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Title :

Ferroptosis evaluation in Parkinson disease

Under the supervision of Professor Mourad ARIBI

Jury

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Foreword

I would like to thank God for giving me the courage and the will to carry out this modest work.

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Dedications

I dedicate this work to

*In memory of my grandmother **Sabria** and my uncle **Nacer**.*

*To the person who gave me life, who sacrificed herself for my happiness and my success, to my mother, **Fatiha**.*

*To my father, **AbdelAllah** who taught me everything, for all the sacrifices he made to see me succeed. No dedication can express my great admiration, my consideration, and my sincere affection for you, my dear parents.*

*To my very dear brothers **Akram, Nadir**, and Rayan.*

To my aunt , uncles, and my cousins

*All my friends **Meriem Ahlem Benmiloud**, **Soumeya BENAMMAR**, **BELGACEM abir**.*

*To my team **Djihane** , **Fareh** et **Amin**. To my Colleagues in master immunology*

I thank my family and my dear friends of ImmunoSpace Club

Finally, thanks to me

Thank you for always being me

CONTENANT

CONTENANT ii

ABSTRACT

BACKGROUND: Parkinson's disease (PD) is a neurodegenerative disorder related to the degeneration of dopaminergic neurons in part of the brain, referred to as substantia nigra (SN). The immune cells infiltration has been observed to induce cell death in neurodegenerative diseases through different ways, including ferroptosis.

OBJECTIVE: In the current clinico-immunological study we investigated for the first time the anomalies of ferroptosis in PD.

MATERIAL AND METHODS: Fourty (40) subjects, including 20 patients with PD, and 20 out age- and sex-matched healthy controls were recruited to the neurological Department of Tlemcen Medical Center University for sectional perspective study.

RESULTS: In contrast to circulating levels of cell protective biomarkers, including, catalase, vitamin C, scavenger total antioxidant capacity (TAC), and albumin, those of ferroptosis biomarkers were increased in patients than in controls, as demonstrated by upregulated levels of iron, malonaldehyde (MDA), conjugated dienes (CD) and carbonyl proteins (CP).

CONCLUSION: In this first report we highlighted that redox status as well as circulating ferroptosis biomarkers was altered in patients with PD. Our outcomes deserve future investigations demonstrating cause-effect relationship of ferroptosis and PD.

KEY WORDS : Parkinson disease, ferroptosis, lipid peroxidation; iron; cell protection markers.

RESUME :

CONTEXTE : La maladie de Parkinson (MP) est une maladie neurodégénérative liée à la dégénérescence des neurones dopaminergiques dans une partie du cerveau, appelée substantia nigra (SN). On a observé que l'infiltration de cellules immunitaires induit la mort cellulaire dans les maladies neurodégénératives de différentes manières, notamment par la ferroptose.

OBJECTIVE : Dans la présente étude clinico-immunologique, nous avons examiné pour la première fois les anomalies de la ferroptose dans la MP.

MATERIEL ET METHODES : Quarante (40) sujets, dont 20 patients atteints de la MP, et 20 témoins sains appariés en âge et en sexe ont été recrutés au service de neurologie du Centre Médical Universitaire de Tlemcen pour une étude de perspective sectionnelle.

RESULTATS : Contrairement aux niveaux circulants des biomarqueurs de protection cellulaire, notamment la catalase, la vitamine C, la capacité antioxydante totale (TAC) et l'albumine, ceux des biomarqueurs de la ferroptose étaient plus élevés chez les patients que chez les témoins, comme le montrent les niveaux accrus de fer, de malonaldéhyde (MDA), de dièns conjugués (CD) et de protéines carbonylées (CP).

CONCLUSION : Dans ce premier rapport, nous avons mis en évidence que le statut redox ainsi que les biomarqueurs circulants de la ferroptose étaient altérés chez les patients atteints de la MP. Nos résultats méritent de futures investigations démontrant la relation de cause à effet entre la ferroptose et la MP.

MOTS CLES : Maladie de Parkinson ; ferroptose ; peroxydation lipidique ; fer ; marqueurs de protection cellulaires.

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ABBREVIATION LIST

A

ACD · *: accidental cell death*

B

BH-4 · *: tetrahydrobioprotein*

C

CNS · *: central nervous system*
co- Q-10 · *: co-enzyme Q-10*
COX · *: cyclooxygenase*

D

DAMPs · *: danger-associated molecular patterns*
DBS · *: deep brain stimulation*
DMT-1 · *: divalent metal transporter*
DNPH · *: dinitrophenylhydrazine, : dinitrophenylhydrazine*

E

ER · *: endoplasmic reticulum*

F

Fe2+ · *: ferrous iron*

G

GPX4 · *glutathione peroxidase 4*
GSH · *: glutathione*

H

H2O2 · *: hydrogen peroxide*

I

IFN-γ · *: interferon-γ*
IL-1β · *: interleukin-1ß*

L

lipid-ROS · *: lipid-reactive oxygen spaces*
LOX · *: Lipoxygenases*

M

MDA · *: Malonic dialdehydes*
MPTP · *: 1-methyl-4-phenyl-1, 2, 3,6-tetrahydropyridine*

N

NSAIDs · *: non-steroidal anti-inflammatory drugs*

O

-OH · *: hydroxyl radicals*
ORAC · *: oxygen reactive antioxidant capacity*

P

PC · *: para compacta*
PD · *: Parkinson's disease*
PE · *: phosphatidylethanolamin*
PLOOH · *: phospholipid hydroperoxides*
PNS · *: peripheral nervous system*
PUFA · *: polyunsaturated fatty acids*

R

ROS · *: reactive oxygen spaces*

S

SN · *: substantia nigra*

T

TBA · *: thiobarbituric acid*
TCA · *: trichloroacetic acid*
TiOSO 4 · *: titanium oxide sulphate*
TLR · *: Tool Like Receptor*
TNF-α · *: tumor necrosis factor-α*

INTRODUCTION

Parkinson's disease (PD) is one of the most popular disorders affecting the brain after Alzheimer's disease, with a prevalence of around 1% in people aged 65 years (Badanjak et al. 2021)**.** It is a neurodegenerative disease characterised by a loss of dopaminergic neurons in the substantia nigra (SN) para compacta (PC), which causes dopamine deficiency and disease progression (Badanjak et al. 2021). Additionally, it has been shown that this disease results by the accumulation of α-syncline protein in neurons forming "Lowy bodies". Dopamine deficiency leads not only to complications in movement and rigidity, but also to mental problems, such as depression. (Schonhoff et al. 2020)

The loss of this part of the brain is associated with neuronal inflammation or neuroinflammation (Kempuraj et al. 2016) due to the infiltration of innate and adaptive immune cells that are activated by Inflammatory mediators including IL-1, IL-18, IL-6 and danger-associated molecular patterns (DAMPs).(Tansey et Romero-Ramos 2018).The source of such mediators' immune response activating molecules has been the subject of numerous scientific studies over years. We also know that, unlike apoptosis that is immunologically silent,(Heckmann, Tummers, et Green 2019), there are several ways of cell death including necrosis, necroptosis, pyroptosis, etosis and ferroptosis, which have the capacity to activate the immune system in the site of tissue damage. Among these modes of death, ferroptosis is the most widely discussed manner of death, it is the most immunogenic, due to its power to release pro-inflammatory molecules and DAMPs.(Sarhan et al. 2018)

Ferroptosis is a programmed cell death that depends on the concentration of intracellular irons, and the increase levels of lipid-reactive oxygen spaces (lipid-ROS) due to up regulation of oxidants and down regulation of antioxidant power of the cell, causing oxidative stress. Therefore, knowing how neuronal cells die remains the most important question in the world, considering that there is no research focusing on the contribution of ferroptosis in the pathogenesis of Parkinson's disease.

To the best knowledge based on the aforementioned, our aims in this study is to evaluate ferroptosis by exploring selected markers of lipid peroxidation (malonaldeyde (MDA) and conjugated diene (CD)), protein oxidation (carbonyl proteins (CP)) as oxidants markers, analysis of the antioxidant activity of the cell (vitamin C, catalase, oxygen reactive antioxidant capacity (ORAC), and albumin), and the determination of iron level in serum, in order to demonstrate the involvement of ferroptosis in patients with PD.

