteine formation and the synthesis of selenoproteins such as GPX4[166]. Se abundance can thus impact upon ferroptosis sensitivity, whereby supplementation promotes ferroptosis resistance and a deficit leads to increased sensitivity, presumably through modulating GPX4 levels and activity [167]. In line with an intrinsically high oxidative environment, the level of Se in the brain is highest in the SN and caudate[168]. Multiple studies report a protective role of Se in several PD models: Se supplementation reduces motor impairments and DNA damage in a rat model in which Paraquat induces parkinsonism [169] and a single dose of Se was sufficient to reverse the depletion of striatal dopamine and its metabolites in the MPTP mouse model [170]. In line with GPX4 expression levels, Selenoprotein P, a peptide with a high content of selenium in the form of selenocysteine, was reportedly reduced in PD SN compared to control brains but increased relative to cell density (Bellinger et al. 2012).

Extensive research has independently linked several components of the ferroptotic pathway to the pathology underlying neuronal degeneration in PD. In serum, the significant diagnostic ability, measured using ROC analysis (AUC: 0,94), has been used to identify the combination of NOX1 and Se as a promising diagnostic biomarker for PD (Hemmati-Dinarvand et al. 2017.

3.4 Implications on the role of α-synuclein in ferroptosis

Aggregated α-synuclein (α-syn), one of the main components in intracellular Lewy Bodies [5], has long been considered a key pathological hallmark of the disease. The relationship of α-syn to disease pathology has been confirmed by genome wide association

studies, where single nucleotide polymorphisms associate with sporadic PD risk whilst confirmed mutations and duplication/triplication in the *SNCA* gene (encoding α-syn) lead to various clinical manifestations ranging from classical to early onset familial PD [173,174]. Despite the involvement of α-syn in PD pathology, the exact physiological function of this protein and the mechanisms linking it to neurodegeneration remain elusive**. Establishing a synucleinopathy link to ferroptosis would strengthen the implication of this novel type of cell death in PD.** To this end, multiple studies have increasingly shown α-syn to have a role in regulating both iron and lipid metabolisms with inference to the ferroptosis pathway (Figure 3). **Interestingly, iron chelators, D-PUFAs, and ferrostatin all suppress cell death induced by toxic α-syn oligomers, meeting the basic criteria set out to define ferroptosis [30,134,175].**

3.4.1 α-synuclein and iron metabolism

Over the past two decades, the interactions between α-syn and iron metabolism have been extensively studied and reviewed (e.g. [113,176,177]). However, since the emergence of ferroptosis, this relationship should be reassessed and placed in the ferroptosis context. Both Fe^{2+} and Fe^{3+} strongly bind to α-syn and promote its oligomerization by converting this intrinsically disordered protein into a β-sheet structure [178–182]. Iron exposure to neuronal cultures overexpressing α-syn with a familial mutation (A53T α-syn) increase aggregate formation and vulnerability to iron induced toxicity [183]. Furthermore, α-syn oligomers interacting with iron in neurons induce ROS and lipid peroxidation production, reduce GSH levels [184], abd have subsequently been shown to induce ferroptosis via iron-dependent oxidation [175]. This is particularly poignant with the knowledge that the dopaminergic neurons, susceptible in PD, are high in iron and have an intrinsically high oxidative environment due to their dopamine metabolism. Iron chelation has not only been shown to

be neuroprotective against PD related neurotoxin insult (i.e MPTP, 6-OHDA and Paraquat) but can reduced α-syn aggregation *in vitro* and rescue behavioural deficits induced by iron exposure in a mouse model of α-syn aggregation [185].

