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Iron Toxicity, Lipid Peroxidation and Ferroptosis among Patient with Alzheimer and Parkinson Diseases

A Thesis

Submitted to the Council of the College of Medicine, University of Kerbala, in Partial Fulfillment of the Requirements for the Master Degree in Clinical Chemistry

By

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بِسْمِ اللهِ الرَّحْمَنِ الرَّحِيمِ

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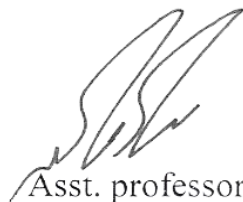
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Was prepared under our supervision at the College of Medicine/University of Kerbala. as a partial fulfillment of the requirement for the Degree of Master in clinical chemistry.

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Dedication

I dedicate this effort to the greatest messenger and honorable family: Amir Almuminin, Alsayida AL Zahra, Imam Al Mujtaba ,Imam Sayed AlShuhada and the nine of his son (May God's peace be upon them) especially Imam AL Mahdi (may God almighty hasten his honorable reappearance).

And to my beloved, dear father, who supported me in my studies but he passed away to God… before saw my success.

To tender mother, the source of my strength and steadfastness.

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Summary

Neurodegenerative diseases (NDDs) are age related conditions described by uncontrolled neuronal death leading to a progressive degeneration in brain functions. NDDs affect millions of people worldwide. Alzheimer's disease and Parkinson's disease are the most common of NDDs. These diseases related to complex and intricate cell death mechanisms and multiple pathways, which include excessive accumulation of iron and lipid peroxidation in different brain regions. The most common NDDs include Alzheimer's disease(AD),Parkinson'sdisease(PD),prion disease, Amyotrophic lateral sclerosis(ALS), Huntington's disease(HD), spinal muscular atrophy, and spinocerebellar ataxia.

 Ferroptosis is a type of programmed cell death dependent on iron and characterized by the accumulation of lipid peroxides,it is genetically and biochemically distinct from other forms of regulated cell death such as apoptosis. ferroptosis initiated by the failure of the glutathione-dependent antioxidant defenses, resulting in lipid peroxidation and eventual cell death.

 This study aimed to evaluate the role of free iron, their transported such divalent metaltransporter1(DMT1), Totalironbindingcapacity(TIBC), 5-lipoxygenase(5LOX) ,Malondialdehyde(MDA)and ferritin level in Alzheimer disease and Parkinson disease .

 A case control study ,80 samples were collected from 40 cases (24)Alzheimer'sdisease(AD)frome psychiatry clinic,(16) Parkinson's disease (PD)frome Neurology clinic aged between (65-90) years and 40 participants as a healthy control with age range (65-87).

 Examination of serum levels of DMT1, MDA, 5LOX were measured using enzyme linked immunoassay (ELISA) technique. serum levels of biochemical tests (lipid

profile, free iron, ferritin, TIBC) were performed using a clinical chemistry analyzer, Complete blood count was done by XP-300™ Automated hematology analyzer Sysmex.

Results indicated that, patients with neurodegenerative disease showned significant increasing in the range level of the TIBC, MDA, Free Iron and Ferritin when comparing to the healthy control groups (p value were <0.05), while the range level of 5LOX and DMT1 were decreased compared to healthy control and only 5LOX was shown a significant differences (p value were <0.05). ROC curve and AUC analysis for the biomarkers were performed. Increased level of Iron showned good performance for prediction Neurodegenerative disease, TIBC was the best prediction for Alzheimer's and Parkinson's disease cases.

It was concluded that, iron forms were linked successfully in the diagnosis of neurodegenerative disease. It is likely that iron's defective homoeostasis and their redox-activity plays a role in the neuropathology of Alzheimer's and Parkinson's disease.

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AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
$A\beta$	Amyloid-beta peptide's
BMI	Body Mass Index
CBC	Complete Blood Count
CNS	Central nervous systems
CSF	Cerebrospinal fluid
CT	Computed tomography
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent assay
GPX4	Glutathione peroxidase 4
GSH	Glutathione
HD	Huntington's disease
HDL	High-density lipoprotein
L-OOH	lipid peroxides
LDL	Low-density lipoprotein
MDA	Malondialdehyde
MRI	Magnetic resonance imaging
NA	Noradrenaline
NDDs	Neurodegenerative diseases
NFTs	Neurofibrillary Tangles
PBMCs	peripheral blood mononuclear cells
PD	Parkinson's disease
PE	phosphatidyl ethanolamine
PLs	Phospholipids
PNs	Peripheral nervous systems
PUFAs	polyunsaturated fatty acids
RBCs	Red Blood Cell
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
SNpc	Substantia Nigra
SPs	senile plaques

TG	Triglycerides
TIBC	total iron binding capacity
WHO	World Health Organization
WHR	World Health Rankings
HB	hemoglobin
LOX	lipoxygenase
FPN	ferroportin
TFR1	Transferrin receptor protein 1

Chapter one

Introduction
and
Literature review

1. Introduction:

Neurodegenerative diseases (NDDs) are neurological disorders that damage the composition and function of the brain's central and peripheral nervous systems (CNS and PNs). NDDs can lead to progressive loss of nerve structure that needed for brain roles **(Ball, *et al*., 2019).** It is a substantial global cause of death and disability **(Collaborators, 2019)**, and a serious danger to human health. Due in part to the recent evolution in the older population, these age-dependent diseases become more and more common **(Reeve, *et al*., 2014).**

Interference that concurrently target many risk factors and disease pathways at an early stage of the illnesses are most likely to be beneficial, given the complicated multifactorial nature of neurodegenerative diseases. aging is the main risk factor for neurodegenerative diseases, while other variables such as cerebrovascular disease, diabetes, and inflammation define phases in this unstoppable complex cascade **(Moldogazieva, *et al*., 2019)**.

Disorder such as oxidative stress, glycation, abnormal protein deposition, inflammation, and progressive neuronal loss are shared by Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and others from kind Neurodegenerative diseases **(Majd, *et al*., 2015)**.

Symptoms of these diseases advance in severity the long-term of the live with the case. While medication can be support and sometimes even slow forward , it can't end it. Some of these diseases have weaken brain function, absence of muscular domination, need more time to learn new skills, loss of memory, depression, social isolation, having undesirable feelings and ideas (**Levenson, *et al*., 2014)**.

1.1. Causes of Neurodegenerative Diseases:

NDDs are thought to be caused by damage to neurons in the brain. The nervous system includes the brain and spinal cord is made up of neuron, when a neuron is injured, it cannot be repaired like other parts of the body and it's unable to replace itself **(Weishaupt & Zhang, 2016)**, as older age, these neurons die, the brain really shrinks **(Peters, 2006)**.

Environmental and Genetic Factors The causes of the majority of neurodegenerative disease are not well understood. Long-term exposure to toxins and specific chemicals are two environmental factors .genetic variables that are known to contribute to the majority of these diseases **(Armstrong, 2020).** In some cases, relatives can pass down mutated genes that can cause developing a neurological disease **(Price, *et al*., 1998)**.