Chapitre 1 Literature review

1. Parkinson disease

The nervous system is the biological command centre that carries out both of voluntary and involuntary functions of the body. Two types of cells composed it ("The nervous system: discovering your inner circuitry" 2019). Neurons that are specializes cells (Kempuraj et al. 2016), they secret neuro-mediators and various chemical messengers ("The nervous system: discovering your inner circuitry" 2019), its specificity is the inability to renew oneself (low self-renewal capacity) within the central nervous system (CNS) (Kempuraj et al. 2016). And support cells among which the glia that surround the neurons ("The Nervous System: Discovering Your Inner Circuitry" 2019).

A number of pathologies can affect the brain especially microglia and astrocytes, such as neurodegenerative diseases like Alzheimer's disease and Parkinson's disease.

1.1. Parkinson disorder

Parkinson's disease is a progressive neurodegenerative disease (Overton et Coizet 2020), which characterize by an accumulation of aggregates of alpha-synuclein protein in the substantia nigra (SN) para compacta (pc) forming the Lewy body, leading to degeneration of dopaminergic neurons (Badanjak et al. 2021).This neuronal death leads to a dopamine deficiency, the hormone responsible for muscle movement(Guérin, s. d.).

1.2. Epidemiology

There are more than 6 million individuals over the age of 65 around the world suffered by PD (Badanjak et al. 2021); it is frequent in men than women with a ratio 3:2 (Tolosa et al. 2021).

1.3. Ethology

PD is triggered by genetic (mutations), environmental or idiopathic factors. In addition, age is consider as the primary factor in the development of the illness (Badanjak et al. 2021)

1.3.1. Genetic factors

Parkinson's disease can be sporadic (not associated with known mutations), or non-sporadic, it is classify as a hereditary disease. (Guérin, s. d.)

23 loci can undergo autosomal mutations contributing to the occurrence of the disease, such as LRRK2 (PARK8), SNCA (PARK1), and PRNN (PARK2), (Badanjak et al. 2021), which can be dominant or recessive (Guérin, s. d.). The most of theme is LRRK2 mutation, it is involved in the modulation of cytokines, through the activation of the Tool like Receptor (TLR) pathway in a Myd88-dependent manner and it is present in dominance in immune cells of the brain, tissue and blood, suggesting that this gene has a role in innate immunity (Badanjak et al. 2021).

1.3.2. Environmental factors

In addition, environmental factors can cause PD, for example, herbicides or pesticides, which induce oxidative stress, DNA damage and neuronal death. (Badanjak et al. 2021)

Furthermore, chemical compounds affect the onset of the disorder, such as 1-methyl-4-phenyl-1, 2, 3,6-tetrahydropyridine (MPTP), present in synthetic heroin (Guérin, s. d.) by the inhibition of complex I of the respiratory chain machinery in mitochondria (Badanjak et al. 2021).

Water pollution can be considered as a trigger of the disease too (Tolosa et al. 2021).

1.3.3. Behavioural factors

Tobacco ; coffee, exercise ; head trauma ;...(Tolosa et al. 2021)

1.4. Symptoms

The sick signs can be divided into motor symptoms that appear after the loss of 50% of the neurons that mean in the late phase of the disease including resting tremors, rigidity, Bradykinesia. and non-motor symptoms like cognitive decline (dementia), psychiatric signs (depression, apathy, anxiety), additionally to constipation and sleep tremors(Badanjak et al. 2021),(Bighametal.2021),(Church 2021).

Table 1.1. Clinical signs (Armstrong et Okun 2020)

Motor symptoms	Non motor symptoms
Bradykinesia	Olfactory loss
Rigidity	Sleep dysfunction
Rest tremor	Autonomic dysfunction
Postural instability	Psychiatric disturbances
	Cognitive impairment
	Fatigue

1.5. Diagnosis

To the present day, the diagnosis of this pathology remains an open point, there are no specific tests to diagnose it except for some genetic tests that are not widely used, or the detection of the α-synuclein protein present whether in the retina, skin, urine or plasma (Cabreira et Massano 2019).

Therefore, the diagnosis is based on motor signs, physical examinations, and patient's history, while the confirmation is done by a positive response to dopaminergic substitution treatments such as ***levodopa*** (Cabreira et Massano 2019).

The history contains the assessment of motor and non-motor symptoms, family history of first-degree relatives. Patients should have a minimum of 2-4 referral factors: (Armstrong et Okun 2020).

1. Restlessness;
2. Response to treatment;
3. Presence of dyskinesia*
4. Presence of loss of function; (Armstrong et Okun 2020).

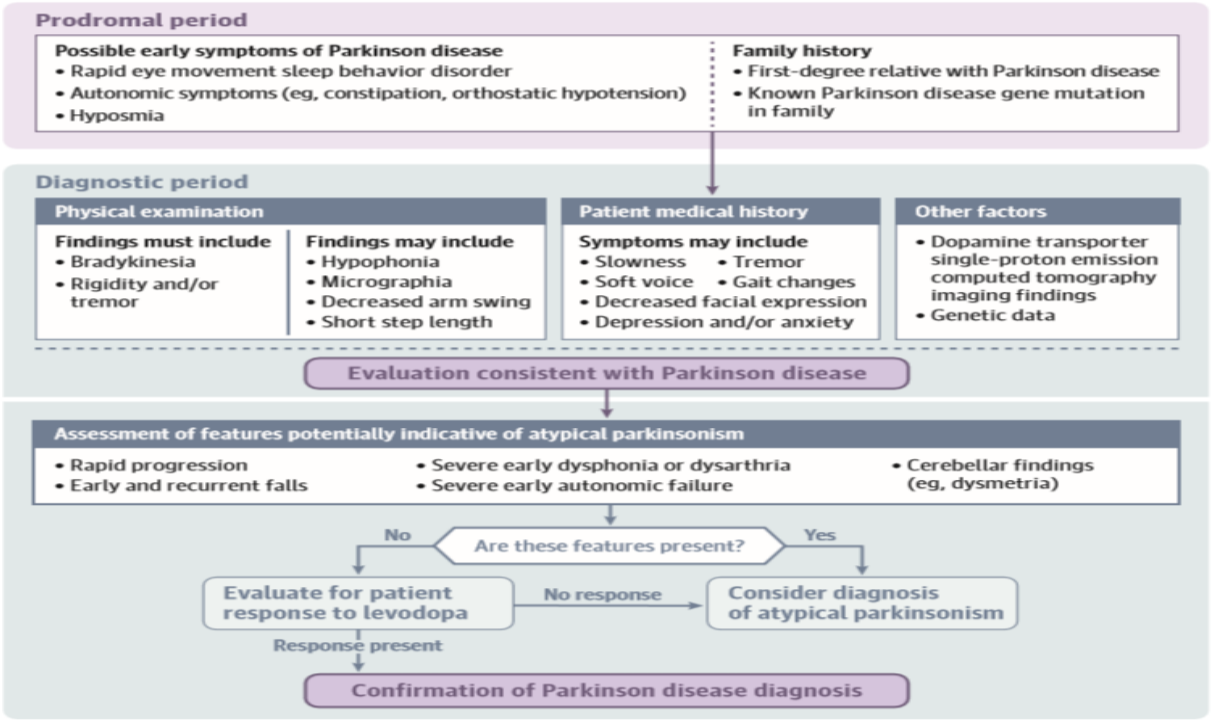

Figure 1.1. Diagnostic of PD (Armstrong et Okun 2020)

1.6. Types of Parkinson

The disease is subdivided into three subtypes, according to diagnosis, prognosis, response to medication and age at the time of discovery of the disease us mentioned in the **(figure 1.2)** (Armstrong et Okun 2020) (Tolosa et al. 2021).

Parkinson Disease Subtype and Estimated Frequency	Disease Presentation	Response of Motor Symptoms to Dopaminergic Medication	Disease Progression
Mild motor predominant 49%-53%	• Young at onset • Mild motor symptoms	Good	Slow
Intermediate 35%-39%	• Intermediate age at onset • Moderate motor symptoms • Moderate nonmotor symptoms	Moderate to good	Moderate
Diffuse malignant 9%-16%	• Variable age at onset • Rapid eye movement sleep behavior disorder • Mild cognitive impairment • Orthostatic hypotension • Severe motor symptoms • Early gait problems	Resistant	Rapid

Figure 1.2. PD types (Armstrong et Okun 2020)

1.7. Physiopathology

Several hypotheses are proposed regarding the pathogenesis of PD, because of the strong connection formed between the different cell lines of the CNS (Lee et Yankee 2021).