While iron can modulate the biophysical nature of α-syn, this protein may also have a role in neuronal iron homeostasis. α-Syn contains an iron response element (IRE) within its 5'UTR mRNA region; a binding site involved in regulating the translation of the protein upon modulation of neuronal iron load [186]. As an iron regulated protein, iron depletion causes a decrease in translation of α-syn[187] whilst overexpression of α-syn in neurons results in higher levels of Fe^{2+} [188]. It has been suggested that α-syn acts as a ferrireductase reducing Fe^{3+} to Fe^{2+} [183] and increases susceptibility to iron-dependent ROS and LOOH production [188]. More recently, Baksi and colleagues have proposed that α-syn directly mediates iron metabolism by facilitating the uptake of transferrin-bound iron, and colocalizes with TfR1 in the plasma membrane. Depletion of α-syn results in TfR retention in recycling endosomes and subsequent depletion of cellular iron stores [189] whilst an increase in α-syn can afect lysosomal activity by disrupting the trafficking of lysosomal hydrolases and impairing ferritinophagy [190]; a process linked to ferroptosis [191,192]. An alternative mechanism in which α-syn is proposed to modulate cellular iron import is through an ability to upregulate the iron transport protein DMT1. Bi *el al*, showed that α-syn-induced p38 mitogen-activated protein kinase (MAPK) phosphorylation of parkin inactivates its E3 ubiquitin ligase activity and reduces DMT1 degradation via ubiquitylation [104].

3.4.2 α-syn and lipid metabolism

Substantial evidence linking α-syn with cellular lipid **metabolism further implicates α-syn in ferroptosis**. Firstly, α-syn has a high degree of sequence homology with apolipoproteins and binds lipids through its N-terminal region. Of particularly high

binding affinity are the PUFAs α-linolenic acid, DHA and eicosapentaenoic acid[193,194]. When exposed to free or phospholipid-bound PUFAs, α-syn undergoes structural changes including an increased propensity to oligomerize [195–198], whereas monosaturated fatty acids (MUFAs) have no effect on α-syn aggregation [194]. Lipid peroxidation products from PUFA, such as HNE, also induce modifications to α-syn and equally promote the formation of toxic oligomers in human neuroblastoma cells [199]. Conversely, α-syn modulates the metabolism of certain membrane PUFAs including **linoleic acid, dihomo-gamma-linoleic acid, AdA and AA. Both the lipid ratio of these PUFAs in the plasma membrane and the membrane fluidity are increased when** α-syn **is overexpressed in neuronal cultures as well as in brain tissue from PD and DLB patients [139]. In contrast, the** cytosolic fatty acid composition is altered and the membrane fluidity reduced in brains of α-syn KO mouse [139]. A separate study also suggests that α-syn contributes to membrane remodelling by sensing lipid packing defects and inducing lateral expansion of lipids [200]. These findings strongly indicate that α-syn has a role in membrane fatty acid compositions and thereby regulating membrane fluidity, vesicle assembly and subsequent synaptic transmission. [201]Finally, technological advances in transmission electron microscopy (TEM) in association with light microscopy imaging has enabled a clearer understanding of Lewy Body composition, revealing a high level of membranous content, fragmented organelles and vesicles at the core of α-syn immunoreactivity [3].

Overall, iron and PUFA dependent studies suggest that α-syn's physiological and/or pathological functions may generate, over time, a pro-ferroptotic environment in dopaminergic neurons. Specifically how α-syn functionally regulates membrane composition remains unclear but Golovko and colleagues have shown *in vivo* that α-syn

plays a key role in the metabolism of brain AA [201]; **the main substrate of LOX-15 and ACSL4, two enzymes implicated in the ferroptosis pathway. Direct or indirect enrichment of cellular membranes with AA, amongst other PUFAs, by** α-syn **under the pathological conditions of elevated free labile iron and oxidative stress, may lead to further lipid peroxidation and drive neurons towards ferroptosis. The recent finding that α-syn oligomers bind the plasma membrane to drive ferroptosis cell death through lipid peroxide generation provide the first direct evidence to support ferroptosis as a pathological mechanism in synucleinopathies [175]. However, further research is essential to strengthen this hypothesis and establish whether α-syn's ability to regulate both iron and lipid homeostasis in neurons are also implicated in the ferroptosis pathway.**

4. CONCLUSION AND FUTURE PERSPECTIVES

New disease modifying therapies and novel therapeutic strategies are in high demand for PD patients. An emerging knowledge on ferroptosis is shedding a different perspective on several physiological and pathophysiological conditions. Indeed, for decades, researchers have been heavily characterising several aspects of PD pathology as independent components, which now may be linked in conferring susceptibility to ferroptosis. These include elevated lipid peroxidation, glutathione depletion**, DJ-1 and CoQ10 deficiency, GPX4 reduction,** mitochondriopathy, iron accumulation and α-synuclein aggregation. Based on this information **it is hard to believe that the extensive similarities between** PD neuropathology **and aspects of the ferroptosis cell death pathway are due to a mere coincidence**. We therefore propose ferroptosis as a key contributor to PD progression with broader implications in synucleinopathies. **Deciphering the role of α-synuclein in the iron and/or lipid components of the ferroptotic pathway now represents an area of increased research focus that is hoped to not only provide a greater understanding to**

the physiological function of the protein but also elucidate one of its neuropathological features in PD.

