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Ferroptosis as a mechanism of neurodegeneration in Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is the most prevalent form of dementia, with complex pathophysiology that is not fully understood. While β-amyloid plaque and neurofibrillary tangles define the pathology of the disease, the mechanism of neurodegeneration is uncertain. Ferroptosis is an iron-mediated programmed cell death mechanism characterised by phospholipid peroxidation that has been observed in clinical AD samples. This review will outline the growing molecular and clinical evidence implicating ferroptosis in the pathogenesis of AD, with implications for disease-modifying therapies.

Keywords: Alzheimer's disease, ferroptosis, iron, phospholipid peroxidation and neurodegeneration

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1 Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia and a leading cause of disability and death in older people (>65 years) worldwide. It is characterised pathologically by extracellular deposition of amyloid-beta (Aβ) that form senile plaques and accumulation of the abnormally modified tau proteins that comprise neurofibrillary tangles. The amyloid cascade hypothesis (first proposed in 1992) is the long postulated pathological model of AD, which positions Aβ as the initial pathological event in AD. More than 30 phase 3 clinical trials of drugs targeting Aβ were unsuccessful in slowing cognitive decline in AD. However, in a controversial decision that has divided the field, the Food and Drug Administration (FDA) in the USA has recently granted accelerated approval of the anti-amyloid immunotherapy, aducanumab, as a disease-modifying drug for AD. The events and discussion points surrounding this approval have been commentated on at length, and it is beyond the scope of this review to reiterate them. But regardless of the potential benefit of anti-Aβ drugs, it is clear that the disease velocity is only marginally slowed by aducanumab and other Aβ antibody-based drugs, and there is a need for alternative or concurrent therapies to deliver substantial clinical impact. Understanding the molecular events that lead to damage downstream of Aβ pathology holds promise for new therapeutic avenues.

Iron elevation was one of the first described chemical changes in AD (Goodman 1953) and is a candidate target for disease-modifying therapies. In the brain, iron has a crucial role in various physiological processes, including oxygen transportation, mitochondrial respiration, DNA synthesis, and the synthesis and metabolism of neurotransmitters (Ward *et al.* 2014). However, as it can undergo redox cycling, labile iron also catalyses the formation of reactive oxygen species (ROS) via the Fenton reaction and facilitates the redox biology of many pro-oxidant enzymes, including lipoxygenase (Ward et al. 2014, Belaidi & Bush 2016).

Iron may also bind to and cause the aggregation of Aβ and tau (Smith *et al.* 2010, Liu *et al.* 2011). While iron has long been implicated in inducing pathology deposition and contributing to toxicity via oxidative stress, a putative role for iron in AD pathogenesis has been revitalised by discovering the cell death mechanism, ferroptosis. Ferroptosis is a unique form of iron-mediated programmed cell death evolutionarily conserved among eukaryotes (Plantae, Fungi and Animalia kingdoms), protozoa, and archaea (Dixon *et al.* 2012, Tang *et al.* 2021). The term "ferroptosis" was coined recently (2012); however, research on this type of cell death has its roots in work pioneered by Harry Eagle in the 1950s and 1960s, who demonstrated that amino acid cysteine deprivation led to cell death (Eagle 1955), while

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endogenous synthesis of cysteine protected against cell death (Coltorti *et al.* 1956, Eagle *et al.* 1961). Similar studies in the 1970s (Mitchell *et al.* 1973, Bannai *et al.* 1977) also demonstrated that cystine starvation reduced glutathione levels and caused cell death, while lipophilic antioxidant, α-tocopherol (a type of vitamin E), rescued cell death without restoring glutathione levels (Bannai et al. 1977), and acetaminophen-induced hepatic necrosis accompanied by glutathione depletion in mice was shown to be rescued by pre-treatment of glutathione or cysteine (Mitchell et al. 1973). Joseph Coyle's group in the late 1980's (Murphy *et al.* 1988, Murphy *et al.* 1989) discovered that glutamate-induced cell death that was dependent on inhibition of cystine transport was later assigned the name oxytosis in 2001 (Tan *et al.* 2001, Maher *et al.* 2020, Ratan 2020), with many now regarding oxytosis a sub-type of ferroptosis.

Ferroptotic cell death results from a redox inequity between iron-induced production of lipid hydroperoxides and several antioxidant defence layers, principally glutathione-dependent glutathione peroxidase 4 (GPX4) that detoxify free radicals and lipid oxidation products (Bersuker *et al.* 2019, Yang *et al.* 2014). In mammals, ferroptosis has been implicated in suppressing tumours and immunity (Tang et al. 2021) and pathologically in degenerative and ischemic diseases (Yan *et al.* 2021). The evidence of iron elevation and lipid peroxidation products in the AD brain implicates the role of ferroptosis in the pathogenesis of AD. While several prior reviews have focused on ferroptosis in Alzheimer's and other neurodegenerative diseases (Maher et al. 2020, Reichert *et al.* 2020, Derry *et al.* 2020, Ashraf & So 2020, Ficiarà *et al.* 2021, Vitalakumar *et al.* 2021, Zhang *et al.* 2021), the rapid accumulation of new findings in this field warrants an updated analysis and contextualisation in the extant literature. Here, we review the evidence for ferroptosis in the pathophysiology of AD and discusses its potential as a therapeutic target.

2 Iron, lipid peroxidation and experimental ferroptosis

Owing to its ability to undergo redox cycling, iron acting alone or as a cofactor in an enzyme can promote radical oxygen species that causes generalised oxidative damage to proteins and lipids. Ferroptosis is a type of oxidative stress that centrally involves the peroxidation of plasma membrane phospholipids. When fully expressed, ferroptosis is a cellular death event ultimately caused by iron-redox reactions but involves a host of feedback and feedforward cellular responses. In this regard, 'ferroptotic stress', which precedes the cell death event, is considered both the aberrant redox chemistry upon membrane phospholipids promoted by iron and also the limitation of defence against this redox imbalance, principally (but not

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exclusively) by glutathione-dependent GPX4. Ferroptosis is not merely iron overload because ferroptosis can be induced by limiting the defence against iron redox reactions without changes to iron levels – indeed, this is the canonical instigator of ferroptosis. So ferroptosis is not simply iron toxicity, but nor is it simply 'oxidative stress'. For example, hydrogen peroxide intoxication, a classical inducer of oxidative stress, responds poorly to classical anti-ferroptotic compounds (Wenz *et al.* 2018). Oxidative stress of a more general nature that is induced by iron can cause peroxidation of proteins and non-membrane lipids, yet these species can conceivably kindle membrane lipid peroxidation or divert the anti-oxidant resources within the cell from protecting against ferroptosis. So, any increase in oxidative load by iron can contribute to chronic ferroptotic stress, and therefore, these concepts cannot be fully delineated. This section discusses the underlying mechanism of iron-mediated redox dyshomeostasis and lipid peroxidation, which, in turn, can contribute to ferroptotic stress.

Polyunsaturated fatty acids (PUFAs; long-chain fatty acids contain more than one double bond), including arachidonic, linoleic and docosahexaenoic acids, are essential components of cell membrane phospholipids (Brand *et al.* 2010) but are also the principal fuel of ferroptosis. PUFAs are highly susceptible to lipid peroxidation due to their reactive hydrogens. They may undergo peroxidation by free 'labile' iron or by iron contained within lipooxygenase enzymes, particularly 12/15 lipoxygenases (Li *et al.* 1997, Khanna *et al.* 2003, Yang *et al.* 2016). Lipoxygenases are ordinarily found in the cytosol but bind the scaffolding protein, PEBP1 (Wenzel *et al.* 2017), which draws these enzymes to the membrane permitting peroxidation of membrane PUFAs.

Lipid peroxidation is categorised into three phases: initiation, propagation, and termination (Lane *et al.* 2018, Dodson *et al.* 2019). In the initiation phase, ROS, reactive nitrogen species and reactive lipid species remove a hydrogen atom from an allylic carbon, specifically in membrane PUFAs, which helps to form a lipid radical (L•). The Fenton reaction, the interaction of ferrous iron (Fe^{2+}) with hydrogen peroxide (H_2O_2), generates the two notable ROS initiators of lipid peroxidation: the hydroxyl radical (OH•) and hydroperoxyl radical (OOH•). Reactive nitrogen species such as peroxynitrite ($ONOO^-$) can also initiate lipid peroxidation because of the interaction between nitric oxide (NO•) and superoxide (O_2•-).

The formed lipid radical rapidly reacts with oxygen to form a lipid peroxyl radical (LOO•) during the propagation phase. LOO• then reacts with another PUFA to generate lipid peroxide (LOOH) and a new L•. The propagation stage persists until a termination reaction occurs by either lack of lipid substrates or endogenous cellular antioxidants such as vitamin E

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or glutathione that donate a hydrogen atom to form a stable non-radical product. Glutathione peroxidases (for instance, GPX4) can reduce the formed lipid peroxides during the propagation phase to lipid alcohols; otherwise, lipid peroxides degrade into hydroxy fatty acids or reactive aldehydes malondialdehyde (MDA) and 4-hydroxy-2-nominal (4-HNE). GPX4 is unique among glutathione peroxidases at being able to detoxify lipid hydroperoxides directly in membranes, and for that reason, it is the main ferroptosis checkpoint. Reactive lipid species that are not detoxified can cause extensive peroxidation of lipids, leading to alteration of the assembly, composition, structure and dynamics of lipid membranes, and ultimately death of the cell.