Several hypotheses are proposed regarding the pathogenesis of PD, because of the strong connection formed between the different cell lines of the CNS (Lee et Yankee 2021).

According to the "Braak" hypothesis, PD starts in the medulla and olfactory bulb, this is stage 1 and 2 of the disease, and the associated symptoms are non-motor. In the 3th and 4th stage (representing the diagnostic stages), the disease progresses to the substantia nigra para compacta (Armstrong et Okun 2020). This progression is associated with reactive oxygen spaces (ROS) amplification, lipid peroxidation and antioxidant depletion, leading to neuronal death and multi-systemic degeneration (Reichert et al. 2020),(Lee et Yankee 2021).

Three types of neuropathological lesions are described in this condition:

1.7.1. Dopaminergic lesions

This neurodegeneration is associated with the presence of Lewy bodies, occurs in the substantia nigra, especially the ventral portion, (Overton et Coizet 2020).

It is a slow and continuous degeneration of dopaminergic neurons, in association with the accumulation of iron, causing a deficiency of dopamine and neuromelanin (Reichert et al. 2020).

This damage is considered an anatomical-pathological criterion that helps in the diagnosis of the disease (Ryman et Poston 2020)**.**

1.7.2. Non dopaminergic lesions

Degeneration spreads to non-dopaminergic neurons, which is coupled to non-motor symptoms, e.g. the loss of norepinephrine neurons causes dementia, the loss of serotonergic neurons gives depression, and the loss of cholinergic neurons causes gait and balance disorders (Thobois et al. 2017).

1.7.3. Lewy bodies

Lewy bodies also have an effect in the onset of PD in association with neuritis, they are an insoluble aggregates which are located in the CNS and peripheral nervous system (PNS) (Garretti et al. 2019). These aggregates present a potent toxicity mediated by enhanced ROS, which causes neuronal loss (Lee et Yankee 2021),(Badanjak et al. 2021) through the activation of both brain resident immune cells "microglia", and peripheral white blood cells (Tansey et Romero- Ramos 2018).(Badanjak et al. 2021).

1.8. Immune system and neuro-inflammation in PD

Our brain is protect by blood-brain barrier, which makes it an immunologically privileged site with limited or reduced immune responses, and a lack of communication with the peripheral immune system outside of pathophysiological conditions. While its dysfunction during the inflammation in the periphery leads to immune cell infiltration into the brain (Kempuraj et al, . 2016).

Other types of resident immune cells that are different from macrophages called microglia protect this organ. They are present in two forms, pro-inflammatory (amoeboid) and anti-inflammatory, they are activated by cervical or peripheral signals, and their main roles are

protection, homeostasis and neuronal plasticity they are mediators of neuroinflammation (Tansey and Romero-Ramos 2018),(Schonhoff et al. 2020).

In 1817, the researchers showed the presence of two types of immune cells (innate and adaptive) in the brain of patients with PD (Garretti et al. 2019),(Schonhoff et al. 2020). where they activate microglia via the liberation of inflammatory mediators like interleukin-1β (IL-1β), IL-6, IL-2, IL-8, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) (Garretti et al. 2019).

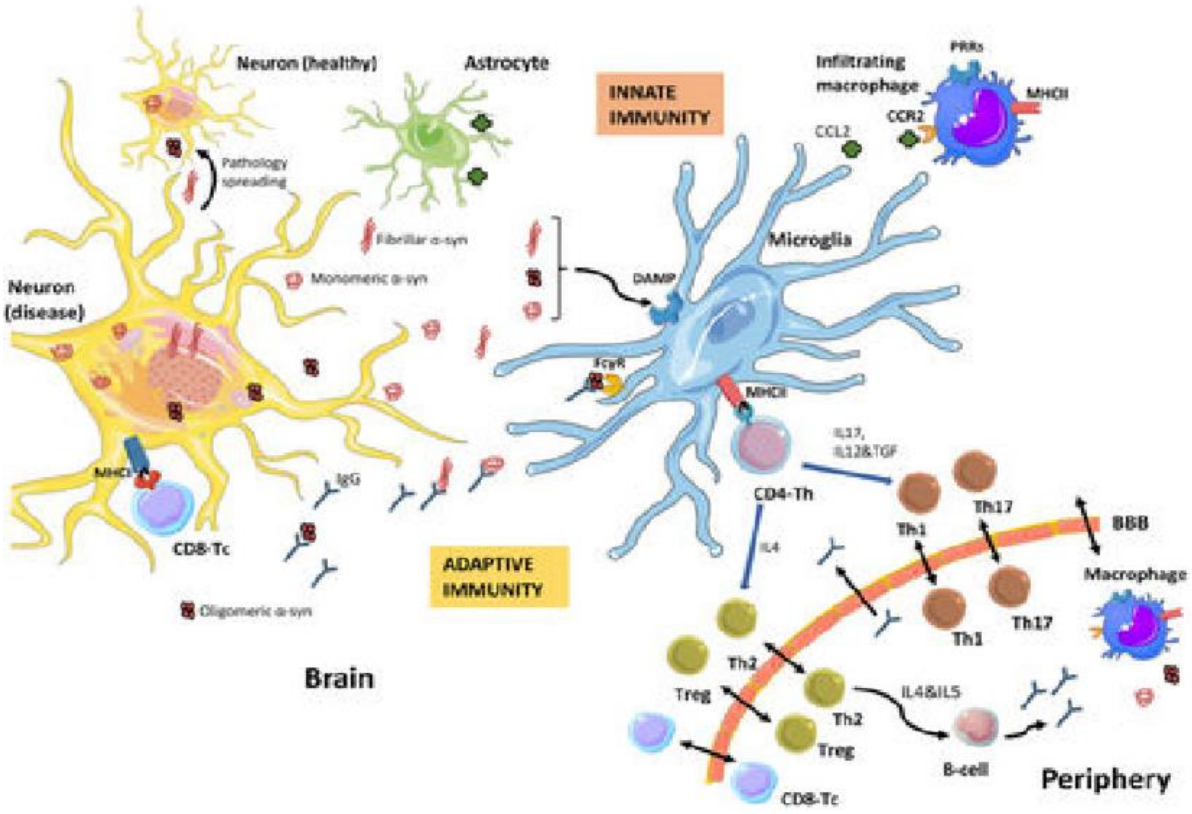

Figure 1.3 innate and adaptive immune response during PD(Tansey et Romero-Ramos 2018).

1.8.1. Neuroinflammation

Neuroinflammation is an immunological response of the cerebral and spinal cord, it is regulated by various inflammatory mediators emitted by glial cells (neurons, astrocytes and microglia), immunity cells (T cells, neutrophils, mast cells), these are represented by cytokines (IL-1α, IL-6 and TNF-α), chemokine, ROS and second messengers (NO and prostaglandins) (Kempuraj et al. 2016) (DiSabato, Quan, et Godbout 2016).

This mechanism is involved in several CNS-related pathologies, as it activates other glial cells, increases peripheral cell infiltration and increases BBB permeability, resulting in neuronal loss and neurodegeneration (DiSabato, Quan, et Godbout 2016).

1.9. Treatments

The treatments used are purely symptomatic, the classical approaches used to treat the disease are usually substitution treatments, to compensate for dopamine, such as levodopa (Badanjak et al. 2021),(Church 2021),(Kumari et al. 2018).

These medications have multiple disadvantages:(Badanjak et al. 2021)

- ✓ Relieve only a few symptoms;
- ✓ Do not slow down the development of the disease ;
- ✓ Present limited efficacy.

The stability of treatments is 5 to 6 years, and then the disease evolves (Reichert et al. 2020).

Treatments used target motor and non-motor symptoms to increase patients' quality of life (Church 2021).

1.9.1. Therapy affecting motor signs

The most used is levodopa; it consists of introducing L-dopamine orally to compensate for the lack of dopamine additionally to carbidopa (Stansley et Yamamoto 2015).

Dopamine receptor agonists in the CNS are also feasible to treat the disease. Inhibitors of the enzymatic activity of the enzymes that lead to the inactivation of dopamine and others are also applied (Church 2021).