Numerous neurodegenerative diseases have also been related to abnormal proteins in the brain. These aberrant proteins have the potential to kill brain nerve cells. With AD, A protein called beta-amyloid has been associated with development of the condition **(Spires-Jones, *et al*., 2017).**

A substantial research suggests NDDS is caused by several factors, including metal toxicity and pesticide exposure, mitochondrial dysfunction, altered bioenergetics,oxidative stress and free radical formation **(Sheikh, et al., 2013)**, Figure(1.1) **(Sheikh, *et al*., 2013).**
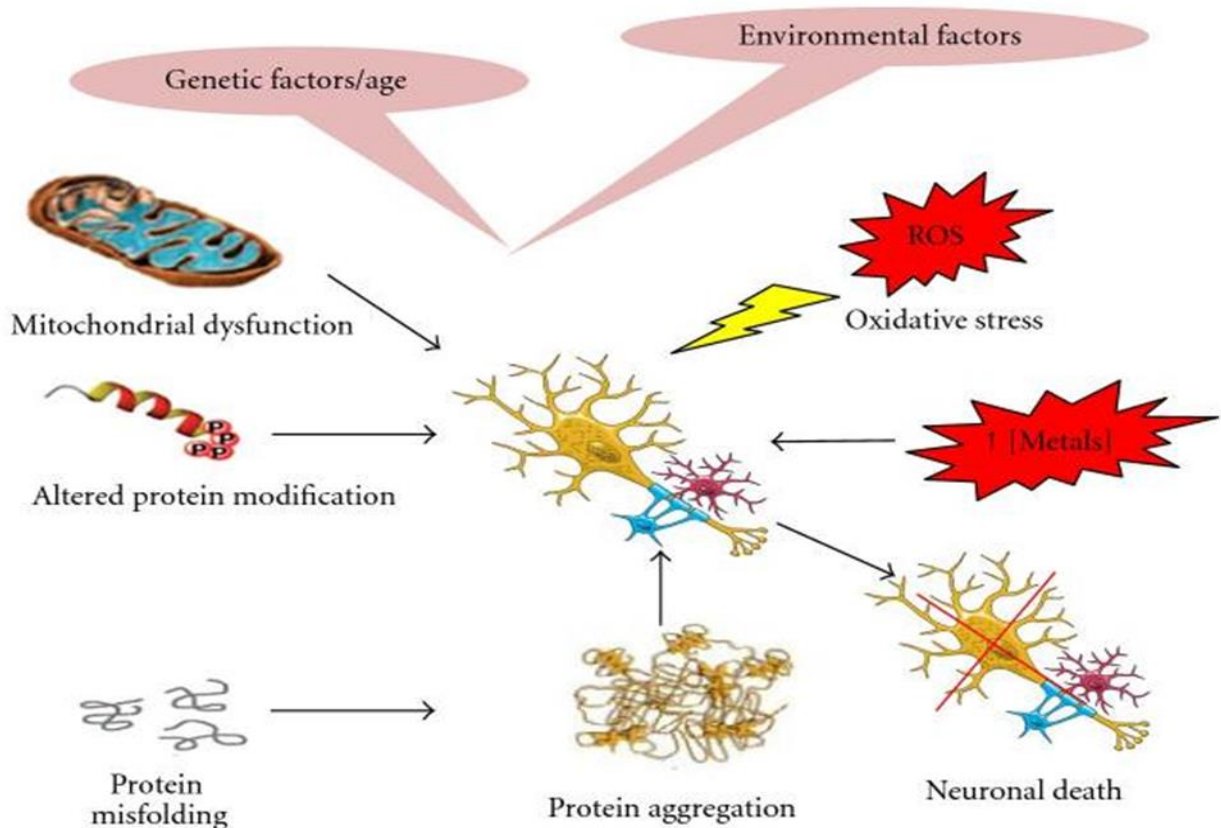
Figure (1.1) Different factors associated with neurodegenerative disease (Sheikh, *et al*., 2013).

1.2.Risk factors for neurodegenerative disease:

1.2.1. Non-modifiable risk factors:

Most of neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease, have aging as their main risk factor. One in ten people ≥65 years old have AD, and the frequency of the condition rises with age. Age-related neurodegenerative disorders have few or no active therapies obtainable (**Hou, *et al*., 2019**).

Sex: Research suggests that there may be a physiological difference in how males and females develop neurodegenerative diseases. The prevalence of many neurodegenerative diseases is biased towards one sex or the other but varies between diseases **(Lotz, *et al*., 2021)**. for example, research show that female is highly at risk for developing AD, with approximately two-thirds of Alzheimer's patients being female **(Pinares-Garcia, *et al*., 2018)**.

Females exhibit a faster rate of hippocampal atrophy and more neurofibrillary tangles as well as a higher rate of cognitive decline.on the other hand, Parkinson's disease is more common among males,it affects males more frequently than females and progresses more quickly in males overall **(Brown, *et al*., 2005).**

Genetic factors: although it is now well established that genetic mutations play a substantial role in AD, the etiology and processes as yet poorly understand. even though it is now well documented that genetic mutations play a significant role in AD. Apolipoprotein E is a polymorphic protein that facilitates lipid transport and damage healing in the brain and is suggested as a cholesterol carrier **(Lambert, *et al*., 2013).**

ApoE mainly has a significant impact on the microglia and astrocytes, which require de novo cholesterol synthesis, The primary genetic factors affecting AD risk are ApoE polymorphic alleles **(Pasqualetti, *et al*., 2022)**.

In PD, up to 60% which is the cause is unknown of PD patients' phenotypes might be interpret by hereditary variables, according to a research that examined the heritability of PD risk in over 500 nuclear families **(Huang, *et al*., 2006).**

1.2.2 Modifiable risk factors:

Some of risk variables that might be changed were examined: Hypertension , obesity, diabetes, depression, cigarette smoking and excess drinking **(Olivari, *et al.*, 2020),** figur(1.2) **(Nazam, *et al.*, 2021).**
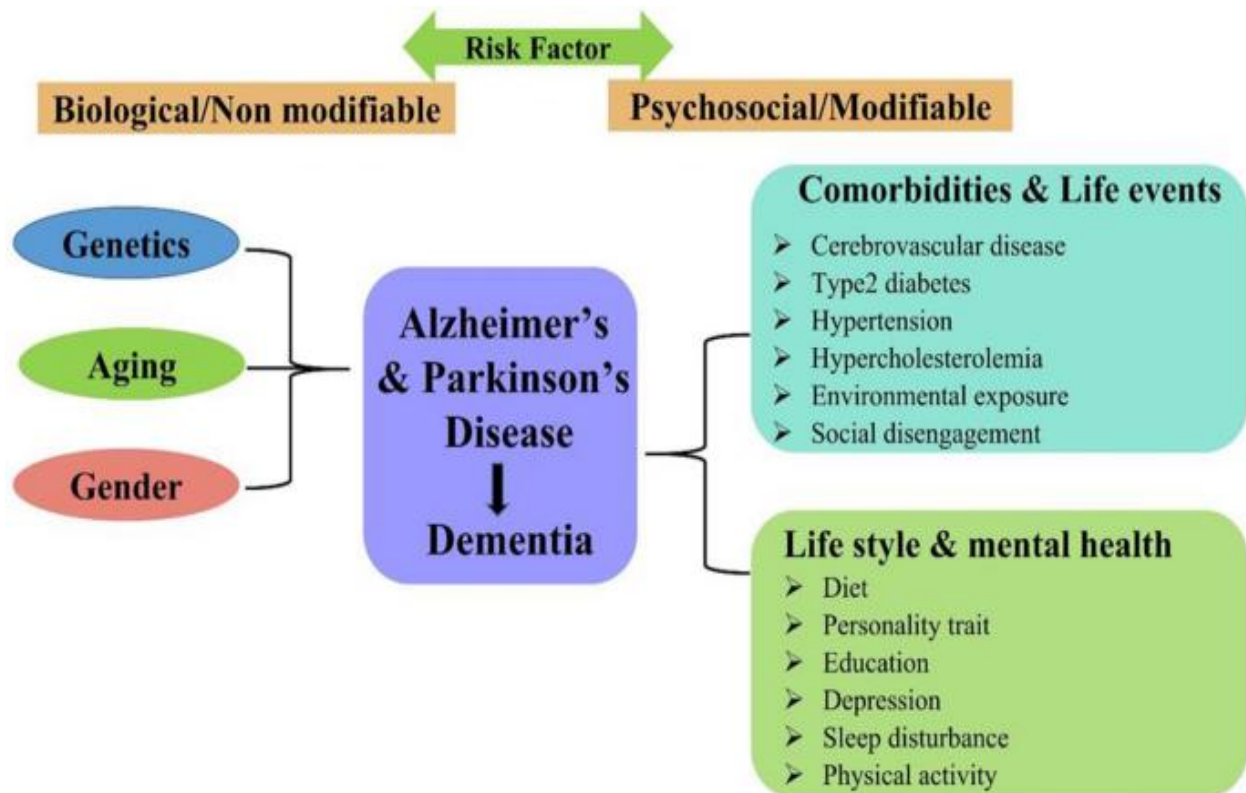
Figure(1.2) Risk factor for neurodegenerative disease