Experimental ferroptosis

Ferroptosis can be induced experimentally by several small molecule compounds that directly or indirectly inhibit GPX4, leading to lipid hydroperoxides accumulation (**Figure 1**). Glutathione depletion via starvation of cysteine, which is rate-limiting for glutathione synthesis, leads to loss of GPX4 activity due to cofactor depletion (Hayashima *et al.* 2021). Experimental ferroptosis inducers are classified into four major groups (Feng & Stockwell 2018). Class 1 ferroptosis inducers block the cystine-glutamate antiporter, system xCT (for example, erastin or glutamate), leading to inhibition of cystine import (Dixon *et al.* 2014). Class 2 ferroptosis inducers cause ferroptosis by directly inhibiting the GPX4 enzymatic activity. RSL3/[1S,3R]-RSL3 induces ferroptosis via covalently interacting with the active site selenocysteine of GPX4, which, in turn, inhibits the enzymatic activity of GPX4, resulting in accumulation of lethal lipid peroxides, and eventually cell death (Yang et al. 2016).

Mevalonate-derived ubiquinone (also known as coenzyme Q10, CoQ10) is an endogenous antioxidant and a vital molecule of mitochondrial electron transport present in the plasma membrane (Hernández-Camacho *et al.* 2018). Class 3 ferroptosis inducers act via depletion of GPX4 protein and concurrent depletion of CoQ10, and examples include ferroptosis inducer 56 (FIN56; N2, N7-dicyclohexyl-9-(hydroxyimino)-9H-fluorene-2,7-sulfonamide) and caspase-independent lethal 56 (CIL56; 2,7-Bis(1-piperidinylsulfonyl)-9H-fluoren-9-one oxime)(Shimada *et al.* 2016). CIL56 may initiate a distinct necrotic cell death signalling cascade, while FIN56 is a specific ferroptosis inducer (Feng & Stockwell 2018). An endoperoxide-containing 1,2-dioxolane, FINO2, is the only class 4 ferroptosis inducer, which

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causes both indirect inhibitions of GPX4 enzymatic function and the direct oxidation of iron (Gaschler *et al.* 2018).

It is clear that iron availability is a factor involved in ferroptotic cell death. Transferrin receptor 1 (TfR1; a type II transmembrane glycoprotein) is ubiquitously expressed on the cell surface and is critically involved in cellular iron uptake (Fillebeen *et al.* 2019, Cui *et al.* 2019). Transferrin, an extracellular glycoprotein, binds extracellular Fe^{3+}, delivered into the cells via TfR1, and Fe^{3+} is reduced to Fe^{2+} via oxidoreductase (STEAP3) in the endosome. Divalent metal transporter 1 (DMT1) is a mammalian transmembrane proton-coupled metal-ion transporter that mediates the transport of multiple divalent metal ions (but highest affinity with iron), and DMT1 transport Fe^{2+} to the cytoplasm (Qian & Shen 2001).

Ferritin, a ubiquitously expressed cytosolic heteropolymer, comprises H-chains (FTH1) and L-chains (FTL), which stores excess iron from the labile iron pool (redox-inactive ferric iron) in the cell to avoid an increase in the size of the labile iron pool that typically follows iron overload (Hou *et al.* 2016, Ito *et al.* 2021). Iron is released from ferritin by a specialised autophagic mechanism termed ferritinophagy, in which nuclear receptor coactivator 4 (NCOA4)-binds to and directs ferritin toward the lysosome for degradation (Ito et al. 2021). Ferroptosis inducers such as erastin can experimentally induce ferritinophagy (Gryzik *et al.* 2021), which accelerates ferroptosis.

While iron has a crucial role in ferroptotic cell death, ferroptosis is not characterised by a poisonous iron elevation. Rather, as discussed in this section, iron available within the cell is liberated to enhance the formation of toxic lipid ROS by two major mechanisms: generation of lipid ROS via the Fenton reaction and/or iron-containing dioxygenase, lipooxygenase, which, in turn, catalyses the peroxidation of lipids. Therefore, a toxic elevation of iron need not occur for ferroptosis (Dixon et al. 2012); rather, the total iron levels dictate *sensitivity* toward ferroptosis.

Ferroptotic cell death can be protected by lipid antioxidants such as liproxstatin-1 (Lip-1), ferrostatin-1 (Fer-1), diacetyl-bis(4-methylthiosemicarbazonato)Cu(II)/Cu^{II}(ATSM), flavonoids such as quercetin, vitamin E and iron chelators such as desferrioxamine (Zilka *et al.* 2017, Southon *et al.* 2020, Wang *et al.* 2021, Hinman *et al.* 2018, Yao *et al.* 2019).

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3 Iron dyshomeostasis and oxidative distress in AD

Iron levels are tightly regulated in the brain to maintain physiological homeostatic balance, while its imbalance leads to oxidative distress (a critical event in ferroptosis) associated with brain atrophy and cognitive decline. Iron elevation does not ordinarily occur during ferroptosis; however, cells/tissues with higher iron levels have increased susceptibility toward ferroptotic death (Lu *et al.* 2015, García-Yébenes *et al.* 2018). In this section, we discuss evidence of iron elevation in AD and, more importantly, the association between iron and disease progression in the context of ferroptosis.

3.1 Iron elevation in AD

Iron is elevated in several cortical areas of the AD-affected brain, evidenced by a meta-analysis of 300 AD cases in 19 investigations (Tao *et al.* 2014). In the largest single study of iron in post mortem AD cases (n=645), iron was recently shown to be elevated, particularly in the inferior temporal cortex of people with pathology-confirmed AD diagnosis, while people with high pathology but without a clinical diagnosis did not have changes to iron levels (Ayton *et al.* 2021). Several recent studies also found that iron is elevated in the cerebral cortex (medial frontal and temporal gyrus) in the AD post-mortem brain (Ashraf *et al.* 2020, Bulk *et al.* 2020).

A recent prospective *in vivo* study with quantitative susceptibility mapping-MRI reliably investigated brain iron levels in AD individuals and healthy control participants (Damulina *et al.* 2020) and found higher iron in the deep grey matter and neocortical regions in the brain of AD patients compared to healthy controls. In addition, several *in vivo* and *ex vivo* MRI studies found that iron is elevated in basal ganglia, specifically in the caudate nucleus, putamen, globus pallidus in AD patients (Bartzokis *et al.* 2000, De Reuck *et al.* 2014, Du *et al.* 2018).

Iron has been found in association with amyloid plaque pathology (Meadowcroft *et al.* 2015, Ayton *et al.* 2017c, Everett *et al.* 2018) and tangle neuropathology (Smith *et al.* 1997, van Duijn *et al.* 2017, O'Callaghan *et al.* 2017, Bulk *et al.* 2018a, Spotorno *et al.* 2020, Ayton *et al.* 2020, Brosseron *et al.* 2021). Ferrihydrite (hydrous ferric oxide) levels (measured by electron paramagnetic resonance) and magnetite/maghemite magnetic moment (measured by SQUID magnetometry) were elevated in the temporal cortex of the AD brain, possibly interacting with Aβ (Bulk *et al.* 2018b). Several *ex vivo* studies revealed myelin-associated cortical iron accumulation and lamination in AD patients (Bulk et al. 2018a, Kenkhuis *et al.*

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2019). The lamination was found to be severely disrupted in AD, which correlated with layer-specific changes in myelin architecture, specifically in the medial temporal lobe.

3.2 Dyshomeostasis in iron regulatory proteins in AD patients

Several iron regulatory proteins were found to be altered in AD. Mitochondrial ferritin is an iron-storage protein found in mitochondria, which is structurally and functionally similar to the well-categorised cytosolic ferritin. Both mRNA and protein levels of mitochondrial ferritin were shown to be significantly elevated in the frontal cerebral cortex in AD patients (Wang *et al.* 2011), possibly due to the elevated oxidative burden in the brain.

Several other iron regulatory proteins were also found to be altered in AD patients, including ceruloplasmin, transferrin and melanotransferrin. Ceruloplasmin is expressed in glia and abundantly found in plasma, which transports iron through the blood to numerous tissues, including the liver, spleen and bone marrow (Ayton *et al.* 2013, Ogun & Adeyinka 2021). Ceruloplasmin is a ferroxidase enzyme that oxidises ferrous iron (Fe^{2+}) to ferric (Fe^{3+}), which is necessary for iron loading onto transferrin. Cerebrospinal fluid (CSF) ceruloplasmin levels predicted cognitive decline and brain atrophy in individuals with underlying Aβ pathology (Diouf *et al.* 2020). High ceruloplasmin levels in CSF correlated with accelerated cognitive decline and ventricular volume enlargement in individuals with MCI and Aβ pathology. In addition, the ceruloplasmin to transferrin ratio and transferrin saturation were elevated in the serum of AD patients (Squitti *et al.* 2010), and the ratio was also associated with H_2O_2 levels and adversely with serum iron levels.