1.9.2. Non-motor symptom treatments

These treatments focus on neuropsychiatric features, depression, digestive problems, sleep disorders, dementia, etc(Church 2021)

Other strategies are proposed in addition, which act on the immune system:

1.9.3. Non-steroidal anti-inflammatory drugs

Studies have shown the promising effect of non-steroidal anti-inflammatory drugs (NSAIDs), such as cyclooxygenase 1 and 2 (COX1 and COX2) inhibitors, and have shown the protective effect of these NSAIDs in chemical experiments, delaying or even preventing the onset of the disease. Recent trials have also targeted inflammatory M1 microglia cells, and transformed them into anti-inflammatory M2 cells, by influencing pro-inflammatory cytokines such as TNF-α, IL-1β and IFN-γ. This was achieved by administering CB2 receptor agonists to epithelial cells, reducing the secretion of pro-inflammatory cytokines, thus preventing neuronal death and glial cell activation (Badanjak et al. 2021).

1.9.4. Anti-α synuclein immunotherapy

this protein is considered as DAMPs, it can activate the immune system, for this they proposed anti-α synuclein antibodies intra and extracellular brain, the results are promising, where they noticed the decrease of microgliosis*, and the increase of the life span of neurons, knowing that the clinical trials are in phase I (Badanjak et al. 2021).

Complications may occur with the treatment such as dyskinesia, for this reason surgical interventions are applied as the deep brain stimulation (DBS), this technique is irreversible and adjusted with the development of the disease (Church 2021).

2. Necroinflammation and ferroptosis

2.1. Necroinflammation

2.1.1. Definition

The chain linkage between innate and adaptive immune responses to cell death by necrosis defines as necroinflammation. Specific processes such as necroptosis, pyroptosis and ferroptosis can regulate this mechanism. (Jing-yan Li, Yao, et Tian 2021).

2.1.2. Origin

The immune system has the ability to recognise self from non-self, but also to distinguish between PAMPs (extraneous danger), and DAMPs (endogenous danger). This recognition allows it to trigger inflammatory reactions.

Cell death by necrosis is driven by an increase in oxidative stress, which activates immune responses by releasing immunogenic substances (DAMPs). “Ferroptosis”, as the most immunogenic type of death, makes the environment of ferroptotic cells more inflammatory and thus causes necroinflammation (Sarhan et al. 2018),(Jing-yan Li, Yao, et Tian 2021).

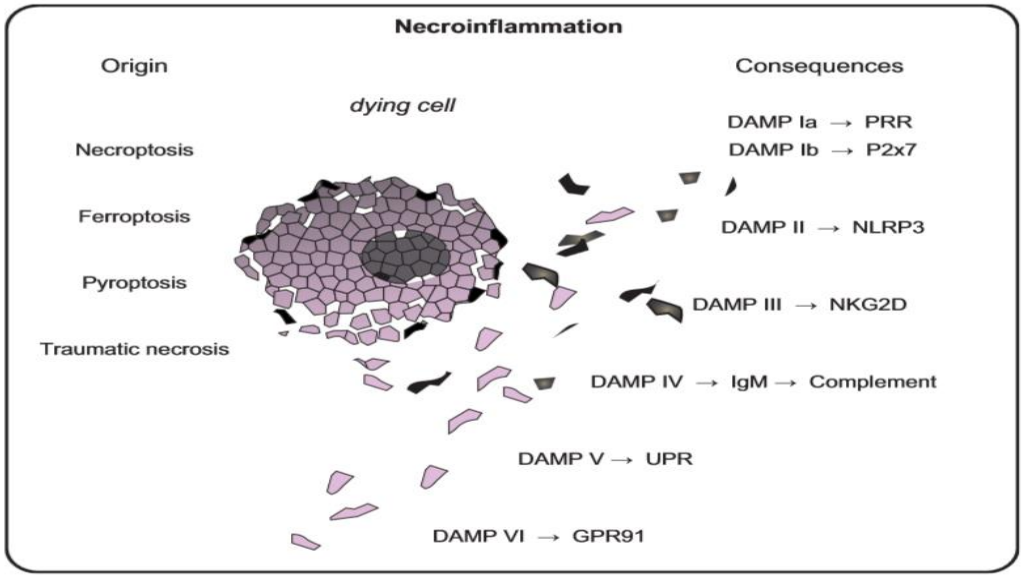

Figure 1.4. Origin and consequences of necroinflammation (Sarhan et al. 2018)

2.2. Ferroptosis

Cell death is a very important process in our organism, whether in normal conditions (physiological), or pathological (diseases)(Jie Li et al. 2020). It allows the regulation of cell destiny, tissue replacement and immune responses (X. Chen et al. 2021). Usually, two types of cell death are distinguished, accidental cell death (ACD), is an uncontrolled death, it is caused by irreparable damage following an exogenous or endogenous stimulation. While the second type, is controlled by signalling pathways, and specific cellular machinery. Pyroptosis, necroptosis, necrosis, etosis and ferroptosis are the main forms representing this category (X. Chen et al. 2020).

2.2.1. What is ferroptosis cell death?

This term "ferroptosis" was first described in 2012,(Jiang, Stockwell, et Conrad 2021) it is defined as a mode of oxidative, non-apoptotic and iron-dependent cell death, and it is characterised by the accumulation of lipid ROS, peroxidised lipids and the production of DAMPs (Jie Li et al. 2020),(X. Chen et al. 2020). It is a controlled necrosis phenomenon regulated through cellular metabolic pathways such us redox homeostasis, iron handling, mitochondrial activity, amino acid, fatty acid and sugar metabolism; but, it can also be regulated by signalling pathways.(Gao et al. 2019) (Jiang, Stockwell, et Conrad 2021).

To sum up, ferroptosis is a particular cell death gathering necrosis, apoptosis and autophagy with differential properties mentioning in the table above (Jie Li et al. 2020).

Table 1.2. The difference between ferroptosis and others types of cell death.

Ferroptosis	Apoptosis	Necrosis	Autophagy
Membrane density Decrease of mitochondria volume.	**Chromatin condensation Apoptotic bodies formation.**	**Cell swelling**	**Vacuoles formation**

2.2.2. Characteristics

2.2.2.1. Morphological

Mitochondrial morphology is one of the judging biomarkers to distinguish ferroptotic cells (Gao et al. 2019). These cells are characterised by a decrease in mitochondrial volume, an increase in membrane density, and a decrease or disappearance of mitochondrial crystals. they keep plasma membrane intact, their nucleus with normal size and without chromatin condensation (Jie Li et al. 2020).

2.2.2.2. Biochemical

During ferroptosis the intracellular amount of glutathione (GSH) is decreased, with a reduction in the enzymatic activity of glutathione peroxidase 4 (GPX4), which leads to a suppression of lipid peroxide reduction. The process is also characterised by oxidation of lipids by Fe2+ and an increase in lipid ROS, (Jie Li et al. 2020).

- **Iron accumulation**

On the above definition, ferroptosis depend on the level of ferrous iron (Fe2+) in cytosol and mitochondria, it is produced when iron (oxidant) increases and thiol (antioxidant) reduces (Wang et al. 2020a).

- **Lipid peroxidation**

Peroxidation of polyunsaturated fatty acids (PUFA) by ROS forming hydroperoxides, is the most important marker of ferroptosis.(Mahmoud, Abbes, et Said, s. d.), (Latunde-Dada 2017)

The markers of peroxidation will be describing below.

- **Loss of antioxidant functions**

Initiators of lipid peroxidation such as erastin and RSL3 have an inhibitory effect on antioxidant systems. Three antioxidants system have been described in the literature, GSH, co-enzyme Q-10 (co- Q-10), tetrahydrobioprotein (BH-4). These systems play an inhibitory role in oxidative ferroptosis(X. Chen et al. 2021).

2.2.2.3. Genetic

On the genetic level, several mutations can be detected and considered as biomarkers of ferroptosis. It has been noticed the activation and up regulation of genes responsible for iron homeostasis, lipid peroxidation (Jie Li et al. 2020), amino acid metabolism (X. Chen et al. 2021), oxidative stress, antioxidants and cellular protection (Jiang, Stockwell, et Conrad 2021), (X. Chen et al. 2021).