(**Nazam, *et al.*, 2021).**

1.3. Types of neurodegenerative diseases (NDDs)

The most common NDDs include Alzheimer's disease, Parkinson's disease, prion disease, Amyotrophic lateral sclerosis, motor neuron disease, Huntington's disease, spinal muscular atrophy, and spinocerebellar ataxia**.**

1.3.1. Alzheimer's disease (AD):

The most prevalent form of dementia,can be defined as a slowly progressive neurodegenerative disease characterized by neuritic plaques and neurofibrillary tangles as a result of amyloid-beta peptide's (Aβ) aggregation in the most influenced region of the brain, the median temporal lobe and neocortical structures **(Breijyeh & Karaman, 2020).**

Ageing, genetics, brain traumas, vascular illnesses, infections, and environmental variables (heavy metals, trace metals, and others) have all been identified as risk factors for AD, which has been described as a complex disease. Alzheimer's disease pathological alterations (Aβ, NFTs, and synaptic loss) have an unknown underlying etiology. Two theories were put forth as potential causes of AD, but it is thought that only one of them a change in the production and processing of amyloid β-protein is the primary starting factor. Some people think that cholinergic dysfunction is a significant risk factor for AD. However, at present, there is no accepted theory for explaining the AD occur **(Breijyeh & Karaman, 2020),** figure(1.3) **(Breijyeh & Karaman, 2020).**
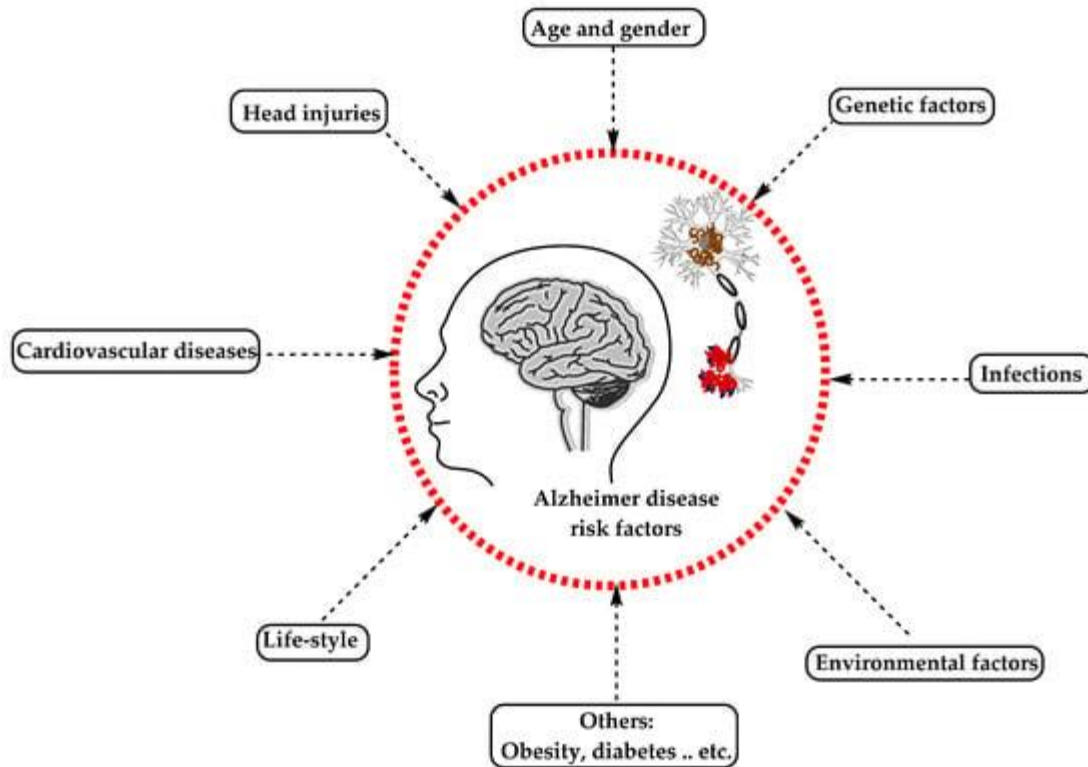
Figure(1.3) The risk factors for Alzheimer's disease (Breijyeh & Karaman, 2020).

There are around 10 million cases of dementia each year, and there are about 50 million individuals with dementia worldwide, Two in three people with dementia live in low- and middle-income countries **(WHO, 2018)**.

In 2006, there were 26.6 million cases of AD in the world. By the year 2050, the worldwide prevalence of AD will grow fourfold to 106.8million **(Brookmeyer, *et al*., 2007).**

According to the most recent WHO data, 2,429 fatalities in Iraq from Alzheimer's and dementia or 1.66% of all deaths occurred in 2020. Iraq is standing 66 in the world with an age-adjusted death rate of 19.16 per 100,000 people (WHR, 2020)**.**

Studies have shown that AD-related brain pathological changes begin 20 years or more before dementia symptoms appear, and it takes years for patients to experience significant memory and language impairments. The reason for this is damage to neurons in the brain associated with thinking and memory **(Lei,** *et al.***, 2021).**

Protein misfolding may be the cause of pathogenic changes in AD **(Calabrò,** *et al.***, 2021)**. Pathologically misfolded proteins may cause normally folded proteins to change their shape, which might lead to the propagation of disease **(Vingtdeux,** *et al.***, 2012).**

There are a number of mechanisms evolved for cell-cell communication, including synaptic transmission, direct contact through gap junctions, and paracrine signaling, therefore it is not impossible for misfolded proteins to spread across cells **(Schiera,** *et al.***, 2015).**

Misfolded protein continue accumulation ultimately lead to the activation of the unfolded protein response, which trigger apoptosis and consequently neurodegeneration **(Diehl,** *et al.***, 2011),** figure (1.4) **(Breijyeh & Karaman, 2020)**.
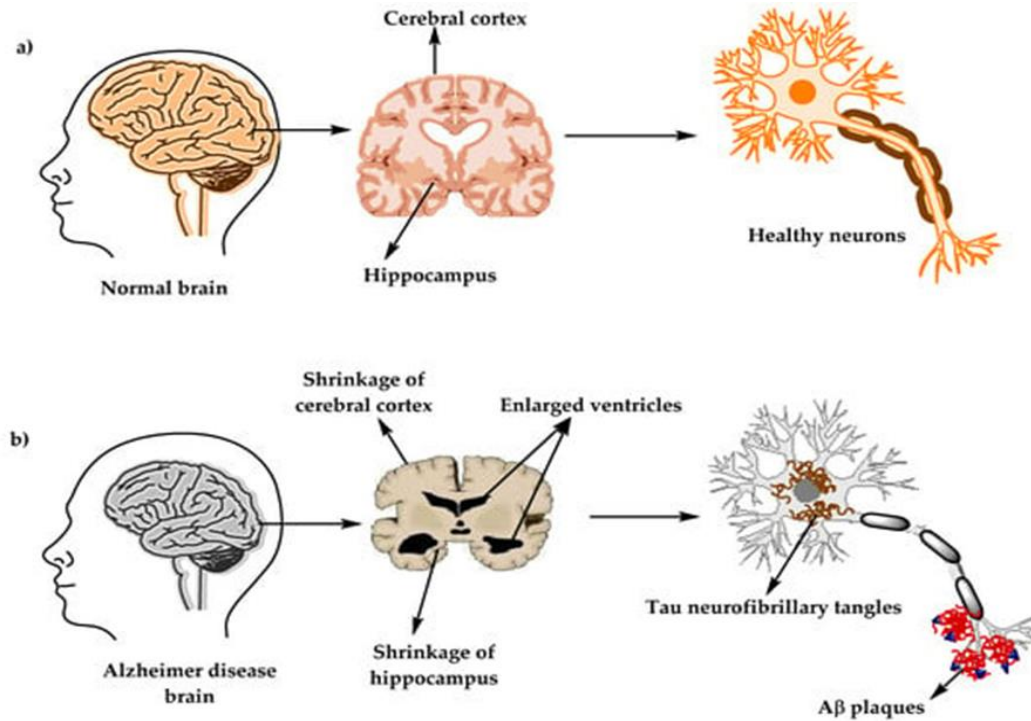
Figure (1.4)The physiological structure of the brain and neurons in (a) healthy brain and (b) Alzheimer's disease (AD) brain (Breijyeh & Karaman, 2020).