Melanotransferrin or melanoma tumour antigen p97, an iron-binding transferrin homolog, was discovered initially at high levels on melanomas and other tumours, cell lines and fetal tissues (Dunn *et al.* 2006). It exists as a plasma membrane glycosylphosphatidylinositol-anchored protein or a soluble and actively secreted protein, and both forms have a physiological function. Melanotransferrin was shown to be expressed in the brain capillary endothelium of cognitively normal individuals, while in people who died of AD, melanotransferrin was found in reactive microglia and senile plaques (Jefferies *et al.* 1996, Rothenberger *et al.* 1996, Yamada *et al.* 1999). Serum melanotransferrin was increased in AD patients (Kennard *et al.* 1996, Kim *et al.* 2001), while CSF melanotransferrin level was diminished in MCI subjects who progressed to AD (Ashraf *et al.* 2019). The evidence suggests that ceruloplasmin, transferrin and melanotransferrin are associated with AD, and they have the potential to contribute to ferroptotic stress through iron dysregulation.

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Altered hepcidin (a crucial peptide hormone in chordates) and ferroportin (a major iron exporter) levels were also found in AD patients. Hepcidin is mainly produced by the liver and secreted into the circulation. The synthesis of hepcidin is increased in response to iron and inflammation while decreased during erythropoiesis (Zhao *et al.* 2013, Collins *et al.* 2008). Hepcidin regulates systemic iron metabolism via interacting with ferroportin (Collins et al. 2008), promoting cellular iron retention and lowering iron in the blood (Zhao et al. 2013). In the healthy human brain, hepcidin and ferroportin were found to be widely distributed and co-localised in neurons and astrocytes (Raha *et al.* 2013), suggesting their role in regulating iron release, while they were found to be downregulated in the hippocampus of AD patients, suggesting a role in an aberrant brain iron regulation in AD brains. Ferroportin was also found to be downregulated in APP/PS1 mouse brain and AD patients in a recent study (Bao *et al.* 2021). Ferroportin gene ablation in principal neurons of the neocortex and hippocampus in mice led to AD-like hippocampal atrophy and memory impairment.

3.3 Risk factors of iron elevation in AD

The cause of iron elevation in AD is unlikely due to the same factors that cause systemic iron overload in the body, including diet or peripheral disorders of iron metabolism, including haemochromatosis (Pirpamer *et al.* 2016). The blood-brain barrier (BBB) dissociates the brain and peripheral iron pools leading to a poor relationship between iron concentrations in the body and brain (Ayton *et al.* 2015). Rather, ageing (Hare *et al.* 2013) and inflammation (Nnah *et al.* 2020) have been reported to elevate brain iron levels and also increase the risk for AD. Brain injuries such as traumatic brain injury (Raz *et al.* 2011, Liu *et al.* 2013, Lu et al. 2015) and ischaemic stroke (Dávalos *et al.* 1994, Garg *et al.* 2020) have also been reported to elevate iron levels in the brain, which may also increase the risk for ferroptosis.

Iron selectively accumulates in several brain regions during ageing, including cortex, cerebellum, hippocampus and amygdala and substantia nigra (globus pallidus, caudate nucleus and putamen) (Connor *et al.* 1990, Aquino *et al.* 2009, Wang *et al.* 2012, Wang *et al.* 2014). The accumulated iron is found mainly bound within ferritin, transferrin and neuromelanin (a dark pigment expressed in the brain structurally similar to melanin) (Zecca et al. 2001, Connor et al. 1990). The BBB permeability is increased with age (Verheggen et al. 2020), which may contribute to raised iron levels in the aged brain.

Ageing is also associated with an elevated inflammatory state in the brain (Raj *et al.* 2017, Wander *et al.* 2020) by elevating glial cells, including astrocytes, oligodendrocytes and

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microglia and their immunoreactivity in the brain (Connor et al. 1990). Iron elevation in AD may be contributed by iron loading in activated microglia (Bulk et al. 2018a), which are a feature of the AD brain (Angelova & Brown 2019). Microglia in AD patient brains were characterised with an elevated expression of ferritin light chain, along with increased expressions of Iba1 (an ionised calcium-binding adapter protein 1, which is specific only for microglia and macrophage expression), decreased transmembrane protein 119 (TMEM119) and purinergic receptor P2Y12 (P2RY12) (Kenkhuis *et al.* 2021), representing iron-accumulating and morphologically dystrophic microglia. Light chain ferritin and Iba1 positive microglia were also found to be increased in patients with high Aβ and tau load. By this mechanism, cellular iron retention is associated with microglial activation to influence AD pathology, especially with Aβ. In contrast, elevated iron may promote a pro-inflammatory state in microglia by the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3)-inflammasome-mediated increase production of the pro-inflammatory cytokine interleukin-1β, and the NLRP3-inflammasome activity was enhanced by elevated iron (Nakamura *et al.* 2016) or heme (Erdei *et al.* 2018). In addition, microglial cells with higher iron were shown to generate more interleukin-1β by activating nuclear factor kappaB (NF-κB) signalling in response to Aβ (Nnah et al. 2020).

3.4 Elevated brain iron links to oxidative distress and cognitive decline

The brain is physiologically enriched with unsaturated lipids and has a high demand for dynamic energy metabolism and redox-active metals such as iron. Yet, neurons have a modest antioxidant defence (Cobley *et al.* 2018), which may make them vulnerable to ferroptosis. Features of ferroptosis, including glutathione depletion and lipid peroxidation in the brain, were shown by several AD post-mortem studies (Ansari & Scheff 2010, Yoo *et al.* 2010, Chiang *et al.* 2017, Jenkins *et al.* 2020). Several other antioxidant enzymes, such as GPX, glutathione-S-transferase and superoxide dismutase, were shown to be reduced in mitochondrial and synaptosomal fractions of frontal cortex tissues in patients with MCI, mild AD and AD, while oxidative distress markers, including thiobarbituric acid reactive substances, 3-nitrotyrosine, protein carbonyls, 4-HNE and acrolein, were found to be significantly increased in AD patients (Ansari & Scheff 2010). In the same study, a negative association was also found between the elevated oxidative markers and Mini-Mental Status Examination (MMSE; a cognitive assessment) scores. The oxidative damage was shown to be localised to the synapses and increased in a disease-dependent fashion (Ansari & Scheff 2010), which implicates lipid peroxidation in AD-related synaptic loss.

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Several lines of evidence have shown that brain iron is associated with accelerated cognitive decline in individuals with AD (Ayton et al. 2015, Ayton *et al.* 2017a, Ayton *et al.* 2017b, Du et al. 2018, Diouf *et al.* 2019, Spotorno et al. 2020, Ayton et al. 2020, Damulina et al. 2020, Ayton et al. 2021). Iron level and cognitive decline are consistent with ferroptosis since iron levels increase susceptibility toward ferroptotic cell death. While iron independently predicts disease progression, it also acts as a partial mediator of cognitive decline and brain atrophy associated with tangles (Spotorno et al. 2020, Ayton et al. 2020). These data position ferroptosis downstream of tangle pathology, proximal to the neurodegeneration phase of the disease.

4 Links between iron and AD pathophysiology

4.1 Iron and APP

The amyloid precursor protein (APP) is increasingly appreciated as a regulator of brain iron and regulated by brain iron (**Figure 2**). Iron regulatory proteins (IRP1/2) regulate cellular iron homeostasis via the iron-responsive elements (IRE) signalling pathway. IRP1/2 bind to RNA stem-loops, IRE, in the untranslated regions (UTRs) of their transcripts (Thomson *et al.* 1999, Anderson *et al.* 2013), which, in turn, control the expression levels of several iron homeostatic proteins, including TfR1 and ferritin, for iron uptake and storage, respectively. When IRP1/2 binds to the 3'IRE of TfR1 mRNA, the translation is facilitated, whereas when these proteins bind to the 5'IRE on ferritin mRNA, the translation is inhibited. IRE is also found on the 5′- untranslated region of APP transcripts (Rogers *et al.* 2002, Rogers *et al.* 2008). In response to increased iron, IRP1/2 are prevented from binding to the IRE on the 5'-untranslated region of the APP transcript, which disinhibits APP translation (Cho *et al.* 2010, Rogers *et al.* 2016).

APP expression is therefore controlled by iron, and conversely, APP influences cellular iron. APP binds to and stabilises ferroportin at the plasma membrane to promote iron efflux (Duce *et al.* 2010, McCarthy *et al.* 2014, Tsatsanis *et al.* 2020), and, accordingly, ferroportin was shown to be downregulated and iron elevated in APP knockout mice brains (Belaidi *et al.* 2018).