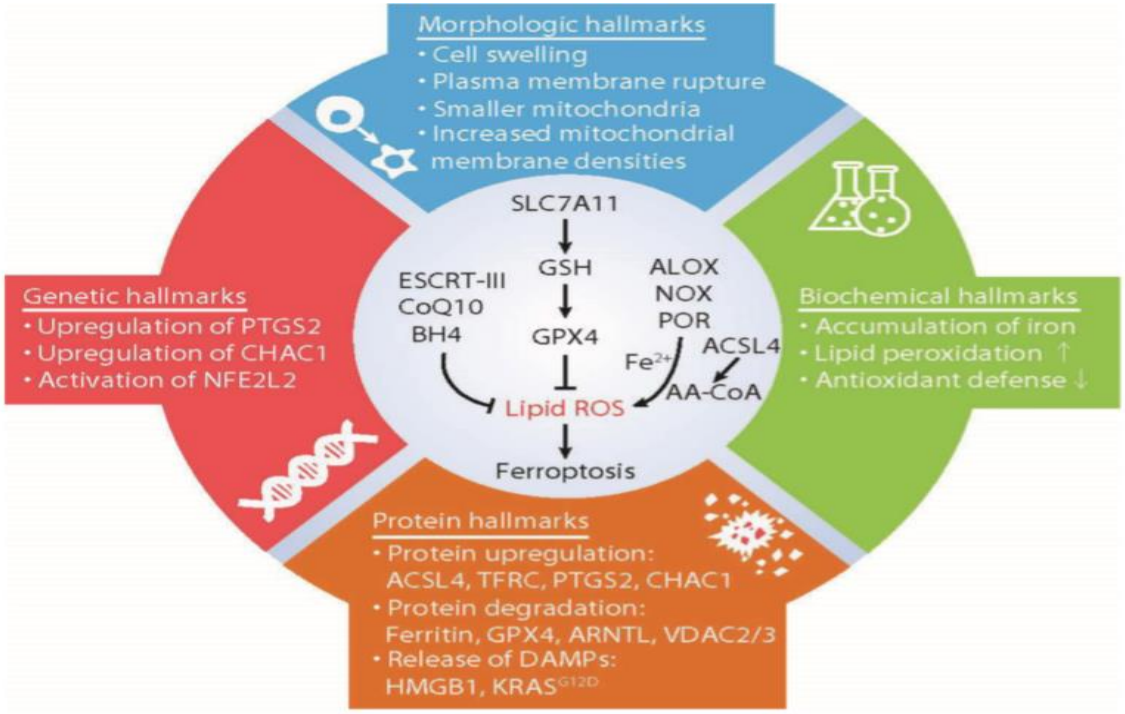

Figure 1.5. Ferroptosis hall marks (Chen et al. 2021).

2.2.3. Mechanism

The initiation and execution of ferroptosis is essentially realized by the intersection of iron, lipid and amino acid metabolism (Wang et al. 2020b).

2.2.3.1. Iron metabolism

Iron is a very important element in our body, it participates in a myriad of redox based metabolic processes, which are involved in the production of ROS, (Jiang, Stockwell, et Conrad 2021). It is also implicated in DNA synthesis, oxygen transport and cellular energy generation (Roemhild et al. 2021). Its poor distribution in the body affects normal physiological processes.

This iron (Fe2+) can be formed into two ways, either from intestinal absorption or from erythrocyte degradation (Jie Li et al. 2020), than it will be oxide by cenuloplasmin forming

fe3+ which will bind with its transporter transferrin (TR) in a pH~7.4 giving holotransferrin (Reichert et al. 2020). This complex (TR-Fe3+) will enter the cell by endocytosis by binding with its transmembrane receptor TRF1 and TRF2 (X. Chen et al. 2020). Then it will be mobilized to the endosomes where Fe3+ (the ferric form) will be released from the TF and converted to Fe2+ (ferrous form) inside the endosome (acidic environment where the pH~5.5) by redox. (Reichert et al. 2020),(X. Chen et al. 2020). Fe2+ is subsequently exported to the cytosol through the divalent metal transporter (DMT-1). This iron either remains labile in the cytosol or is stored in ferritin/hemosiderin, (Reichert et al. 2020).

The increase in the level of labile iron present high cytotoxic power due to its ability to catalyse the Fenton reaction, allowing the formation of hydroxyl radicals (-OH), from hydrogen peroxide (H2O2),(Reichert et al. 2020). These radicals have the power to react with biological molecules such as proteins, DNA, and fatty acids, resulting in damage to cell organelles and thus cell death by ferroptosis (Reichert et al. 2020; Roemhild et al. 2021).

2.2.3.2. Amino acids metabolism

The metabolism of amino acids is done by three key components

a) Xc- system

Is an amino acid antiporter, ensures the exchange of extracellular L-cystine (cysteine dimer) and intracellular L- glutamate, and thus participate in the regulation of the influx of intracellular L-cystine essential for GSH synthesis, this type of exchange plays crucial roles in the CNS including:(Bridges, Natale, et Patel 2012)

- Oxidative protection ;
- Blood-brain barrier function;
- Neurotransmetteur release ;
- Synapse organisation ;
- Chemo-resistance and chemo-sensitivity. (Bridges, Natale, et Patel 2012)

b) Glutathione Peroxidase 4 (GPX4)

One of the members of the selenoprotein glutathione peroxidase family. it is an enzyme that catalyses the reduction and thus detoxification of cytotoxic hydro peroxides (L-OOH) to the corresponding alcohols (L-OH), using the selenocysteine residue and two electrons provided by GSH or low molecular weight thiol, which allows the convention of (reduced) GSH to (oxidised) GSSH (Jie Li et al. 2020; Jiang, Stockwell, et Conrad 2021; Yoo et al. 2012). It has a high affinity for lipid hydro peroxides which makes it the only one that can reduce them (hydro peroxides) (Yoo et al. 2012).

c) Glutathione (GSH)

GSH is the most abundant reducer in mammals, it is very necessary for the biogenesis of iron-sulphur clusters, and it acts as an enzymatic cofactor for GPX4 and glutathione-s-transferase. Its biosynthesis requires cystine, which comes from two environmental sources, either through the Xc system or through the natural amino acid transporter, otherwise it will be synthesised by the transsulfuration pathway using methionine and glucose (Jiang, Stockwell, et Conrad 2021).

Its level in the cell is important for detoxification of xenobiotic, prevention of oxidative stress and cellular damage, especially in the CNS where O_2 consumption is high, the wealth of enzymes and metabolites that are likely to generate ROS, and its reduced antioxidant capacity (Bridges, Natale, et Patel 2012).

d) Ferroptosis enhancing

Inhibition of this system leads to inhibition of cystine uptake, which limits the rate of GSH biosynthesis. This leads to a reduction in GPX4 activity and a decrease in the antioxidant capacity of cells, causing an accumulation of lipid ROS and the occurrence of oxidative damage leading to cell death by ferroptosis, (Jie Li et al. 2020, 202)

2.2.3.3. Fatty acids metabolism (lipid roxidation)

Lipid peroxidation occurs in three main stages, initiation, transmission and termination.

The first phase can be manifested by both enzyme and non-enzyme controlled mechanisms, non-enzymatic peroxidation is mediated by iron and oxygen which catalyse a reaction cascade, the so-called Fenton reaction, forming phospholipid hydroperoxides (PLOOH), and allowing the propagation of lipid peroxidation (Jiang, Stockwell, et Conrad 2021). Lipoxygenases (LOX) and/or cytochrome P450 oxidoreductase are responsible for the enzymatic peroxidation (POR) (Jiang, Stockwell, et Conrad 2021)

LOX is an enzyme that selectively catalyses two types of phospholipids, found in phosphatidylethanolamin (PE), which are arachidonoyl-PE and adrenoyl-PE (Wang et al. 2020a).

In this first step, PUFAs located in the lipid bilayer will lose a hydrogen (H) molecule, belonging to the 1,4-cis-pentadiene structure, forming a carbon-centred phospholipid alkyl radical (PL-).(Guichardant et al. 2006),(Jiang, Stockwell, et Conrad 2021).

The second step consists of the intersection of this radical with another PUFA to give another radical (PI-), and rearrange into a conjugated diene (CD), the newly formed radical will be attacked by oxygen forming a peroxyl radical (PLOO-) (Guichardant et al. 2006).

PLOO- will subsequently remove a hydrogen molecule from another PUFA giving hydroperoxides (PLOOH), which are toxic to the cell (Guichardant et al. 2006);(Jiang, Stockwell, et Conrad 2021).