1.3.2 Diagnosis of alzheimer's disease

It is include loss of memory, Physical and neurological exam like; reflexes,coordination,muscle tone and balance.also lab tests for example blood tests may help rule out other potential causes of memory loss and confusion, such as a thyroid disorder or vitamin levels that are too low.mental status and imaging of brain magnetic resonance imaging (MRI) and amyloid PET imaging **(Podcasy & Epperson, 2022)**.

1.3.3. Parkinson's disease (PD)

It is a common, progressive neurodegenerative disorder, affecting approximately 1% of people over 65 years of age (**Wirdefeldt,** *et al.***, 2011**).

It is a movement condition characterized by slow motion, solidity and shiver in body. Its appearance can vary, though, and may include non-motor symptoms including cognitive decline and sleep difficulty. The diagnosis is made clinically by the recognition of these key features and the exclusion of other causes of parkinsonis (**Stoker & Greenland, 2018**).

The basal ganglia, a region of the brain that regulates movement, experiences nerve cell impairment and/or death, which results in the most noticeable signs and symptoms of Parkinson's disease. These nerve cells, or neurons, normally generate the crucial brain neurotransmitter dopamine. movement problems linked to the condition are brought on by decreased dopamine production as a result of neuronal death or impairment (**Poirier,** *et al.***, 2016**) ,(figure 1.5) (**Miljkovic,** *et al.***, 2016**).
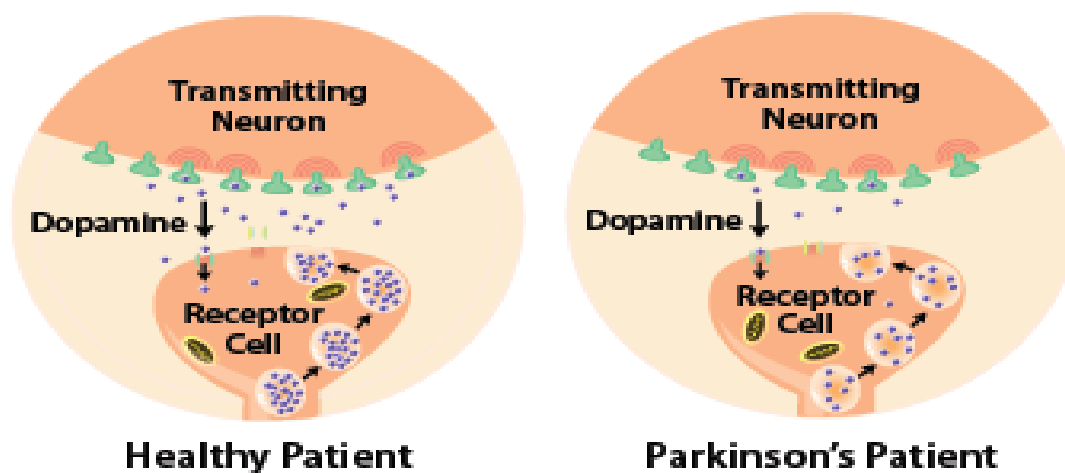
Figure(1.5) Different between healthy patient and Parkinson's patient
(**Miljkovic,** *et al.***, 2016**).

There is growing evidence that additional loss of noradrenaline (NA) neurons of the locus coeruleus, the principal source of NA in the brain, could be involved in the clinical expression of motor as well as in non-motor deficits (**Delaville, *et al*., 2011**).

More people worldwide are becoming disabled and dying from Parkinson's disease (PD) than from any other neurological condition. In the last 25 years, PD prevalence has doubled. according to 2019 estimates, there were approximately 8.5 million people worldwide who had PD. According to current estimates, PD caused 329000 deaths in 2019, an increase of over 100% since 2000 (**Schiess, *et al*., 2022**).

According to the latest WHO data published in 2020 Parkinson's Disease Deaths in Iraq reached 0.45% of total deaths. The age adjusted Death Rate is 4.74 per 100,000 population Iraq is standing 59 in the world (**WHO, 2020**).

The risk of PD is influenced by age. With an average starting age of 60, advanced age is the biggest recognized risk factor for Parkinson's disease. Young-onset PD is the term used to describe PD that develops before the age of 50 (**Reeve, *et al*., 2022**).

There may be a physiological difference in how males and females acquire Parkinson's disease, according to a research males experience PD more commonly than females. While females have a greater death rate and a faster rate of illness development than males, males are twice as likely to get PD (**Cerri, *et al.*, 2019**).

10% to 15% of all PD cases are hereditary in nature. The likelihood of getting PD is almost two times higher for people who have a parent or familial who has the condition (**Klein & Westenberger, 2012**).

Toxin exposure, in the opinion of some scientists and medical professionals, may marginally raise the chance of PD development. Agent Orange, pesticides, herbicides, solvents, manganese and other metals, and organic pollutants are just a few examples

of the items that might cause exposure in this category **(Collier,** *et al.***, 2011)**,(figure 1.6) **(HealthTech, 2018)**.
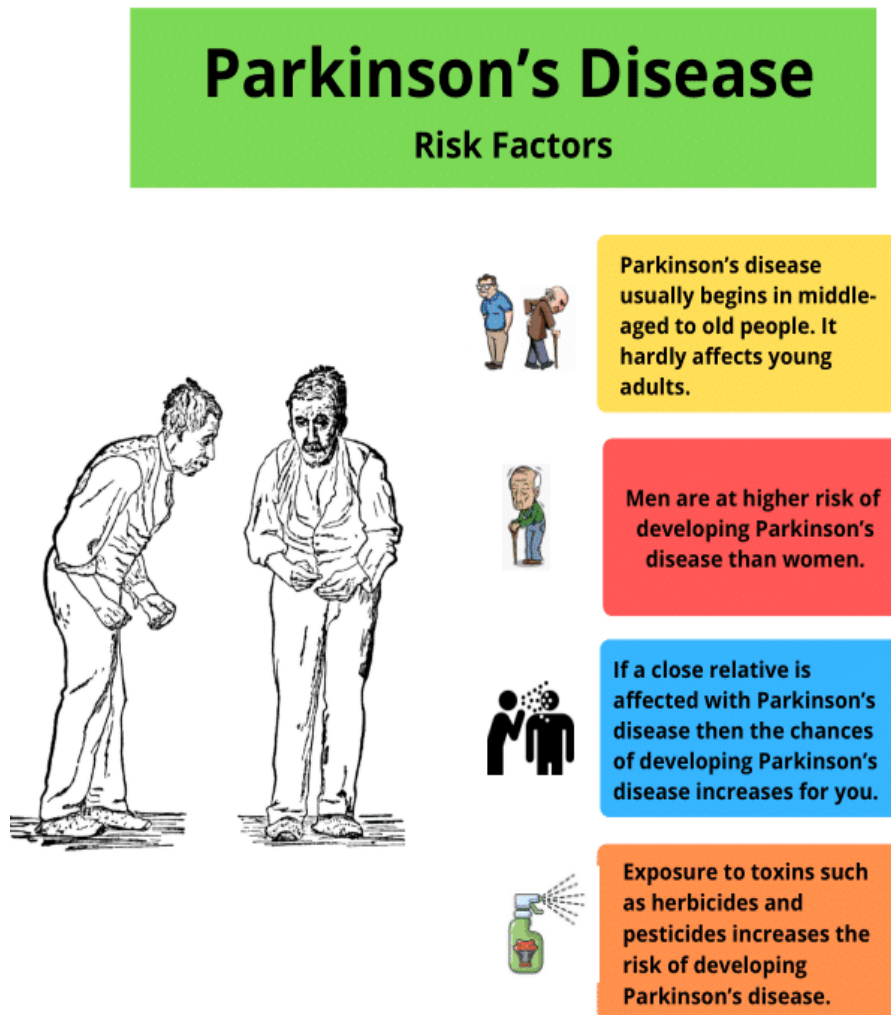
(Figure1.6) Risk Factors for Parkinson's Disease (HealthTech, 2018).