The influence of APP on iron export depends on how APP is processed, and iron also influences APP processing. APP is processed by two alternative pathways: amyloidogenic and non-amyloidogenic. Amyloidogenic processing involves sequential cleavage by β- and γ-secretase at the N and C termini of APP, respectively (Joshi & Wang 2015). β-secretase

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(BACE1)-mediated cleavage of APP generates the 99-amino acid CTF (C99), which becomes internalised and is then processed by γ-secretase at multiple sites to generate cleavage fragments of 43, 45, 46, 48, 49 and 51 amino acids. The fragments are then cleaved again by γ-secretase that yields the final Aβ species ($A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{43}$) in endocytic compartments (Takami *et al.* 2009, Olsson *et al.* 2014). Non-amyloidogenic processing involves α-secretase-mediated APP cleavage that generates soluble amyloid precursor protein (sAPP)α and an 83-amino-acid CTF (C83) (Haass *et al.* 1995). Iron was shown to affect APP processing in retinal pigment epithelium cells (Guo *et al.* 2014), thereby increasing the generation of APP processed products such as C83, C99 and $A\beta_{42}$. Iron treatment also altered APP processing by increasing BACE-1 activity, which, in turn, augments $A\beta_{42}$ release in BV-2 microglial cells (an immortalised mouse glial cell line) (Gong *et al.* 2019) and the medium of SH-SY5Y cells (an immortalised human neuroblastoma cell line) (Banerjee *et al.* 2014). In addition, non-amyloidogenic processing of APP was found to be affected by iron treatment, which increased α-secretase activity and sAPPα distribution in primary cortical neurons (Chen *et al.* 2018).

APP familial mutations that alter the APP proteolytic processing were shown to affect intraneuronal iron by changing ferroportin location (Tsatsanis et al. 2020). The pathogenic Italian-APP mutation (favours β-cut) was shown to elevate intracellular labile iron content compared to wild type-APP, which was attributed to the destabilisation of membrane-associated APP and ferroportin (Tsatsanis et al. 2020). Conversely, the protective Icelandic-APP mutation (favours α-cut) lowered the intracellular labile iron content by maintaining membrane-associated ferroportin in neuronal cells.

Pharmacological manipulation of APP processing caused the same consequence to iron as these genetic lesions. Inhibition or depletion of BACE-1 was shown to downregulate intraneuronal labile iron levels (Tsatsanis et al. 2020), in contrast, promoting the amyloidogenic APP processing by inhibiting α-secretase activity or siRNA knockdown of the predominant α-secretase, ADAM10, led to a rise in neuronal labile iron.

APP trafficking may also influence neuronal iron status. β-secretase processing of APP, which occurs in the endocytic pathway, is clathrin-dependent and requires lipid rafts. Disrupting lipid rafts within the membrane reduced the intracellular labile iron in response to iron treatment while noticeably elevating APP and ferroportin on the cell surface (Tsatsanis et al. 2020). To achieve cleavage of APP, BACE1 also needs to be trafficked with APP in this

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compartment. The GTPase, ADP-ribosylation factor 6 (ARF6), stimulates APP and BACE1 internalisation, promoting APP cleavage by β-secretase that favours ferroportin internalisation. Accordingly, siRNA-mediated depletion of ARF6 stabilised ferroportin and reduced intracellular iron levels (Tsatsanis et al. 2020). In addition, posttranslational modification of APP trafficking to the cell surface alters neuronal iron homeostasis (Tsatsanis *et al.* 2019).

4.2 Iron and Aβ

While APP has a vital role in brain iron physiology, iron has also been shown to act pathologically with Aβ that may affect AD pathogenesis. Several *in vitro* studies found that iron binds to Aβ (Liu et al. 2011, Bousejra-ElGarah *et al.* 2011, Lermyte *et al.* 2019), and the binding affinity of iron to Aβ was eight times higher than that of transferrin (Jiang et al. 2009), which causes Aβ to aggregate (Mantyh *et al.* 1993, Huang *et al.* 2004, Huang *et al.* 1999) and engenders toxicity (Rottkamp *et al.* 2001, Rival *et al.* 2009, Liu et al. 2011). Iron treatment was also shown to elevate Aβ levels in senescent microglia that were co-cultured with SH-SY5Y cells (Angelova & Brown 2018), and the elevation was linked to a decrease in release of the insulin-degrading enzyme (IDE), insulysin (a thiol zinc-metalloendopeptidase). While it has been assumed that this interaction with Aβ and iron is pathological, it is possible that this has a physiological role. Indeed, Aβ was shown to elevate intraneuronal Fe^{2+} by capturing and reducing Fe^{3+} from the ferritin core (Balejcikova *et al.* 2018), which may be a physiological mechanism of iron release from ferritin.

Iron in AD cortex measured using histochemistry was shown to be correlated with the severity of amyloid pathology (van Duijn et al. 2017), and, similarly CSF ferritin (a reporter of brain iron) was shown to predict longitudinal changes in CSF $A\beta_{42}$ levels (predicting plaque load) (Ayton *et al.* 2018). Several *in vivo* rodent model studies implicate iron with Aβ deposition and cognitive impairment. In an APP/PS1 mouse model, x-ray microscopy techniques at submicron resolution revealed a direct association between the morphology of Aβ plaque and iron (Telling *et al.* 2017), suggesting the development of an iron–amyloid complex. Magnetite iron species were also revealed in plaques (Telling et al. 2017), implying an aberrant iron redox chemistry. Iron treatment was shown to impair cognitive functions in APP/PS1 mice, accompanied by increasing Aβ accumulation and phospho-tau expression (Becerril-Ortega *et al.* 2014, Chen *et al.* 2019). Accumulated iron with Aβ deposition was also found in microglia of APP/PS1 mice and postulated to contribute to microglial dysfunction (McIntosh *et al.* 2019). Iron treatment was shown to cause a genotype-related

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elevation in glycolysis in APP/PS1 mouse microglia (Holland *et al.* 2018), accompanied by elevated 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 and ferritin expression. The above findings suggest that elevated brain iron interacts with Aβ to cause oxidative distress and cognitive deficits in AD.

4.3 Iron and tau

Tau is a microtubule-associated protein and is the major component of neurofibrillary tangles. Iron has been shown to mediate the association between tangle pathology with cognitive decline and brain volume loss when measured by direct measurement of iron in the postmortem brain (Ayton et al. 2020) and quantitative susceptibility mapping MRI as a reporter of iron (Spotorno et al. 2020). This finding suggests that iron may act downstream of tau to cause damage. Indeed, treatment of an iron chelator, deferiprone, to a mouse model of tauopathy (rTg(tau_{P301L})4510) lowered sarkosyl-insoluble tau and improved cognitive function (Rao *et al.* 2020, Rao *et al.* 2021).

Iron was also shown to promote tau hyperphosphorylation (Lovell *et al.* 2004, Rao & Adlard 2018) via iron-mediated induction of cyclin-dependent (Cdk5)/P25 complex, glycogen synthase kinase 3 beta (GSK-3β) kinase, and protein phosphatase 2A. In cell culture models, iron caused aggregation of hyperphosphorylated tau (Yamamoto *et al.* 2002, Wan *et al.* 2019) via an iron-binding motif in the tau protein and possibly by dysregulating insulin signalling (Wan et al. 2019). The co-localisation of iron and tau in NFT-bearing neurons was also associated with progressive neurodegeneration in a recent post-mortem study (Hansra *et al.* 2019).

Tau protein may also have a physiological role in iron homeostasis by promoting cellular iron efflux through the trafficking of APP to the cell surface, which acts to stabilise ferroportin (Lei *et al.* 2012, Lei *et al.* 2017). It is possible that this surface trafficking of APP may be impaired by the hyperphosphorylation and aggregation of tau (thus lowering the soluble fraction of tau) during AD pathogenesis (Wong *et al.* 2014, Yan & Zhang 2020). Tau was also found to be suppressed in a transient middle cerebral artery occlusion rat model of ischemic stroke. Tau loss preceded iron accumulation in this model, and APP treatment lowered iron and attenuated the infarct (Tuo *et al.* 2017). The above evidence suggests that iron interacts with tau to cause neurodegeneration in AD and related conditions; conversely, tau maintains cellular iron homeostasis, but a putative role of an iron-tau interaction in ferroptotic stress needs further investigation.

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4.4 Iron and apoE

Allelic variation to apolipoprotein E (*APOE*- gene; apoE- protein) is the major genetic risk factor for sporadic AD, but the reason is uncertain and may involve iron (Mahoney-Sanchez *et al.* 2016). The *APOE* ε4 isoform increases risk, the ε2 isoform decreases risk, while the ε3 isoform is benign. *APOE* gene knockout was shown to cause progressive iron accumulation in the liver and spleen of aged mice (Ma *et al.* 2021). The attributed mechanism explaining iron elevation in *APOE* knockout mice was increased phosphorylation of extracellular regulated protein kinase (ERK1/2) that led to up-regulation of TfR1 (promotes iron import) and nuclear factor erythroid 2-related factor-2 (Nrf2)-dependent downregulation of ferroportin (promotes iron export). In contrast, iron treatment was shown to upregulate intracellular apoE levels in neurons and astrocytes *in vitro* (Xu *et al.* 2016), while neuron- and astrocyte-secreted full-length apoE was reduced upon iron treatment.