In the normal case, PLOOH will be converting to the corresponding alcohol (PLOH) by GPX4, (Jiang, Stockwell, et Conrad 2021). In pathological cases or ageing the activity of this enzyme is reduced which results in the increase of the lifetime of PLOOH, and the degradation of cellular products, (Guichardant et al. 2006). Also they will react with PUFAs in biological membranes such as mitochondria, peroxisomes, endoplasmic reticulum (ER), lysosomes and plasma membrane, in cooperation with free radicals (PLOO-) and alkoxyl phospholipid radicals, leading to the formation of by-products such as oxidised proteins, causing the degradation of these membranes and cell death (Jiang, Stockwell, et Conrad 2021).

Non-enzymatic lipid peroxidation is the key mechanism in the induction of ferroptosis, as it leads to a breakdown of biological membranes within the cell (Jie Li et al. 2020). In addition,

accumulated (PLOOH) has the ability to alter the structure and function of nucleic acids and proteins (Wang et al. 2020a).

- **Markers of lipid peroxidation**

- Conjugated dienes are structures present in most natural and biological molecules. (Dumonteil et Berteina-Raboin 2022).
- Malonic dialdehydes (MDA) Is the result of lipid peroxidation, they form during the degradation of hydroperoxides. MDAs are not specific to lipids, as they can be formed during the interaction between the hydroxyl radical and vitamin C, and between hydroxyl and deoxyglucose (Guichardant et al. 2006).
- F2-isoprostanes are eicosanoids, resulting from the oxidation of arachidonic acid (Guichardant et al. 2006).
- 4-hydroxy-alkenals they result in the same way as MDA, following the degradation of hydro peroxides (Guichardant et al. 2006).

2.2.4. Oxidative stress and cell death

2.2.4.1. Oxidative stress

Reactive oxygen species (ROS) are free radicals (superoxide anion, hydroxyl radical), and their precursors such as hydrogen peroxide (H2O2) (Garait, s. d.), Which are produced in balance with antioxidants (catalase, ascorbic acid, etc.) in normal conditions. The alteration of this balance (oxidative stress) represents a pathophysiological mechanism that leads to damage of the different components of the cell such as lipids, proteins, and DNA, causing cell death (Curtis et al. 2012).This disturbance results either in an increase of ROS without an augmentation of antioxidants, or in a decrease of antioxidants (Garait, s. d.).

They are generate in the presence of oxygen through three manners:(X. Chen et al. 2020)

- the Fenton reaction via iron degradation of H2O2 giving radical hydroxyl;(Latunde-Dada 2017)
- the mitochondria;
- NADPH oxidase (NOX).

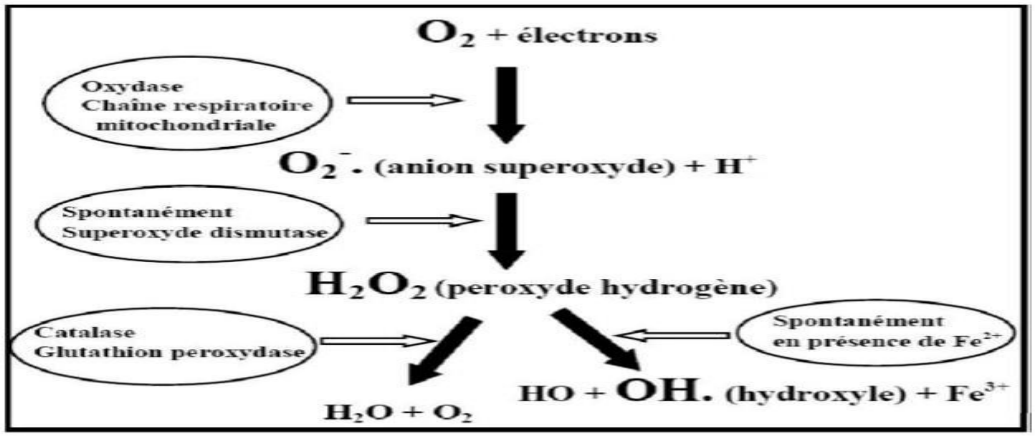

Figure 1.6.the principals ROS and antioxidants enzymes

(Mahmoud, Abbes, et Said, s. d.)

Among the consequences of this disturbance

- **Lipid peroxidation**
- **Protein oxidation**

The oxidation of amino acids of proteins by ROS leads to the loss of their function and their fragmentation (proteolysis) to form aggregation in extracellular compartments of cells(« Stress oxydant, RMLg, 2007.pdf » s. d.), and generating carbonylate proteins (CP).(Mahmoud, Abbes, et Said, s. d.)

Other study has shown that the products of lipid peroxidation affect proteins inducing their carbonylation. MDA is one of them, its level is higher during ferroptosis, it promotes protein carbonylation and the amplification of ferroptosis signal(Y. Chen et al. 2018). This phenomenon is involved in many neurodegenerative diseases together with inflammation and oxidative stress (Curtis et al. 2012).

2.2.4.2. Antioxidants and cell protection

To detoxify the oxidants in the different compartments of the cell, and to protect the cells from ROS accumulation, the cell has developed a biochemical defense system provided by antioxidants (Santos-Sánchez et al. 2019a)

a) Enzymatic antioxidants system

- **Catalase**

Is an antioxidant enzyme that can be found in most aerobic cells specially in peroxisomes(Santos-Sánchez et al. 2019a), it catalyzes the degradation reaction of two hydrogen peroxide molecules into one oxygen molecule and two water molecules.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The disruption of its function or the decrease of its expression in the cell is associated with the development of several metabolic diseases such as the two types of diabetes, neurological diseases such as parkinson's disease and even cancer and autoimmune diseases.

The enzyme has therapeutic roles as a detoxifying agent of ROS (Nandi et al. 2019).

b) Non enzymatic antioxidants system

They are free radicals scavenger’s compounds including

- **Vitamin C**

Vitamin C is an antioxidant, synthesised in the liver and kidneys, plays an inhibitory role in lipid peroxidation, giving vitamin E when it reacts with lipid radicals (« Stress oxydant, RMLg, 2007.pdf » s. d.).

- **Albumin**

Is a protein synthesized in the liver, it is considered an antioxidant in plasma, blood and intestinal fluid. It interacts with free radicals avoiding their interaction with the different components of the blood causing damage to this protein.

In fact, the protein can bind iron during a pathological overload. It also neutralizes ROS (Belinskaia et al. 2020)

The total antioxidant capacity of the cell can be measured by Oxygen radical absorbance capacity (**ORAC**) assay.(Santos-Sánchez et al. 2019b)

2.2.5. Ferroptosis in disease

The process of ferroptosis implicates in many disorders such as systemic diseases (affect organs), like cardiovascular, digestive system, nervous system diseases, and cancer. (Jie Li et al. 2020)

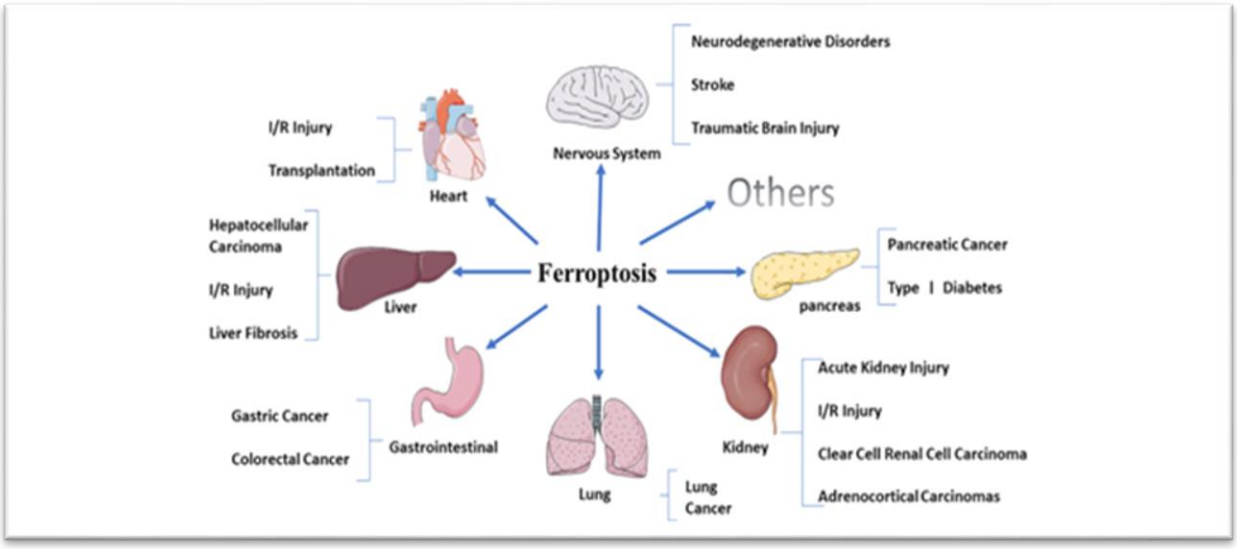

Figure 1.7. Ferroptosis in various systems' disease (Jie Li et al. 2020).