The fact that iron chelation, an established anti-ferroptotic strategy, has shown the first clinical benefits in two independent clinical trials on early-PD [98,114] should encourage further progress in targeting ferroptosis. Ultimately, the role of ferroptosis in neurodegenerative disorders will only be confirmed when additional anti-ferroptotic therapies advance successfully to clinical trial.

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advisory boards, served as a consultant and given lectures for pharmaceutical companies such as Orkyn, Aguettant, Abbvie, Medtronic, Novartis, Teva, UCB, Lundbeck.

Laura Mahoney-Sanchez, Hind Bouchaoui, Scott Ayton and Jean Christophe Devedjian have nothing to declare.

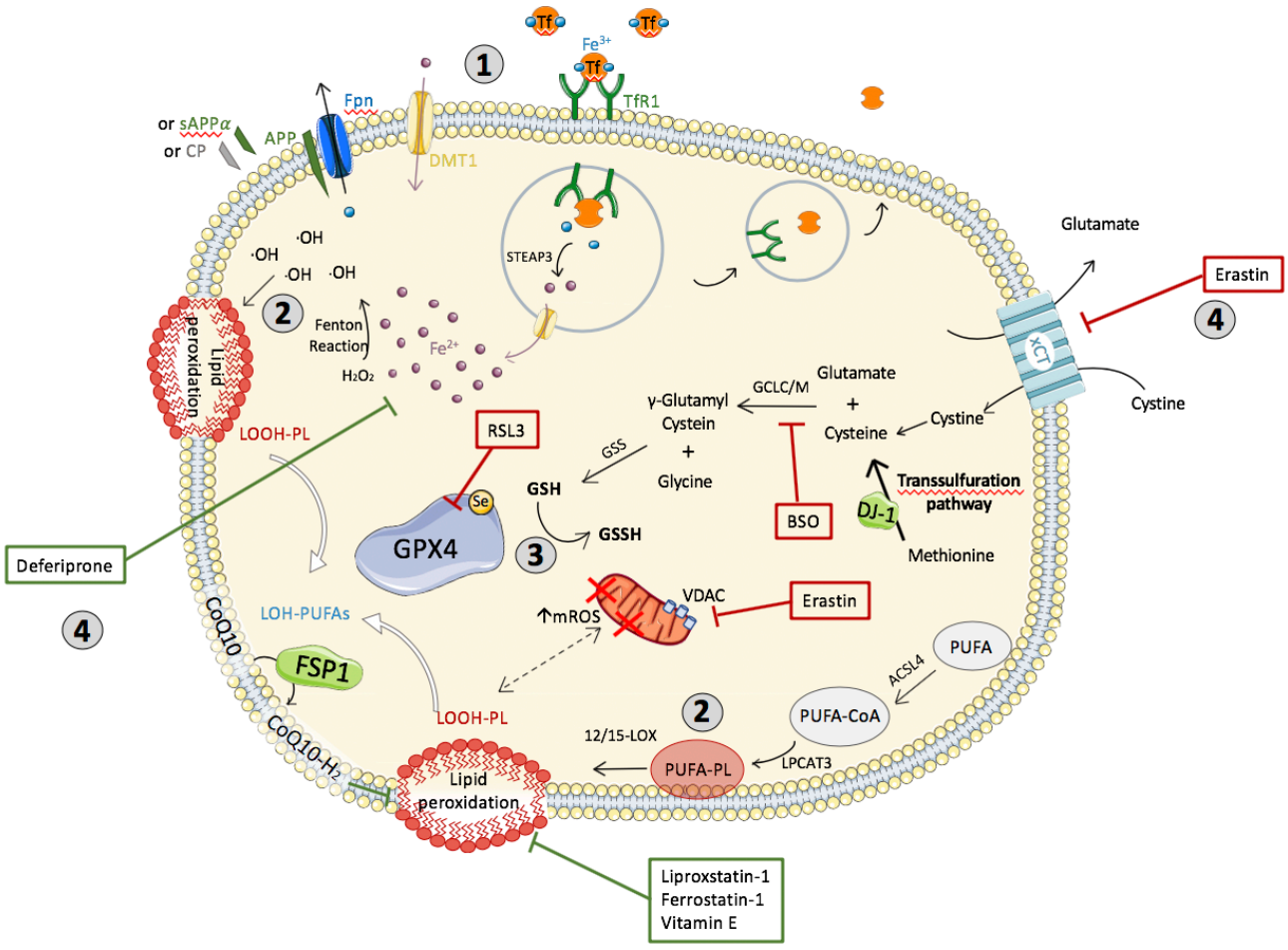

Figure 1: The ferroptosis pathway. Alterations in the iron regulatory pathway and phospholipid oxidation are implicated in Parkinson's disease pathology. **1.** Increased intracellular iron occurs by enhanced import of iron within transferrin (Tf) through Transferrin receptor (TfR), and increased import of Fe^{2+} through the divalent metal transporter 1 (DMT1). In addition, iron export is impaired through the destabilization of

ferroportin (Fpn) on the cell surface by β-amyloid precursor protein (APP) or ceruloplasmin (CP). **2.