Several clinical studies have also investigated the link between *APOE* isoforms and iron (Ayton et al. 2015, van Bergen *et al.* 2016, Ayton et al. 2017a, Kagerer *et al.* 2020). An association between CSF ferritin and apoE levels was found, and *APOE4* was reported to elevate ferritin levels in the longitudinal Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort (Ayton et al. 2015). The association of ferritin with longitudinal cognitive decline was increased in ε4 carriers compared to non-carriers (Ayton et al. 2017a).

The default mode network DMN is an MRI-determined distinctive connectivity model of synchronous cortical neuronal activity activated at resting state and involves spatially distant brain regions such as the medial prefrontal cortex and lateral parietal cortices, posterior cingulate and hippocampus (Kagerer et al. 2020). The default mode network is impaired in the early stages of AD (Sheline *et al.* 2010) and in *APOE4* carriers (Hahn *et al.* 2019, Kagerer et al. 2020). This change in default mode network activity in *APOE4* carriers was found to be correlated to the MRI-determined cortical iron load (Kagerer et al. 2020), signifying a synergistic interaction between *APOE4* and cortical iron with brain function. These laboratory and clinical studies show a surprising relationship between iron and *APOE* and await further mechanistic evaluation.

5 Iron and ferroptosis as therapeutic targets for AD

The previous discussion supports that iron and ferroptosis may contribute to neurodegeneration in AD; therefore, targeting iron and ferroptosis could be a promising

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therapeutic option for AD. Different classes of anti-ferroptotic agents that are of potential benefit are described below.

5.1 Iron chelators

Iron chelators currently in clinical use such as desferrioxamine, deferasirox and deferiprone, have been shown therapeutic promise in preclinical and clinical AD models (Gleason & Bush 2020). Desferrioxamine is hydrophilic and a relatively large compound, which predominantly acts on extracellular iron (binding ratio: desferrioxamine and iron are 1:1) with subsequent poor oral bioavailability and BBB permeability, and a short half-life (Neufeld 2006). In contrast, deferasirox (binding ratio: 2:1) and deferiprone (binding ratio: 3:1) are orally bioavailable and have a high affinity for intracellular iron (Neufeld 2006, Vlachodimitropoulou *et al.* 2017). The main advantage of deferiprone is that it penetrates the BBB and chelates intracellular iron but has less affinity than deferasirox; therefore, it has less tendency to deplete stored body iron. Deferiprone mechanistically penetrates cell membranes, forms a complex with iron, exits cells, and redistributes iron to transferrin for recycling (Boddaert *et al.* 2007, Sohn *et al.* 2008).

Intranasal administration of desferrioxamine was shown to ameliorate high iron diet-induced altered pathology and cognitive deficits in APP/PS1 mice (Guo *et al.* 2013b, Guo *et al.* 2013a). Iron-mediated enhanced phosphorylation, amyloidogenic processing of APP and deposition of Aβ in APP/PS1 mouse brain were ameliorated by desferrioxamine treatment (Guo et al. 2013b). Desferrioxamine also reduced high iron diet-induced hippocampal tau phosphorylation (at the Thr205, Thr231 and Ser396 sites) in APP/PS1 mouse via CDK5 and GSK-3β kinase pathways (Guo et al. 2013a). Desferrioxamine treatment via intraperitoneal injection also prevented apoptosis in the brain and resulted in M2 activation and inhibition of M1 activation in microglia in the same mouse model (Zhang & He 2017). In a recent study, desferrioxamine via intraperitoneal injection also demonstrated neuroprotective activity, possibly by preventing ferroptosis in a rat model of spinal cord injury (Yao et al. 2019), and the activity was attributed to the amelioration of impairment due to iron dyshomeostasis, lipid peroxidation, gliosis, which, in turn, increased neuronal survival.

Moreover, an oral administration with deferasirox demonstrated neuroprotective activity by preventing age-related iron accumulation, reducing ferritin and TfR1 expression and reversing altered Aβ metabolism in the brain conducted in an Albino Wistar rat model (Banerjee *et al.* 2016). The study also found that iron accumulation resulted in oxidative

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stress and NF-κB activation in the rat brain, which deferasirox treatment ameliorated. While deferasirox demonstrated promising neuroprotective effects, the BBB penetration property of deferasirox is limited but improves when conjugated with lactoferrin (Kamalinia *et al.* 2013). Lactoferrin-deferasirox conjugates mitigated Aβ-induced learning deficits in a rat model of AD (Kamalinia et al. 2013).

Another iron chelator, deferiprone, also demonstrated neuroprotective activity in several preclinical studies (Molina-Holgado *et al.* 2008, Prasanthi *et al.* 2012, Fawzi *et al.* 2020, Rao et al. 2020). It protected against H_2O_2- and $A\beta_{1-40}$-induced death in primary cortical neurons and SH-SY5Y cells (Molina-Holgado et al. 2008) and demonstrated (administered orally) neuroprotective activity in rodent models (Prasanthi et al. 2012, Fawzi et al. 2020, Rao et al. 2020). Deferiprone rescued against hypercholesterolemia-induced AD pathology by reducing Aβ and tau phosphorylation levels in the hippocampus, plasma iron and cholesterol levels (Prasanthi et al. 2012). It also attenuated scopolamine-induced cognitive impairment, increased acetylcholinesterase activity, Aβ levels and iron deposition in rats (Fawzi et al. 2020), and significantly ameliorated anxiety-like behaviour and improved cognitive function in a mouse model of tauopathy (rTg(tauP301L)4510) (Rao et al. 2020).

Deferiprone also conferred potential therapeutic activity against several neurodegenerative diseases in clinical trials, which was found to be well-tolerated in a 12-month trial in neurodegeneration with brain iron accumulation (NBIA) (Abbruzzese *et al.* 2011). In a pilot study in Friedreich's ataxia, followed by a 6-month randomised controlled trial, deferiprone was shown to be safe and mitigate brain iron deposition (Pandolfo *et al.* 2014). Deferiprone improved motor performance in a phase II clinical trial of PD (Devos *et al.* 2014). The phase II clinical study of deferiprone in AD, the Deferiprone to Delay Dementia (3D Study; clinicaltrials.gov/ct2/show/NCT03234686), is currently recruiting. Besides deferiprone, desferrioxamine (intramuscular administration) was tested in an early-stage clinical trial reported to slow cognitive decline in AD patients by 50% over 24 months in 1991, but this was never followed up (Crapper McLachlan *et al.* 1991). The available studies indicate that iron chelators could be promising therapeutics for AD.

Clioquinol (CQ; an iodinated 8-hydroxyquinoline) is a copper/zinc ionophore and a mild iron chelator withdrawn from the market due to a potential side effect: subacute myelo-optico neuropathy in Japanese patients in the early 1970s (Mao & Schimmer 2008). Development of this drug was stopped due to the complications with large-scale manufacture (Gleason &

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Bush 2020). CQ (oral administration) was shown to decrease iron-induced Aβ42 aggregation *in vitro* and inhibit Aβ accumulation in AD transgenic mice (Cherny *et al.* 2001). In a placebo-controlled phase II trial of 32 patients, CQ ameliorated cognitive deficits and lowered the level of plasma Aβ-42 (Ritchie *et al.* 2003). Oral administration with CQ also demonstrated neuroprotective activity, accompanied by antiferroptotic activity, via alleviation of MPTP-induced iron dysregulation and lipid peroxidation in substantia nigra studied in a monkey model (Shi *et al.* 2020). The activity was also possibly attributed to the activation of protein kinase B/mechanistic target of rapamycin survival pathway and prevention of p53-mediated cell death.

5.2 Dexmedetomidine

Dexmedetomidine, an α2-adrenoceptor agonist, is commonly used in the perioperative period for critical intensive care unit patients for sedation, analgesia and anxiolysis. It demonstrated neuroprotective activity in several preclinical studies, including against neonatal brain injury (Sanders *et al.* 2010, Degos *et al.* 2013, Sifringer *et al.* 2015, Endesfelder *et al.* 2017, Perez-Zoghbi *et al.* 2017, Wang *et al.* 2019a, Sun *et al.* 2020a), traumatic brain injury (Schoeler *et al.* 2012, Wu *et al.* 2018, Zhang *et al.* 2018a, Li *et al.* 2019a, Feng *et al.* 2021) and stroke (Wang *et al.* 2016, Wang *et al.* 2020, Yang *et al.* 2021). The administration with dexmedetomidine via tail vein injection protected against $A\beta_{1–42}$-induced memory impairment by increasing miR-129 expression and reducing hippocampal apoptosis in a mouse model of AD (Sun *et al.* 2020b). Dexmedetomidine was also shown to protect against $A\beta_{1–42}$-induced apoptosis in hippocampal neurons and astrocytes *in vitro* (Wang *et al.* 2019b); however, the neuroprotection was also attributed to the amelioration of $A\beta_{1–42}$-induced deacetylation of histone H3 by promoting the accumulation of histone deacetylase (HDAC)-2 and HDAC5 in the cell nucleus and the reduced production of brain-derived neurotrophic factor.