Chapitre 2 Material and methods

1. The studied population

Our analysis focused on people over 55 years of age, followed up in the neurological Department of Tlemcen Medical Center University; and who carried out their consultation at the Boudghen-Tlemcen center.

The study will target two populations:

- A group of healthy individuals (n =20) who are not affected by Parkinson's disease or other autoimmune diseases.
- A group of patients with Parkinson's disease (n = 20) who have suffered from the disease alone or in combination with other diseases.

Patient inclusion criteria

- The cases were from the regions of TLEMCEN.

The inclusion criteria for the controls are as follows

- The controls are from the same region;
- They are in the same age category as the patients;
- They do not present any chronic illnesses.

The characteristics of these populations are summarized in the table about.

1.1. Individual patient interview

Patient information is collected using a detailed background questionnaire, which includes questions on

- Surname/ first name/age/ gender/ geographical location;
- Age of occurrence of the disease;
- Start of treatment;
- Clinical signs developed;
- Accompanying diseases;
- Medication;

1.2. Ethical considerations

The recruited persons are informed by the aim of the study, and they have signed a detailed informed consent presented in the annex.

2. The samples

2.1. Sampling

Blood samples were taken from the vein in the elbow, on an empty stomach; the blood is put into "Dry, EDTA and Heparin" tubes, which are already labelled and coded.

The samples then centrifuge at 3000 rpm for 15 minutes to obtain the EDTA plasma heparin, and serum from the dry tube (Figure 01).

The EDTA plasma uses for the assay of vitamin C (in the same day), MDA, catalase, carbonyl proteins ORAC, and the serum uses for the determination of conjugated diene, and

heparin plasma uses to determine iron and albumin. Whereas, the pellet from the EDTA tube uses for the preparation of erythrocyte lysate, which has been used in the determination of erythrocyte MDA, erythrocyte catalase, and erythrocyte carbonyl proteins. (Sari 2014)

For the red cell lysate, 2 ml of physiological water (9 g NaCl) mixed with 1 ml of pellet for washing, and then it centrifuged at 2000 rpm for 5 min. The supernatant removes, and 1 ml of pellet mix to gather with 2 ml of ice-cold distilled water to lyse the erythrocytes, vortexes, incubated for 10 min in the refrigerator, and then centrifuged at 4000 rpm for 10 min. The supernatant represents the erythrocyte lysate (Sari 2014).

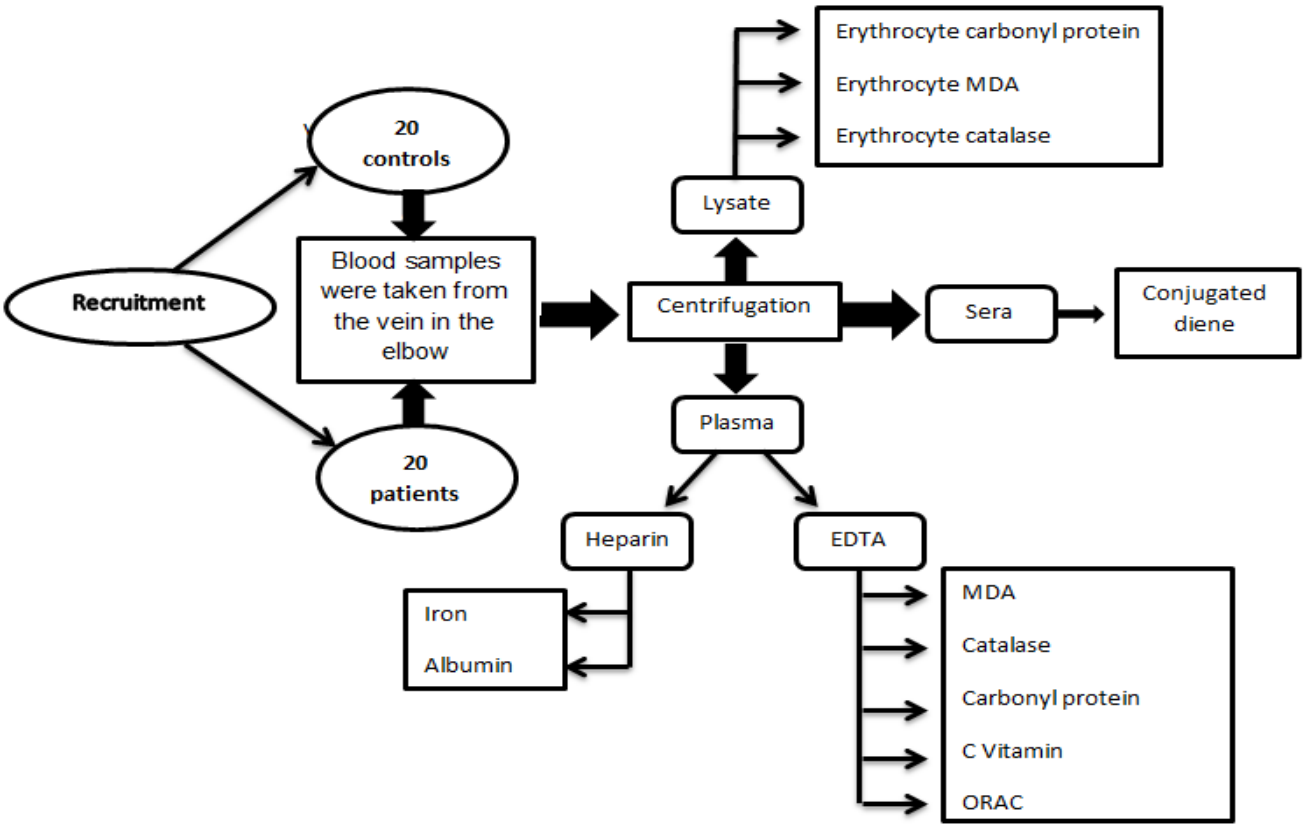

Figure 2. 8. Study design

2.2. Determination of ROS markers

2.2.1. Lipids peroxidation hallmarks

2.2.1.1. Determination of plasma Malondialdehyde (MDA) (Nourooz-Zadeh et al. 1996)

MDA is the most widely used marker for lipid peroxidation, mainly because of the simplicity of the technique and its sensitivity. The principle consists of the reaction between the aldehyde and the thiobarbituric acid (TBA) molecule, after a hot acid treatment, forming a chromogenic condensation product, consisting of one MDA molecule and two TBA molecules. The intensity of this chromogenic product reads at 532nm, and the concentration of MDA in the sample calculates using the extinction coefficient of the MDA-TBA complex.
(ε=1.56.10 mol-1 .l.cm-1 at 532 nm).

2.2.1.2. Determination of erythrocyte Malondialdehyde (MDA) (Nourooz-Zadeh et al.1996)

The same principle and determination of plasma MDA uses for the determination of erythrocyte MDA, the same method uses, except that erythrocyte lysate made instead of plasma.

2.2.1.3. Determination of conjugated diene (Ito, Sono, et Ito 2019) modified

The formation of conjugated dienes (CDs) results from the rearrangement of the double bonds of polyunsaturated fatty acids (PUFAs) following the radical abstraction of a malonic hydrogen. CDs are considered the primary products of lipid oxidation and exhibit ultraviolet absorption at 234nm.

2.2.2. Protein's oxidation hallmarks

2.2.2.1. Determination of plasma carbonyl proteins)(Levine et al. 1990)

Are markers of protein oxidation, they measure in the presence of u 2-4 dinitrophenylhydrazine (DNPH), the reaction results in the formation of coloured dinitrophenylhydrazone.

The reading is taken at 2 wavelengths, 350 nm and 375 nm, to determine the plasma concentration of the proteins, the extinction coefficient ε= 21.5 m mol-1.l.cm-1 is used, and they are presented in µmol/l.