1.3.4.Diagnosis of Parkinson's disease

Parkinson's disease is a brain disorder that causes unintended or uncontrollable movements, such as shaking, stiffness, and difficulty with balance and coordination. From motor symptoms which includes four cardinal features: bradykinesia, rest

tremor, rigidity, and postural and gait impairment **(Rodriguez-Oroz, *et al*., 2009)** In addition, even to an unskilled observer, motor signs may appear immediately **(Gallagher, *et al*., 2010)** . Brain structural imaging uses magnetic resonance imaging (MRI) or computed tomography (CT) **(Heim, *et al*., 2009) .**

1.4. Iron (Fe)

It is essential component for human survival and one among the important heavy metals for human nutrition **(Hsu, *et al*., 2020)** .Iron plays very important roles for the transfer of oxygen and electrons, cell division, differentiation and gene regulation **(Boldt, 1999)**.

The red blood cells' hemoglobin, which is where 70% of the body's iron is bound, that gives blood its red pigment. The remaining iron is either stored in cells or is bound to other proteins including myoglobin, transferrin, and ferritin. Regular iron equilibrium is influenced by the reticuloendothelial system, which is responsible for clearing damaged red blood cells (RBCs) via macrophages of the spleen, liver, and bone marrow **(Abbaspour, *et al*., 2014).**

Iron is involved in many fundamental biological processes in the brain including oxygen transportation, DNA synthesis, mitochondrial respiration, myelin synthesis, and neurotransmitter synthesis and metabolism **(Crichton, 2002).**

Iron homoeostasis is needed to maintain normal physiological brain function, whereas misregulation of iron homoeostasis can cause neurotoxicity through different mechanisms.homoeostatic mechanisms provide the conditions for optimum cell function by maintaining an equilibrium of available iron concentrations between cellular compartments and buffering molecules, and preventing toxic effects caused by excessive concentrations of free iron **(Wang & Pantopoulos, 2011).**

The quantity of iron in the labile iron pool can rise when iron concentrations are higher than the cellular iron retention capacity of storage proteins or other molecules, which might be detrimental and result in oxidative damage and cell death **(Kruszewski, 2003)**.

The iron accumulation noted in neurodegenerative diseases is a primary event or a secondary effect is unclear. Age-related accumulation of iron might be an important factor that contributes to neurodegenerative processes **(Tian,** *et al.***, 2022).**

1.4.1. Iron transport, utilization, and storage:

After being absorbed by the intestinal mucosa, iron is either transferred through the mucosal cells immediately to the plasma, where it is specially delivered incorporation with transferrin, or change into ferritin and stored by the mucosal cells. When mucosal cells have damage, any iron they had absorbed is excreted from the body. The body's iron case affects mucosal cell detention, which is reduced in iron deficiency and enhanced in case of iron excess. Normal levels of total iron in circulation that is linked to transferrin range from 50 to 70 mol (3 to 4)mg. Iron in plasma is taken up by cells and either incorporated into haem or stored as ferritin haemosiderin, probably formed by the condensation of several molecules of ferritin. Iron released by the break down of Hemoglobin(Hb), at the end of the erythrocyte ' s life, is normally efficiently conserved and later reused**(Beckett,** *et al***., 2010)**,(figure1.7) **(Yiannikourides & Latunde-Dada, 2019)**.
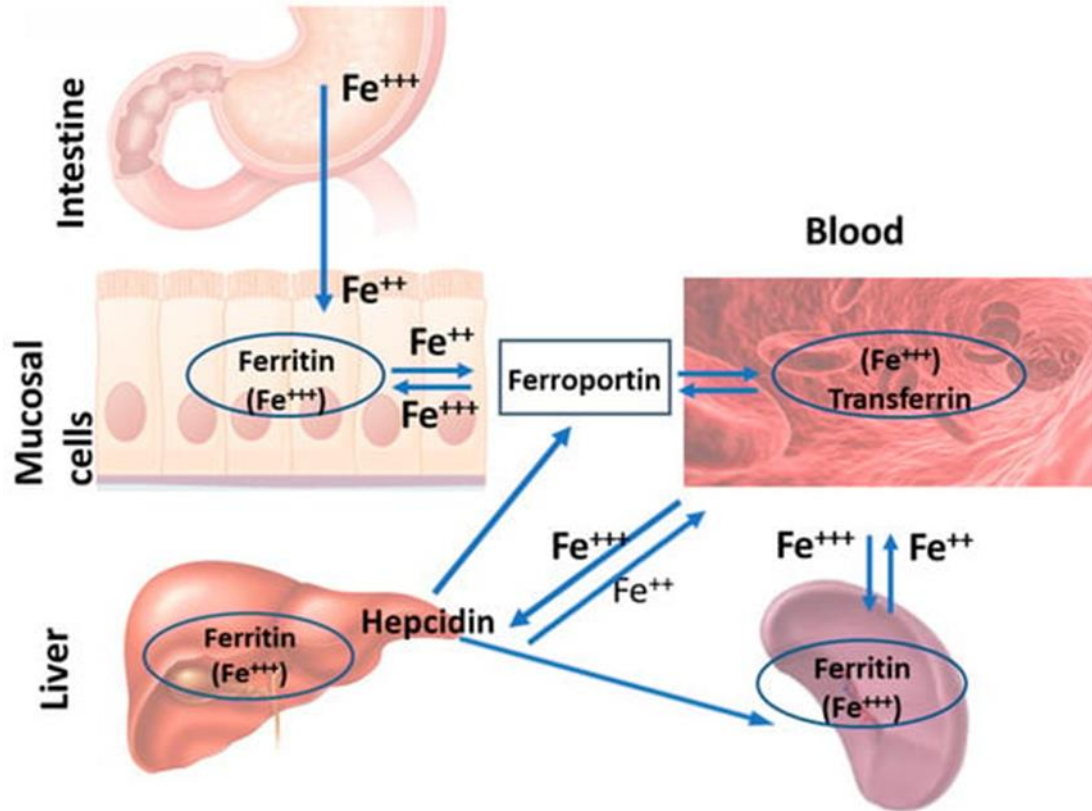
Figure(1.7) the main tissues where iron metabolism occurs. (Yiannikourides & Latunde-Dada, 2019)

1.4.2. Iron in the brain

For healthy brain action, physiological iron levels must be preserve. In effect, a number of neurodegenerative diseases, including Friedreich's ataxia (FA), Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), and neurodegeneration with brain iron aggreation (**Cheng,** *et al.***, 2022**). Due to the aggreation of iron in the aging brain, levels of labile, possibly toxic iron rise (**Ward,** *et al.***, 2014**). When iron builds up in neurons at dangerous amounts, as in neurodegeneration, this can cause cell death by apoptosis, autophagy, necrosis and ferroptosis (**Dixon,** *et al.***, 2012**).