Several studies demonstrated that dexmedetomidine activates the Nrf2 signalling pathway to protect against inflammation and oxidative stress (Li et al. 2019a, Lan *et al.* 2020, Feng et al. 2021, Yang et al. 2021). Dexmedetomidine was also found to be protective against ferroptosis, demonstrated by recent cell culture studies (Qiu *et al.* 2020, Chen *et al.* 2021). It prevented tert-butyl hydroperoxide-induced cell death in SK-N-SH cells (an immortalised human neuroblastoma cell line) by reducing iron accumulation and ferroptosis (Qiu et al. 2020). The antiferroptotic activity of dexmedetomidine involved regulating iron importers and exporters via c-Jun NH2-terminal kinase (JNK)- and signal transducer and activator of

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transcription 4 (STAT4)-Sp1 signalling. Dexmedetomidine was also found to be protective against methotrexate-induced neurotoxicity in HT-22 cells (an immortalised mouse hippocampal cell line) via amelioration of neuroinflammation, oxidative stress and iron dysregulation (Chen et al. 2021). The demonstrated protective effect by dexmedetomidine was found to be attenuated by NCOA4 siRNA transfection, suggesting that dexmedetomidine-mediated antiferroptotic activity was largely dependent on the prevention of ferritinophagy.

In addition to its promising neuroprotective effects in the preclinical AD models, the phase II clinical study of dexmedetomidine in dementia, Sub-Lingual Dexmedetomidine in Agitation Associated With Dementia (TRANQUILITY); clinicaltrials.gov/ct2/show/NCT04251910), is currently recruiting. Dexmedetomidine (200 μg or 400 μg, continuous infusion) in patient-controlled analgesia (a method of pain control) was also shown to significantly decrease the incidence of postoperative delirium and early postoperative cognitive dysfunction 7 days after major surgery without increasing any side effects in a randomised, double-blind clinical trial (Zhao *et al.* 2020).

5.3 Antioxidants

Vitamin E

Vitamin E (tocols) represents a family of compounds categorised into two subgroups as tocotrienols (four unsaturated analogues) and tocopherols (four saturated analogues α, β, γ and δ) (Singh *et al.* 2013). These tocol species, and many of their derivatives, act as lipophilic radical trapping antioxidants (RTAs; α-tocopherol is the most biologically active form of vitamin E) to prevent phospholipid hydroperoxide formation (Burton *et al.* 1980, Liebler *et al.* 1990, Yamauchi 1997, Zilka et al. 2017, Angeli *et al.* 2017). The antiferroptotic activity of vitamin E species may also involve the prevention of lipoxygenases such as 5 and 15 lipoxygenases (Maccarrone *et al.* 2001, Hinman et al. 2018). Alpha-tocopherol hydroquinone, an endogenous metabolite of vitamin E, demonstrated potent antiferroptotic activity via reduction of the non-heme iron in 15-lipoxygenase from its active Fe^{3+} state to its inactive Fe^{2+} state (Hinman et al. 2018).

Several pre-clinical studies have been suggested the therapeutic promise of vitamin E against ferroptotic stress (Wortmann *et al.* 2013, Hambright *et al.* 2017, Hu *et al.* 2021). Gpx4BIKO mice (conditional deletion of *Gpx4* in forebrain neurons) supplemented a vitamin E deficient diet showed an accelerated rate of hippocampal neurodegeneration and dysfunctional

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behaviours compared to vitamin E-supplemented mice (Hambright et al. 2017). An *in vitro* study found that α-tocopherol protected against ferroptosis in *Gpx4*-deficient hematopoietic stem and progenitor cells via ameliorating lipid ROS (Hu et al. 2021). These laboratory findings suggest that loss of vitamin E may lead to neurodegeneration, while treatment with vitamin E may potentially protect against ferroptotic stress.

The levels of vitamin E were shown to be reduced in plasma, serum and CSF of AD patients (de Wilde *et al.* 2017). Some clinical trials have found that high vitamin E supplementation slowed cognitive deterioration in AD patients (Devore *et al.* 2010, Basambombo *et al.* 2017); however, other trials found that vitamin E did not decrease AD risk or slow down the progression of AD (Gray *et al.* 2008, Kryscio *et al.* 2017). In a recent *ex vivo* clinical study conducted on 113 deceased participants from the Memory and Aging Project (de Leeuw *et al.* 2020), higher levels of α- and γ-tocopherols were found to be associated with lower activated microglia density in cortical brain regions, suggesting that brain α-tocopherol levels may generate an anti-inflammatory environment to reduce total microglia density.

Selenium

Selenium is a trace element essential for GPX4 synthesis (Conrad & Proneth 2020) and also inhibits ferroptosis (Alim *et al.* 2019, Ingold *et al.* 2018). Treatment of sodium selenate (an inorganic compound of selenium produced by selenium oxidation) via intracerebroventricular injection in a mouse model of stroke was shown to protect neurons by augmenting GPX4 via coordinated activation of transcription factor AP-2 gamma and specificity protein 1 (Sp1) (Alim et al. 2019); however, it also defended against GPX4-independent excitotoxicity- or ER stress-mediated cell death.

Several clinical studies have demonstrated the beneficial role of selenium against cognitive decline. Supplementation of Brazil nuts (containing high selenium) for six months was shown to replenish selenium levels and improve verbal fluency and constructional praxis in MCI patients (Rita Cardoso *et al.* 2016). A recent Randomized Controlled Pilot Trial found that a high or super nutritional supplementation (24-week) of sodium selenate increased selenium uptake into the CNS. While the treatment did not cause an improvement to clinical function in this small study, cognitive function was associated with selenium levels when stratifying the study groups as either responsive or non-responsive to selenate supplementation (Cardoso *et al.* 2019). Conversely, selenomethionine (a selenoamino acid) did not reduce the incidence

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of dementia in cognitively healthy males (aged >60 years) in the vitamin E and selenium (PREADVISE) clinical trial (Kryscio et al., 2017); however, the study subjects were not stratified corresponding to their CSF selenium status.

N-acetylcysteine

N-acetylcysteine (NAC, a thiol-containing redox modulatory dietary supplement) is a precursor of L-cysteine, which can penetrate the BBB. NAC was shown to increase glutathione levels, protect against oxidative stress, stimulate redox-regulated cell signalling and enhance immune responses (Hara *et al.* 2017, Faria *et al.* 2019). Intraperitoneal injection with NAC restored brain glutathione levels and prevented lipid peroxidation in an AD mouse model (Fu *et al.* 2006). A recent study demonstrated the antiferroptotic activity of NAC against hemin-induced hemorrhagic stroke (Karuppagounder *et al.* 2018) by neutralising arachidonate-dependent generation of toxic lipids.

NAC was found to be well-tolerated in probable AD patients studied in a 6-month randomised controlled trial (Adair *et al.* 2001). In several secondary measures, including the Wechsler Memory Scale and letter fluency tests, NAC therapy significantly improved cognitive functions in the treatment group (n=23) compared to placebo (n=20). However, NAC therapy did not alter several primary outcome measures, such as the MMSE score in this preliminary study, and larger sample sizes may be required to determine whether NAC effectively improves cognition in AD patients.

Polyphenols

Polyphenols are naturally occurring antioxidants that can prevent oxytosis and ferroptosis due to their ROS scavenging property in preclinical studies (Darvesh *et al.* 2010, Zheng *et al.* 2021). Polyphenols such as quercetin and fisetin can also regulate several signalling pathways to provide neuroprotection (Ehren & Maher 2013, Jakaria *et al.* 2019). The BBB penetrating and iron-binding properties of curcumin (an active hydrophobic polyphenol) suggest its potential role in AD therapy (Mishra & Palanivelu 2008, Jiao *et al.* 2006). Curcumin demonstrated promising effects in 32 AD preclinical studies (Voulgaropoulou *et al.* 2019); however, the effects were largely dependent on its antioxidant and anti-inflammatory properties. Curcumin also ameliorated erastin-induced cell death in MIN6 pancreatic β-cells by lessening iron accumulation and lipid peroxidation (Kose *et al.* 2019).

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Numerous curcumin derivatives, including coumarin-quinoline hybrids, were also shown to possess acetylcholinesterase inhibitory and iron chelation activities (Duarte *et al.* 2019), and hybrids of hydroxypyridinone and coumarin were shown to have a protective effect against H_2O_2-induced cytotoxicity in U-251 cells (an immortalised human glioma cell line) and ameliorate cognitive impairment in a scopolamine-induced AD mouse model (Zhang *et al.* 2019). While curcumin shows potential effect in preclinical investigations, current clinical evidence is not positive, with one significant limitation being the low bioavailability of curcumin (Ringman *et al.* 2012, Voulgaropoulou et al. 2019).