2.2.2.2. Determination of erythrocyte carbonyl proteins (Levine et al., 1990)

The same method uses, just using erythrocyte lysate instead of plasma.

2.3. Determination of antioxidants activities

2.3.1. Determination of plasma and erythrocyte catalase activity (Aebi 1974)

The assay consists to measure the rate of decomposition of hydrogen peroxide.

The medium contains the sample (lysate or plasma) as the source of catalase, the hydrogen peroxide solution (H2 O2), and the staining reagent: titanium oxide sulphate (TiOSO 4).

The catalase leads to the decomposition of H_2O_2, causing the absorption of the H 202 solution to decrease with time.

We read the results at 420 nm. The concentrations of the remaining H202 are determined from an n H202 standard.

We followed these steps to calculate enzyme activity rates:

A= (Log of starting concentration - Log of remaining H202 concentration). Therefore:

Plasma catalase activity expressed in U /ml / min = A x Vi / Ve / T.

Erythrocyte catalase activity expressed as U/ /ml / min = A x Vi x D / Ve / T.

Where:

Vi: incubation volume; Ve: sample volume; T: incubation time; D: Lysate dilution.

2.3.2. Determination of the total antioxidant capacity of plasma (ORAC) (Blache and Prost, 1992)

The total antioxidant capacity of plasma, that mean its capacity to absorb free oxygen radicals (ORAC: Oxygen Radical Absorbance Capacity) is estimated by the capacity of red blood cells to resist free radical-induced haemolysis in vitro in the presence of plasma. This method is based on monitoring the haemolysis of red blood cells induced by a free radical generator as a function of time.

This involves bringing a suspension of red blood cells into contact with free radicals under controlled and standardized conditions. All the enzymatic and chemical systems of the sample are mobilised to protect the integrity of the cells until their lysis. Thus, haemolysis occurs gradually over time. Measuring the increase in absorbance at 450 nm every ten minutes allows the kinetics of haemolysis to be monitored.

The addition of a determined quantity of an antioxidant, vitamin E (Trolox) or vitamin C (ascorbic acid) allows the neutralisation of a quantity of free radicals in the incubation medium and thus allows the protection of the red blood cells against the attack of the free radicals and haemolysis. The lysis kinetics curve of the red blood cells is therefore deviated and a shift in the curve is observed as a function of time.

Plasma contains several antioxidant defence systems and thus allows the protection of red blood cells against free radical attack. In the presence of plasma, a shift in the kinetics of red cell haemolysis is also observed. The total antioxidant capacity of plasma thus represents its capacity to neutralize the free radicals produced in vitro and thus to delay the hemolysis of the attacked red blood cells, thus indirectly slowing down the increase of the optical density at 450 nm. In order to quantify this total antioxidant power, the use of purified antioxidants (Trolox, vitamin C) at known concentrations allows calibration. Haemolysis is therefore monitored over a time interval ranging from two to 6 hours. The optical densities (D0) are read every ten minutes at 450 nm.

The ORAC of each sample is calculated by the following formula:

1U ORAC = ∑ (OD Blank- OD Standard) / OD number.

ORAC Sample = [∑ (OD Blank - OD Sample) / OD number] / [∑ (OD Blank - OD Standard) / OD number] x one U ORAC.

Blank = incubation of red blood cells with free radical generator

Standard = Trolox (1µM) or vitamin C (2µM).

2.3.3. Determination of vitamin C (Jacota and Dani, 1982)

Plasma vitamin C is determined using Folin's reagent and a standard range of ascorbic acid. After precipitation of plasma proteins with trichloroacetic acid (TCA) and centrifugation, the supernatant incubates in the presence of diluted ciocalteau folin staining reagent. The vitamin C contained in the plasma reduces the folin reactive giving a yellow coloration. The

intensity of the color obtained is related to the concentration of vitamin C present in the sample at a wavelength of 769 nm. The concentration is determined from a standard curve obtained from an ascorbic acid solution.

2.3.4. Albumin concentration (SPINREACT,S.A./S.A.U. Ctra.Santa Coloma, 7 E-17176 SANT ESTEVE DE BAS (GI) Espagne)

Albumin combines with bromocresol green at slightly acidic pH, resulting in a change in color of the index, from yellow-green to bluish-green, and proportional to the concentration of albumin present in the sample tested.

2.4. Iron determination (BioSystems S.A. Costa Brava, 30. 08030 Barcelona (Spain))

Ferric ions bound to transferrin in the sample are released by guanidinium and reduced to ferrous by means of hydroxylamine.

The ferrous ions react with ferrozine to form a coloured complex, which can be measured by spectrophotometer.

The reading is taken at 560 nm, and calculations are made using the following equation:

$C_{sample} = (A_{sample} - A_{blank}) / (A_{standard}) \times C_{standard}$

C: the concentration.

A: absorbance.

Table 2.3 the Demographics characteristics of population study

Parameters	Controls	patients
Number	20	20
Age	62.85 ± 1,3	69,2 ± 2,2
Size (cm)	163,2 ± 1,809	164,55 ± 2,817
Weight (Kg)	71,25 ± 1,9	65,65 ± 2,4
IMC	27,025 ± 0,94	24,155 ± 0,67
Sex (M/F)	12/8	12/8

The values represent means ± error standard

Chapitre 3 Results and interpretation

Chapitre 4 Discussion

The oxidative balance is a physiological process of the cells, it allows maintaining the normal biological functions of the living cells, and their disturbance leads to their death and thus the damage of tissues and the dysfunction of organs (Yang et Lian 2020).

Several studies have shown the involvement of oxidative stress in peripheral inflammatory diseases, which can be as far away from the body as the brain.

In our study, which is carried out on Parkinson's disease, we found an increase in the level of oxidants in patients compared to controls, whereas the level of ROS-detoxifying antioxidants has been shown in other research to be simulated in diseases related to oxidative stress. Our results show the opposite, where an increase in some antioxidant parameters was observed in patients compared to controls.

This increase level in ROS leads to two major conclusions, which are lipid peroxidation and protein oxidation.

Lipid peroxidation is an important marker of cell death by ferroptosis and is evaluated through two principal biomarkers, which are MDA and conjugated diene in a number of studies where they have been found to be elevated during ferroptosis.

The results obtained in our laboratory show a non-significant increase of both variables in patients compared to controls.

The oxidation of protein generate carbonyl proteins at the end of the reaction, it is consider as oxidant marker , its level is important in many disorders as motioned in (Mahmoud, Abbes, et Said, s. d.).

In our study, we have found significant difference in the concentration of erythrocyte CP in patients suffered from PD than healthy control.

Iron is heavy metals, it is considered as pro-oxidant, when its concentration is overload in the blood cause an inflammation and ROS augmentation (Zhai et al. 2021).

Its level is higher in plasma of patients that of controls with significant difference as markers of oxidative stress.

Antioxidants hallmarks like albumin that play an important role both in normal conditions and during oxidative stress, it react with iron when it is overload in the blood, leading to albumin's damage and the reduction in its concentration (Santos-Sánchez et al. 2019a).

In this study, we have found that albumin's concentration is decreased in patients than controls as result of the high level of iron in the same groups.

Many others studies have been shown that the decrease level of catalase is associated with the development of many disorders including neurodegenerative disease (Nandi et al. 2019).

Catalase activity is higher in patients than in control conversely to other results, and it is the same results regarding vitamin C levels.

Chapitre 5 Conclusion

The origin of Parkinson's disease and the mechanism in which it develops remains an opaque point. The results obtained are promising and give much hope as to the involvement of ferroptosis in this illness, where we have shown the presence of lipids peroxidation biomarkers (MDA, CD) and oxidants markers (PC and iron).

knowing that other studies are interested in this new form of cell death and its mechanism, and the possibility of using several therapies, which pushes us to deepen our work in the same sense and to evaluate other parameters of the of ferroptosis not only in the blood but also in the neuronal cell itself. Aiming at therapeutic strategies, that targets ferroptosis in the brain rather than in the periphery.

Several questions remain unanswered regarding necroinflammation and the different types of cell death during the pathogenesis of the disease,

Is ferroptosis the cause or the consequence of necroinflammation?

Are the markers of ferroptosis sufficient to diagnose the disease?

If ferroptosis is involved in the development, it becomes easy to prevent degeneration?

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