1.5.Ferroptosis:

The term 'ferroptosis', first proposed in 2012, refers to a programmed cell death resulting from accumulation of iron and lipid peroxidation. the regulation of ferroptosis is associated with multiple signal pathways, and there is increasing evidence to suggest its participation in the regulation of numerous diseases **(Hu,** *et al.***, 2022).**

Unlike apoptosis, autophagy, necroptosis, and pyroptosis, ferroptosis has distinct morphological, biochemical and genetic characteristics **(Li,** *et al.***, 2020).**

Cells that undergo ferroptosis generally show a necrosis-like morphological transformation, including cell membrane rupture, cytoplasmic swelling, and moderate chromatin condensation **(Tang,** *et al.***, 2021)** .

Iron buildup and lipid peroxidation are two biochemical features of ferroptosis, an oxidative cell death. Through the Fenton reaction(is one of the advanced oxidation processes , which generates highly reactive hydroxyl radicals by decomposition of hydrogen peroxide using ferrous ions), excessive intracellular iron can directly generate substantial levels of reactive oxygen species (ROS), hence accelerating oxidative damage. Additionally, it can interfere with oxygen homeostasis by enhancing the activities of lipoxygenases (LOXs) for example are oxidases **(Zhou,** *et al.***, 2020).**

 Ferroptotic cells usually present mitochondrial shrinkage, an increase in membrane density, reduced or absent cristae, and rupturing of the outer membrane. Since autophagy promotes ferroptosis, autophagy-related ultrastructures, such as double-membrane autophagosomes and various lysosome-related vesicles, are often seen in ferroptotic cells or tissue **(Chen,** *et al.***, 2021).**

1.5.1.Ferroptosis pathway:

The iron-dependent mechanism of ferroptotic cell death is accompanied by the production of highly reactive free radicals, intense peroxidation of membrane phospholipids (PLs) rich in polyunsaturated fatty acids (PUFAs), primarily of arachidonic or adrenic acids from phosphatidyl ethanolamine (PE) molecules, and an increase in (ROS) **(Sun, *et al*., 2020)**.   The central nervous system also has a complicated equilibrium between (ROS) and the antioxidant system, which protects cells from harmful stimulit and regulates oxidative stress through a number of mechanismsm these prosess includes the loss of intracellular antioxidants such as reduced glutathione (GSH), which is followed by deadly lipid peroxidation. Glutathione peroxidase 4 (GPX4), a significant gatekeeper of intracellular redox homeostasis, stimulant inhibits ferroptosis **(Mao, *et al*., 2020)**, (figure1.8) **(Dodson, *et al*., 2019).**
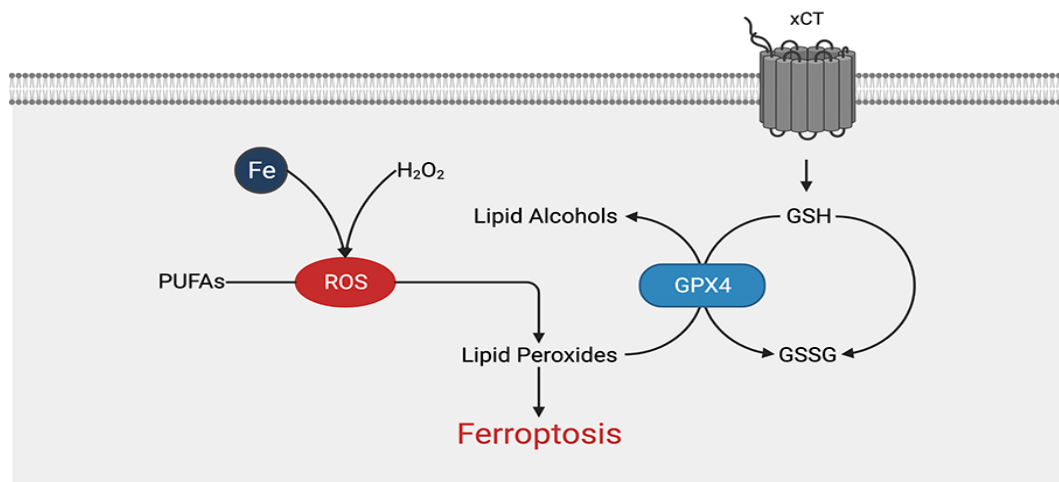
(Figure1.8): Cascade of ferroptotic simplification. Ferroptosis is started by a buildup of free iron. Normal functioning of GPX4 involves limiting the amount of lipid peroxides produced by ROS. (Dodson, *et al*., 2019)

1.5.2. **Ferroptosis and Lipid Peroxidation**

The initiation of ferroptosis is caused by lipid peroxidation **(Stockwell & Jiang, 2020).** lipid peroxides (PL-OOH) mainly lipid hydroperoxides (L-OOH) increase the oxidation of membrane lipids and produce ferroptosis, they have the potential to harm the lipid bilayer of the plasma membrane. The structure and operation of proteins and nucleic acids can be changed by an increase in the concentration of lipid peroxides. it can generate additional toxicity due to its degradation products **(Wang, *et al.*, 2020).**

The degree of lipid hydroperoxidation damage and ferroptosis increases with the amount of free polyunsaturated fatty acids (PUFAs) present in the cell **(Bayır, *et al*., 2020)**. Because the methylene groups' C-H bonds are among of the weakest C-H bonds known, PUFAs are excellent substrates for autoxidation **(Conrad & Pratt, 2019).**

The structure of the PUFA molecule contains bis-allyl hydrogen atoms that can be abstracted. Then, there is a rearrangement of the resonance radical structure, with subsequent addition of molecular oxygen, giving rise to the peroxyl radical and the formation of the primary molecular product, lipid hydroperoxide (L-OOH). Soon after, the cleavage of the L-OOH molecule occurs, giving rise to highly electrophilic secondary oxidation products, including epoxy, oxo- or aldehyde groups, which are highly reactive and toxic to membranes and cells **(Tyurina, *et al*., 2019).**

Both enzymatic and non-enzymatic PUFA oxidation processes that result in ferroptosis are possible **(Konstorum, *et al*., 2020).** The ROS and hydroxyl radical produced by the Fenton reaction are responsible for the non-enzymatic oxidation process. This process is both non-selective and non-specific. Since the amount of readily abstractable bis-allyl hydrogens in the PUFA molecule determines oxidation

rates, a highly diverse pattern of oxidation products collect, with oxygenated PUFA-PLs with 6, 5, 4, and 3 double bonds prevailing **(Conrad & Pratt, 2019)**.