** An elevated labile iron pool catalyzes the formation of phospholipid hydroperoxides. Free cytosolic polyunsaturated fatty acids (PUFA) are conjugated to coenzyme-A (CoA) by acyl-CoA synthetase long-chain family member 4 (ACSL4) allowing PUFA-CoA to be incorporated into the phospholipids in the plasma membrane. Phospholipid-PUFA are oxidised by lipoxygenases 12/15, contributing to the accumulation of phospholipid hydroperoxides at the plasma membrane level. Mitochondrial dysfunction result in increased ROS production which may also contribute to lipid peroxidation in the plasma membrane. **3.** Cystine uptake through the X_{c-} antiporter is required for biosynthesis of glutathione (GSH). Glutathione peroxidase 4 (Gpx4) uses 2 GSH molecules to safely reduce phospholipid hydroperoxides to their corresponding lipid-alcohols, producing H_2O and glutathione disulphide (GSSG) as byproducts. Elevated levels of intracellular iron with depletion of Gpx4, as evidenced in models of PD, promotes the accumulation of phospholipid hydroperoxides leading to a disruption in membrane integrity through a ferroptotic pathway. **4.** Ferroptosis is induced by Erastin that blocks Cystine import, GSH biosynthesis and mitochondrial VDAC, and/or directly inhibiting GPX4 with RSL3. Conversely, reducing the labile iron pool (i.e deferiprone) or depleting the phospholipid hydroperoxides (i.e. liproxstatin-1, ferrostatin-1or vitamin E) are promising targets for inhibiting ferroptosis in PD pathology. FSP1 serves as a ferroptosis suppressor by regenerating CoQ10, whose reduced form – CoQ10-H_2 traps membrane lipid hydroperoxides preventing lipid peroxidation propagation.