Some other polyphenols with the ability to penetrate the BBB, such as gastrodin (Zeng *et al.* 2021) and baicalein/5,6,7-trihydroxyflavone (Wei *et al.* 2014), demonstrated anti-ferroptotic activity in cell culture models (Jiang *et al.* 2020a, Li *et al.* 2019b). Gastrodin protected against H_2O_2- and glutamate-induced ferroptotic lethality (Jiang et al. 2020a, Jiang *et al.* 2020b), possibly via upregulation of Nrf2, heme oxygenase (HO)-1, glutathione and GPX4 and downregulation of MDA levels *in vitro*. Glutamate-induced increase in acyl-CoA synthetase long-chain family member 4 (ACSL4), prostaglandin-endoperoxide synthase 2 (PTGS2) expressions were shown to be downregulated by gastrodin treatment in HT-22 cells (Jiang et al. 2020a). Gastrodin treatment also attenuated glutamate-induced iron dysregulation in HT-22 cells (Jiang et al. 2020a) by increasing ferroportin and decreasing iron levels. Several mechanistic studies also demonstrated its neuroprotective activity against $A\beta_{42}$-induced neurotoxicity in SH-SY5Y cells (Zhang *et al.* 2016, Zeng et al. 2021) and transgenic AD mouse models, including Tg2576 (Zhang et al. 2016) and APP/PS1 (Zeng et al. 2021) by alleviating oxidative stress, neuroinflammation and AD-like pathology.

Baicalein also demonstrated neuroprotective activity in AD models. It protected against heparin-induced Tau40 (2N/4R, the longest isoform of human tau) aggregation by enhancing the formation of SDS-stable oligomers and preventing fibril formation *in vitro* (Sonawane *et al.* 2021). The treatment with baicalein also prevented $A\beta_{1\text{-}40}$-induced memory impairment in a rat model of AD (Wei et al. 2014) by promoting energy metabolism and neurotransmission and preventing apoptosis and oxidative stress. In addition, it attenuated cognitive impairment in the APP/PS1 mouse model by preventing the activation of NLRP3 inflammasomes and the toll-like receptor 4/NF-κB signalling pathway (Jin *et al.* 2019). Baicalein conferred antiferroptotic activity in several cell lines such as pancreatic cancer cells (Xie *et al.* 2016), HT-22 cells (Li et al. 2019b), PC12 cells (an immortalised differentiated rat

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pheochromocytoma line) and primary cortical neurons (Duan *et al.* 2021), and the anti-ferroptotic activity was mainly attributed to the prevention of lipid peroxidation.

Moreover, a diet containing high polyphenols (26 polyphenol subclasses) was associated with reduced risk of dementia in the Three-City (3C) Study, a large prospective French cohort of older persons (1,329 adults) (Lefèvre-Arbogast *et al.* 2018). However, a systemic review on 24 studies (18 clinical and six observational trials) of polyphenols did not provide supportive evidence of clinical benefit (Colizzi 2018). Further clinical studies on larger cohorts may be required to determine whether polyphenols may benefit AD patients more definitively.

5.4 Alpha-lipoic acid

Alpha-lipoic acid, an organosulfur compound, is found naturally in fruits and vegetables, which can also be synthesised in animals and humans, and is a key player in mitochondrial energy production. It demonstrated neuroprotective activity in preclinical experiments by preventing inflammation (Kamarudin *et al.* 2014, Ahuja *et al.* 2019, Choi *et al.* 2020), apoptosis (Zara *et al.* 2013) and oxidative stress (Kamarudin et al. 2014, Ahuja et al. 2019, Uppakara *et al.* 2020, Camiolo *et al.* 2019).

Alpha-lipoic acid treatment demonstrated the formation of chelates with iron in human mesenchymal stem cells and zebrafish models (Camiolo et al. 2019). It ameliorated copper metabolism via translocation of copper from the extracellular to intracellular space in the SH-SY5Y cell line (Metsla *et al.* 2021). Alpha-lipoic acid treatment reversed ferric ammonium citrate-induced increase in tissue iron accumulation and oxidative stress (Camiolo et al. 2019). Several recent studies have demonstrated antiferroptotic activity of alpha-lipoic acid in cell culture models (Liu *et al.* 2020, Liu *et al.* 2021). The treatment with alpha-lipoic acid was shown to alleviate MPP^+ -induced ferroptosis in PC12 cells by activating the PI3K/Akt/Nrf2 pathway (Liu et al. 2021) and ameliorate AD-like pathology in animal models (Zara et al. 2013, Rodriguez-Perdigon *et al.* 2016, Liu *et al.* 2017, Zhang *et al.* 2018b, Zhang *et al.* 2020). Consistent with these preclinical data, alpha-lipoic acid has shown promising effects in small AD clinical studies (Hager *et al.* 2007, Fava *et al.* 2013, Shinto *et al.* 2014).

6 Conclusion

Understanding the complicated pathophysiology of AD is a priority for identifying new therapeutic targets for AD drug discovery. Iron dyshomeostasis may contribute to ferroptotic stress associated with AD pathogenesis, evidenced by several preclinical and clinical studies.

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Therefore, iron and ferroptosis could be possible targets for AD therapy. However, iron- and ferroptosis-mediated aberrant cellular signalling pathways that may cause neurodegeneration in AD need further investigation. Several AD-implicated proteins, including APP, tau and apoE, have been shown to regulate brain iron homeostasis, and disease-related changes to these proteins may affect iron biochemistry and associate with ferroptotic damage. Therefore, the role of these proteins needs to be examined in ferroptosis signalling pathways to understand AD pathophysiology and provide opportunities for developing disease-modifying therapeutics.

More than 30 failed phase 3 clinical trials of drugs targeting β-amyloid have yet to provide compelling evidence that reducing this pathology is an effective therapeutic strategy, yet there are lessons from these trials that could be applied for other drug targets such as iron and ferroptosis. For example, enrolling only subjects who have biomarker-confirmed AD, utilising target engagement biomarkers to prioritise drugs (possibly selecting patients who only have biomarker evidence of high iron), and using additional biomarkers of disease progression such as neurofilament light in plasma and brain volume using MRI.

Figure legends

Fig. 1 Schematic representation of the mechanism of ferroptosis induction

The regulatory pathways of ferroptosis are interlinked and tightly regulated, including glutathione (GSH)/GPX4 pathway, iron and lipid metabolism. Erastin, sorafenib, glutamate, and/or sulfasalazine blocks the system xCT (Dixon et al. 2014, Sato *et al.* 2018, Tang & Tang 2019), BSO inhibits gamma-glutamylcysteine synthetase (γ-GCS; the rate-limiting enzyme for the synthesis of glutathione) (Reliene & Schiestl 2005), and RSL3, altretamine and/or ML162 blocks the GPX4 activity (Sui *et al.* 2018, Hassannia *et al.* 2018), which results in lipid peroxidation-mediated ferroptotic cell death. Transferrin (Tf)-bound Fe^{3+} is delivered into the cells via transferrin receptor 1 (TfR1), which is then reduced to Fe^{2+} via oxidoreductase (STEAP3) in the endosome, followed by divalent metal transporter (DMT1)-mediated Fe^{2+} transportation into the cytoplasm (Qian & Shen 2001). Excess iron from the labile iron pool is stored in ferritin (redox-inactive ferric iron), which can be degraded by ferritinophagy and releases a free iron pool (Hou et al. 2016). Low cysteine levels promote ferroptosis by depleting glutathione and fostering ferritin degradation to release cytoplasmic iron to fuel the peroxidation reaction (Hayashima et al. 2021). Iron chelators (such as desferrioxamine/DFO or curcumin) prevent iron from participating in the Fenton reaction

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(Rainey *et al.* 2019). Acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) participate in incorporating polyunsaturated fatty acids (PUFAs) into cellular membranes, sensitising them toward ferroptosis initiation. Lipoxygenase (LOXs; iron-containing dioxygenases) can oxidise phospholipids containing polyunsaturated fatty acid chains (PUFA-PLs) to polyunsaturated-fatty acid-containing-phospholipid hydroperoxide (PUFA-OOH) to accumulate lipid peroxides and their degradation products, which, in turn, initiates ferroptosis possibly via membrane destabilisation, cytoskeletal changes, and altered proteostasis (Kuhn *et al.* 2015, Dodson et al. 2019). Erastin- and RSL3-induced PUFAs peroxidation-mediated ferroptosis can be inhibited by several antioxidants such as liproxstatin-1 (Lip-1) and ferrostatin-1 (Fer-1), flavonoids and Cu^{II}(ATSM). Created with BioRender.com

Fig. 2 APP exports iron via maintenance of ferroportin

APP promotes the stabilisation of surface ferroportin (FPN1). Disrupted/decreased APP translation is controlled by IRP/IRE iron-dependent signalling, which may inhibit efflux of intraneuronal iron export. APP proteolytic processing interferes with iron efflux by influencing ferroportin, while APP α-Secretase-dependent processing raises APP binding to ferroportin on the cell surface to aid iron efflux. The amyloidogenic processing of APP via clathrin and lipid raft reliant endocytosis and ARF6 reliant internalisation of the BACE1 represses APP on the cell surface (Tsatsanis et al. 2020). Thus, destabilisation of ferroportin leads to degradation of internalised ferroportin and impairs iron export. Long-term amyloidogenic processing of APP in some forms of familial AD (Tsatsanis et al. 2020) may lead to an elevated neuronal iron burden and associate neurotoxicity. Created with BioRender.com

Abbreviations

AD: Alzheimer's disease

AICD: APP intracellular domain

APP: Amyloid precursor protein

ARF6: ADP-ribosylation factor 6

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Aβ: amyloid-beta

BACE1: beta-secretase 1

BBB: blood-brain barrier

CoQ10: Coenzyme Q10

CTF: c terminal fragment.