   PUFAs are subjected to enzymatic oxidation by lipoxygenases (LOXs). The dioxigenation of polyunsaturated fatty acids with at least two isolated cis-double bonds is promoted by LOXs. In humans, there are different isoforms of LOX (5-LOX, 12S-LOX, 12R-LOX, 15-LOX-1, 15-LOX-2 and eLOX3**(Yang, *et al.*, 2016),** (figur1.9) **(Wan, *et al*., 2019).**
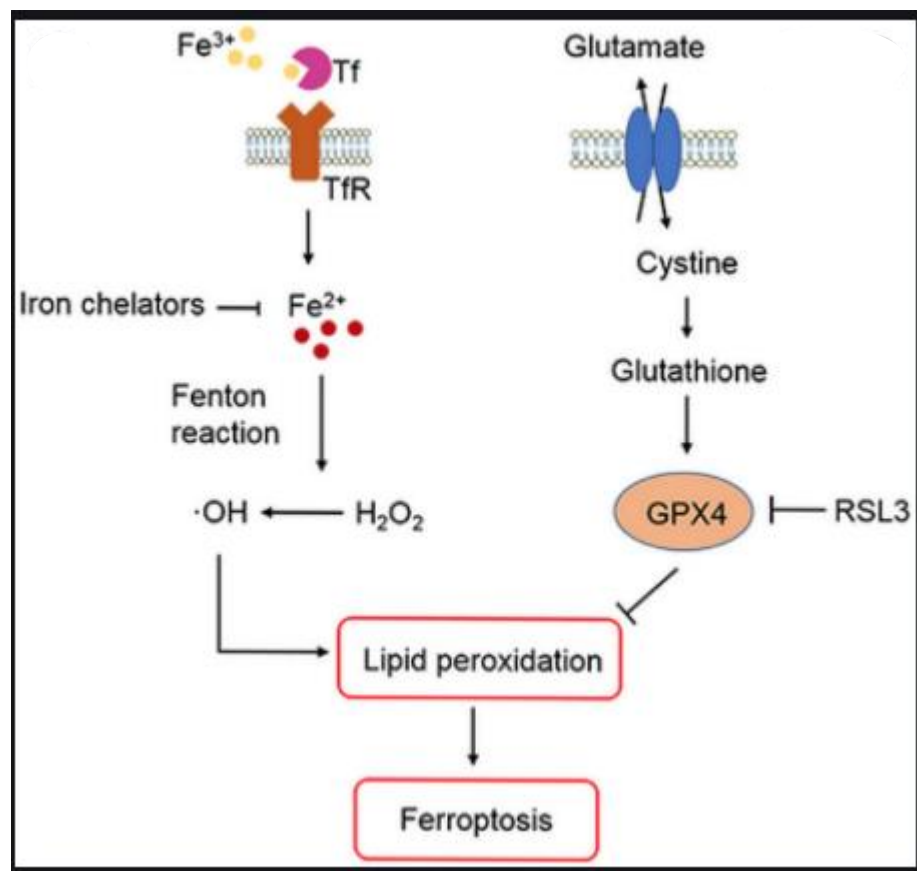
(Figur1.9) Ferroptosis and Lipid Peroxidation (Wan, *et al*., 2019).

1.5.3. Ferroptosis in Neurodegenerative diseases:

All human tissues need iron to function, it can be hazardous in some circumstances, particularly for the brain. Nervous tissue is susceptible to oxidative damage caused by increase iron and less antioxidant mechanisms, despite the fact that the cellular metabolism of the CNS requires iron as a redox metal for energy synthesis, chiefly the creation of ATP (**DeGregorio-Rocasolano,** *et al.***, 2019**). In ferroptosis, brain iron builds up along with glutathione depletion and lipid peroxidation at the same time. This sets off a series of reactions that result in inflammation, neurotransmitter oxidation, impaired neuronal communication, myelin sheath degeneration, dysregulated astrocytes, and cell death. Additionally, it has been demonstrated that ferroptosis in motor neurodegeneration is linked to poor GPx4 and glutathione system activity (**Gu,** *et al.***, 2015**). the non-oxidative form of dopamine is a strong inhibitor of ferroptotic cell death. Dopamine reduced erastin-induced ferrous iron accumulation, glutathione depletion, and malondialdehyde production. Moreover, dopamine increased the stability of GPx4(**Wang,** *et al.***, 2016**).

1.5.4. Ferroptosis in Alzheimer's Disease

Alzheimer's disease (AD) is regarded as a neurodegenerative condition that causes several difficulties in the brain (**Koukoulitsa,** *et al.***, 2016**).

AD is characterized by a gradual dysfunction in the cortical and hippocampus neuronal regions that results in both the loss of neuronal function and cell death. it is the most prevalent form of dementia. The distinct of AD is the histopathological presence of an extracellular β-amyloid (Aβ) deposition in senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs) formed from the hyperphosphorylation of the tau protein. These changes are due to the increase in oxidative stress, mainly an

increase in ROS and intra and extracellular hydrogen peroxides(**Siddappaji & Gopal, 2020**),(figure1.10) (**Reichert,** *et al***., 2020**).
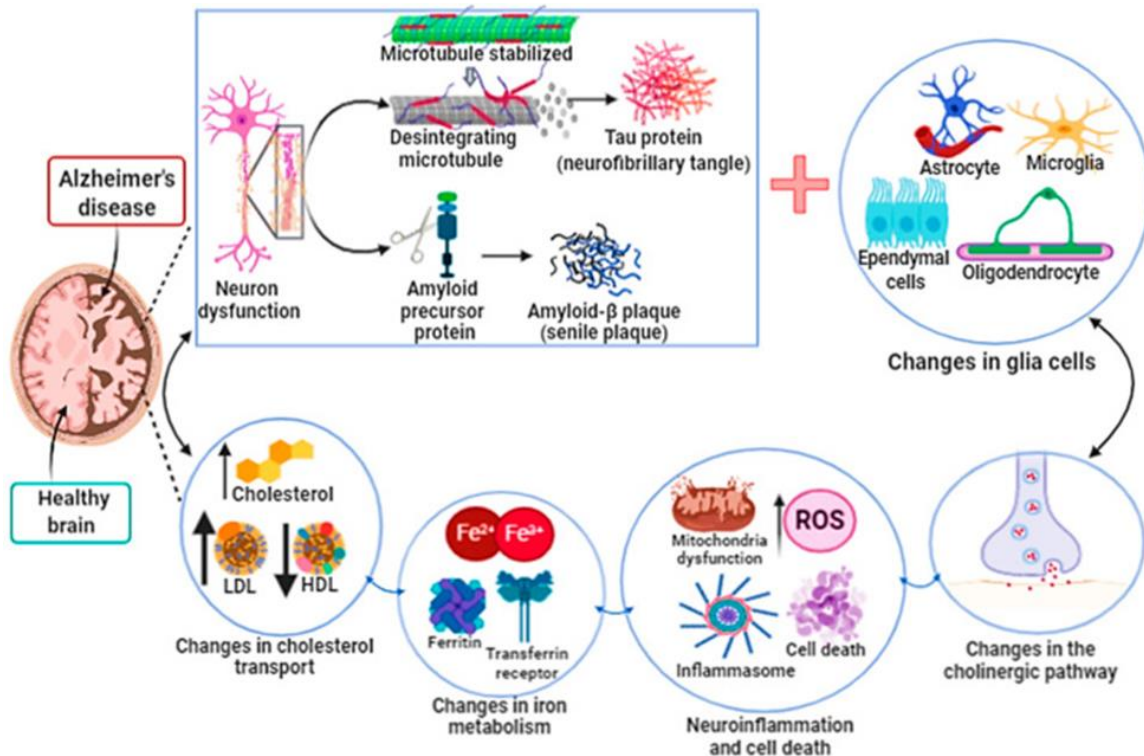
(Figure1.10) Ferroptosis in Alzheimer's Disease (Reichert, et al., 2020).

The formation of ROS and neurodegeneration in AD are related to iron dyshomeostasis (**Kocahan & Doğan, 2017**).

Additionally, Aβ plaques and NFTs have been linked to aging and alterations in iron metabolism (**Svobodová,** *et al.***, 2019**). iron deposition has been linked to the misfolding of Aβ plaques and NFTs (**Yan,** *et al***., 2020**).

 NFTs and Tau protein formation are both influenced by iron. Tau phosphorylation is induced and controlled by iron (**Nikseresht,** *et al.***, 2019**).

Tauopathy is the name for the relationship between NFT and neurodegenerative dysfunctions (**Bond, *et al*., 2011**).