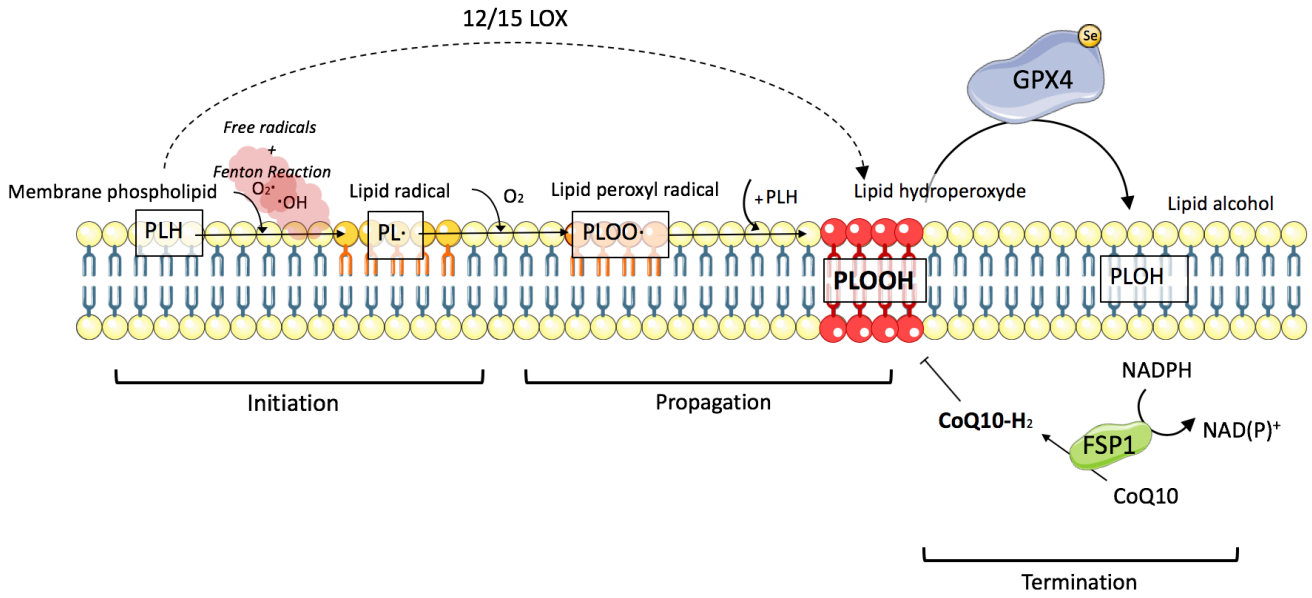

Figure 2: Membrane lipid peroxidation.

The process of membrane phospholipid peroxidation takes place via three steps: initiation, propagation and termination. The Fenton reaction and free reactive radicals abstract a hydrogen atom from the phospholipid carbon chain forming a lipid radical (PL·). These radicals rapidly react with oxygen and form lipid peroxyl radicals (PLOO·), which can subsequently react with neighbouring lipids to propagate the generation of new lipid peroxyl radicals and lipid hydroperoxides (PLOOH). The lipid peroxidation reaction is terminated when antioxidant elements or enzymes, such as GPX4, reduce the lipid peroxides to lipid alcohols (L-OH). The FSP1 - CoQ10 - NAD(P)H system works in parallel to GPX4 in suppressing lipid peroxidation at membranes and subsequent cell death by ferroptosis.