DMT1: divalent metal transporter

Fer-1: ferrostatin-1

GPX4: glutathione peroxidase 4

GSGG: glutathione disulphide

GSH: glutathione

4-HNE: 4-hydroxy-2-nominal

HO-1: heme oxygenase 1

IRE: iron-responsive element

LAMP2: lysosomal membrane-associated protein 2

Lip-1: liproxstatin-1

LPCAT3: lysophosphatidylcholine acyltransferase 3

MDA: malondialdehyde

NAC: N-acetylcysteine

Nrf2: Nuclear factor erythroid 2-related factor 2

PTGS2: prostaglandin-endoperoxide synthase 2

PUFA-OOH: polyunsaturated-fatty acid-containing-phospholipid hydroperoxide

PUFA-PLs: phospholipids containing polyunsaturated fatty acid chains

ROS: reactive oxygen species

sAPP: soluble amyloid precursor protein

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Sp1: specificity protein 1

system Xc-: cystine/glutamate transporter

TfR1: transferrin receptor 1

γ-GCS: gamma-glutamylcysteine synthetase

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Author contributions

MJ and SA drafted the manuscript; MJ, SA, AAB and AIB critically evaluated and edited the manuscript

Conflict of interest statement

AIB is a shareholder in Alterity Biotechnology Ltd, Cogstate Ltd, and Mesoblast Ltd. He is a paid consultant for, and has a profit share interest in, Collaborative Medicinal Development Pty Ltd.

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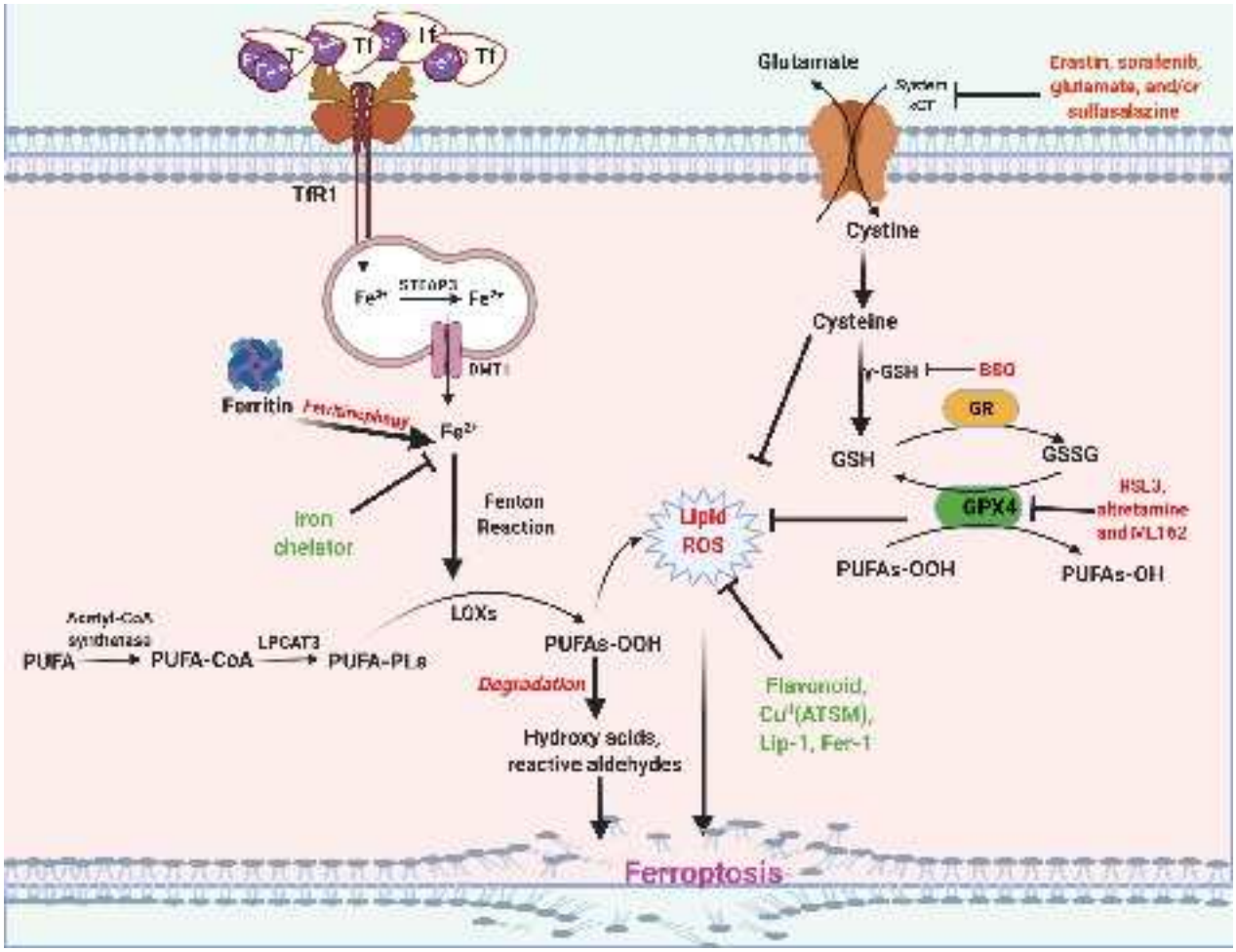

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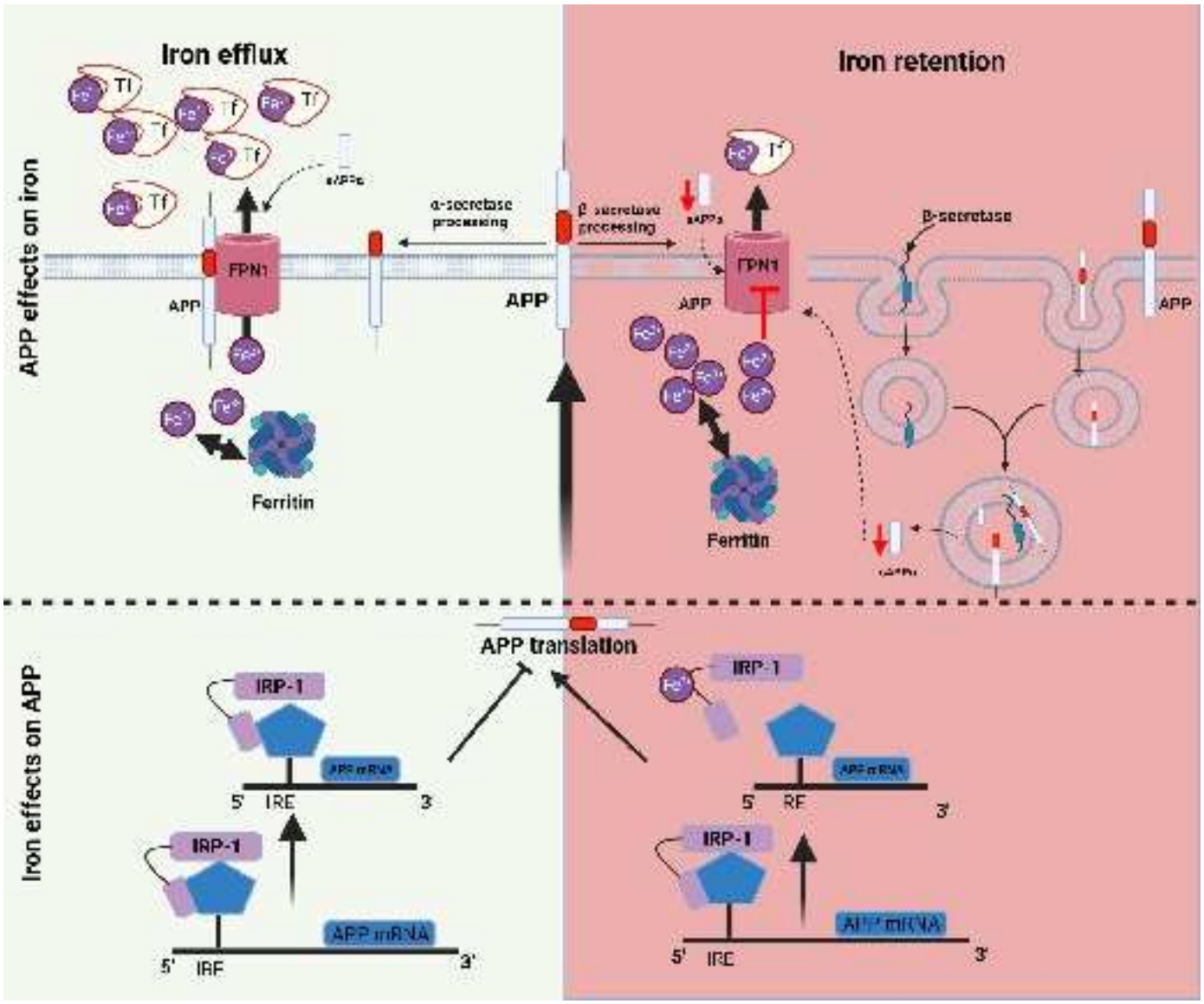

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