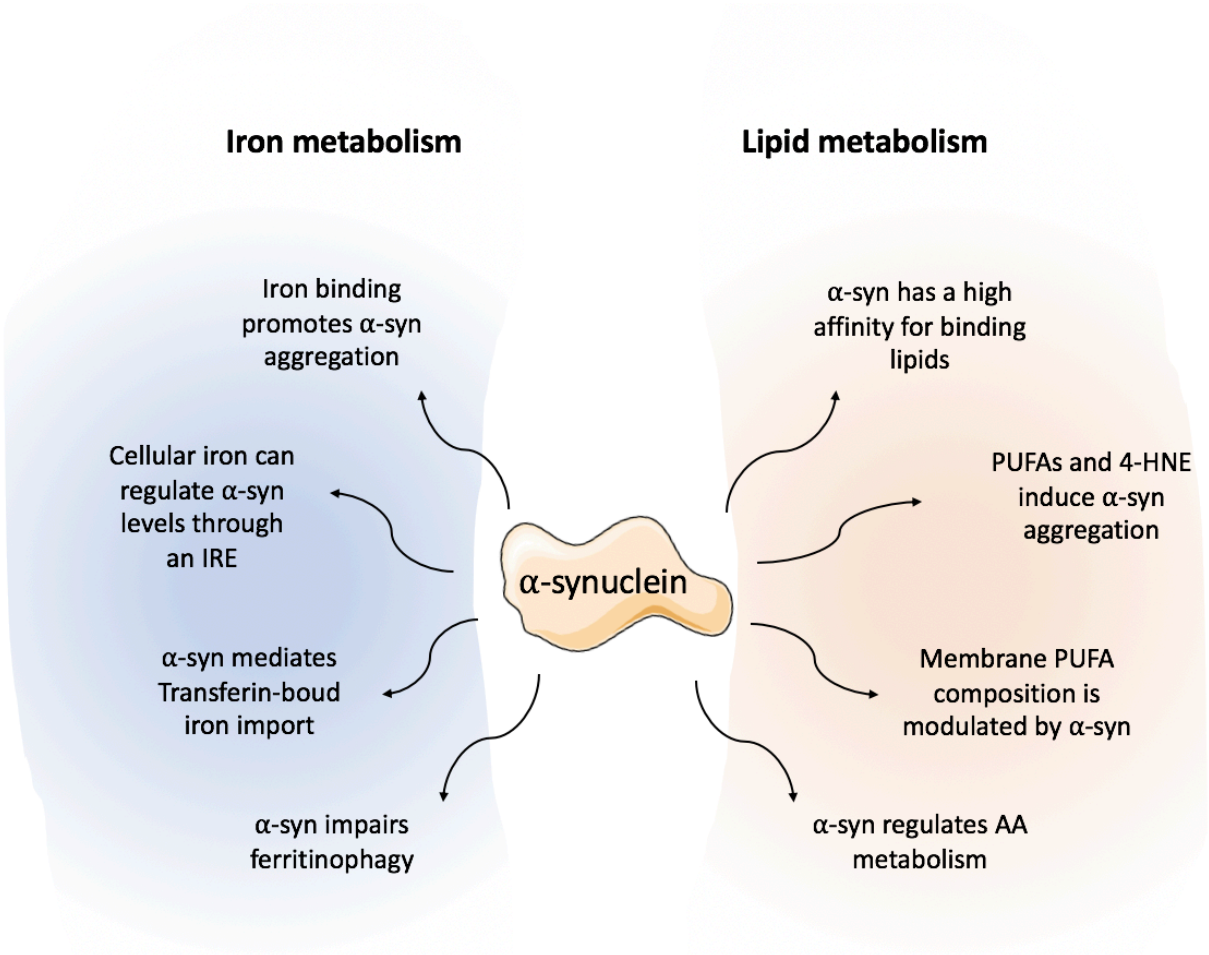

Figure 3: The iron and lipid metabolism interplay with α-synuclein.

Increasing studies are linking α-syn to metabolism of iron and lipid, in particular PUFAs, suggesting a possible role of α-syn in ferroptosis.

Table 1: Features of Parkinson's Disease Pathology consistent with Ferroptosis

A list of known Parkinson's disease pathology hallmarks common to Ferroptosis that support the role of this novel cell death in the disease pathogenesis.

Feature	Comment	References
Decreased XcT- and GSH	DNA methylation analysis revealed downregulation of SLC7A11 gene. Measures in post mortem brain regions from PD revealed reduction in GSH levels in the SN of PD patients	(Vallerga et al, 2020 ; Pearce et al, 1997; Sian et al, 1994; Sofic et al, 1992)
Altered brain PUFA composition	Post-mortem analyses reveal a reduction of PUFAs in the SN of PD patients	(Dexter et al, 1989)
Elevated lipid peroxidation products	HNE and MDA are elevated in the SN of PD brains and associated with iron accumulation. HNE is equally elevated in the CSF of PD patients	(Dexter et al, 1989; Domenico et al, 2017; de Farias et al, 2016)
Decrease GPX4 in Substantia Nigra	In post mortem analysis: reduced GXP4 levels in the SN in PD brains, but increased relative to cell density of surviving neurons	(Bellinger et al, 2011)
Increased iron in SN	MRI and QSM analyses confirm iron accumulation in the SNpc in PD patients. Iron concentrations correlate with disease severity	(Dexter et al, 1987, 1988; Hirsch et al, 1991; Hopes et al., 2016; Wang et al., 2017)
Clinical benefits of Iron Chelation	A double-blind, randomized, placebo-controlled clinical trial of early-stage PD showed a decreased motor handicap progression and reduced iron deposits in the SN of PD patients taking DFP. An ongoing phase 3 multicentre clinical trial will assess DFP as a disease modifying treatment	(Devos et al, 2014)
Decreased CoQ10 levels	Levels of the antioxidant CoQ10 are reduced in PD animal models and PD patients	(Battino et al., 1996; Mischley et al., 2012)

DJ-1 depletion	DJ-1 loss of function mutations are associated with early-onset PD. DJ-1 is a negative ferroptosis regulator as it maintains the cysteine and GSH biosynthesis through the transsulfuration pathway	(Burbulla et al, 2017; Cao et al, 2020)

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