The oxidation process slows down or excludes the regular action of the Aβ and tau protein (**Pohanka, 2014).**

Amyloid precursor protein (APP) is a type 1 transmembrane protein, and in healthy people, it seems to have a role in the growth of synaptic activity. The pathogenesis of AD is linked to the proteolytic cleavage of the APP to produce the β-amyloid peptide (Aβ), since APP mutations that affect this process increase the risk of AD or cause familial AD (**Serrano-Pozo, *et al*., 2011).**

The Aβ plaques effectively contribute to the production of ROS In the presence of free iron, which increases DNA damage, protein oxidation, and lipid peroxidation (**Butterfield & Boyd-Kimball, 2019**).

1.5.5. Ferroptosis in Parkinson's Disease:

The pathophysiological characteristics of PD include the slow and progressive degeneration of dopaminergic neurons in the pars compacta of the substantia nigra (SNpc), which is associated with a systematic and progressive accumulation of iron, leading to dopamine depletion, disappearance of neuromelanin and the appearance of intracellular Lewy bodies having aggregated α-synuclein as the main component (**Bloem, *et al*., 2021)** . As PD progresses, oxidative stress, lipid peroxidation, and mitochondrial dysfunction rise along with the glutathione systems' loss of antioxidant enzymes. All of these related conditions cause neuronal death and impair the organism's capacity to operate (**Lees, *et al*., 2009).**

It has long been known that iron and PD are related. Daily exposure to high iron levels raises the likelihood of developing Parkinson's disease (PD). Magnetic

resonance imaging (MRI) also showed that PD patients' substantia nigra and globus pallidus have higher iron concentrations. This rise was related to the progression of the illness, neurodegeneration, and the degree of moving weekness **(Rossi, *et al*., 2013).**

Although an iron-dependent cell death mechanism had not yet been proposed at that time. several genes and proteins related to iron metabolism of brain cells have been found to be mutated in PD patients, strengthening the correlation between iron metabolism and Parkinson's disease **(Valko, *et al*., 2016)**.

In some earlier studies on PD , increased oxidative stress was linked to PUFA peroxidation, a decline in GPx4 function, and depletion of the glutathione system **(Van, *et al*., 2016) .**

The evidence is clarifying the molecular mechanisms involved in the interaction of PD and ferroptosis cell death. α-Synuclein, a protein abundantly expressed in the nervous system and a main component of Lewy bodies, has been widely studied in PD as its pathogenic effects are strongly correlated with PD's pathophysiology**(Stefanis, 2012) .**

Additionally, it has recently been demonstrated that α -synuclein aggregation, a characteristic of (PD), causes the generation of ROS, which is then followed by lipid peroxidation in an iron-dependent way and ultimately cell death **(Plamena R Angelova, *et al*., 2020)** ,(figure1.11) **(Reichert, *et al*., 2020)**.
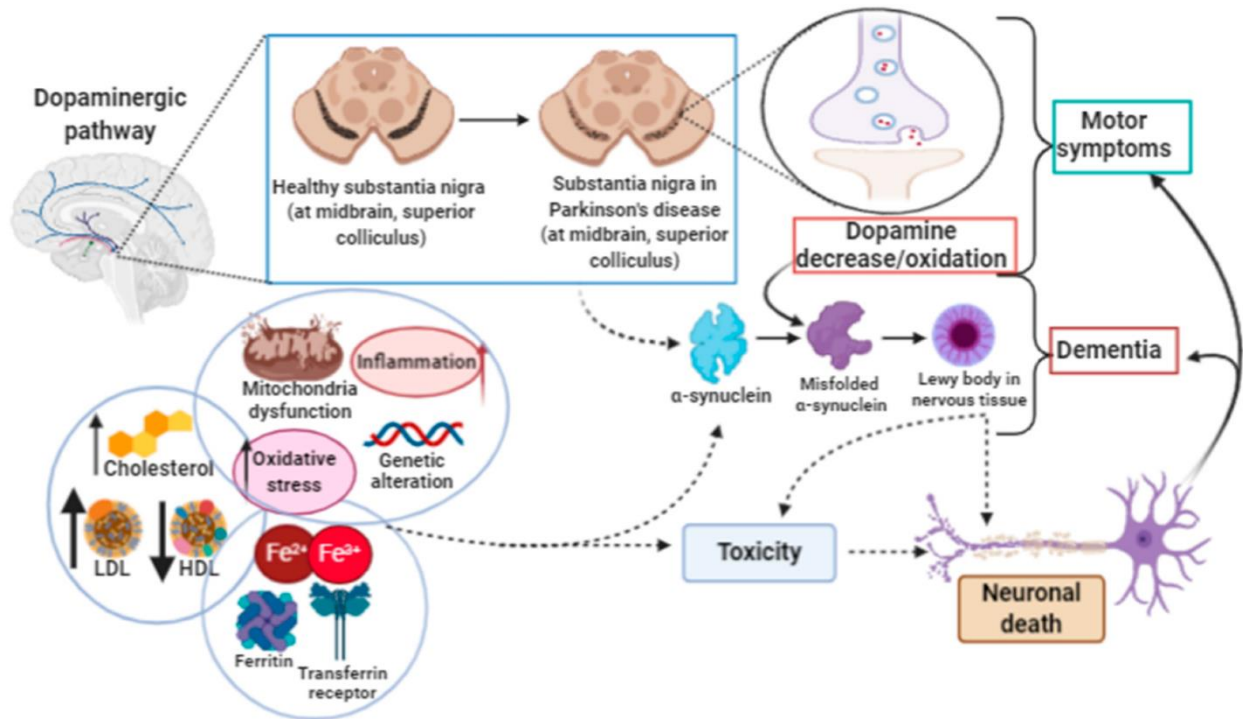
(Figure1.11) Ferroptosis in Parkinson's Disease (Reichert, *et al*., 2020).

1.6. Biomarkers

1.6.1. Iron forms:

The creation of neurotransmitters and the myelination of nerve fibers are only two examples of the biological processes in the brain that iron participates in **(Ward, *et al*., 2014).**

Normal iron homeostasis disturbances can increase harmful excess radical species production **(Kell, 2010).** Because alterations in regional or cellular iron content, or in the proteins in charge of closely controlling iron metabolism, may signify susceptibility to the damage caused by oxidative stress, which is seen in the pathophysiology of neurodegenerative disorders. **(Rouault, 2013)**.

1.6.1.1. Serum iron

Although plasma [iron] is low in iron deficiency and is raised in iron overload, these changes occur relatively late when iron stores have already become either completely depleted or seriously overloaded. In addition, serum [iron] also alters in conditions not associated with changes in iron store. serum [iron] determination is only required for diagnostic purposes for a few conditions, for example in suspected cases of acute iron poisoning and in the assessment of individuals with an increased risk of haemochromatosis **(Beckett, *et al.*, 2010).**

1.6.1.2. Serum ferritin

The main protein involved in storing iron is ferritin. Blood ferritin levels are used to measure how much iron is stored in the body and can increase in response to circumstances with substantial inflammation **(Knovich, *et al.*, 2010).**

The liver, spleen, marrow, duodenum, skeletal muscle, and other anatomical areas of the human body store iron in the form of ferritin **(SAITO, 2014)** .

No of the reason, elevated serum [ferritin] is seen in iron overload, as well as in a large number of cancer or liver disease patients. untreated iron overload is essentially excluded by a normal serum [ferritin] level. Currently, measuring serum [ferritin] offers the most helpful indicator of iron status that is regularly accessible **(Beckett, *et al*., 2010).**

1.6.1.3. Serum transferrin, total iron binding capacity(TIBC)

The primary blood protein that binds to iron and carries it across the body is called transferrin. Its blood level is immediately measured by a transferrin test. Alternatively, transferrin may be calculated to transform its level into the quantity of

iron it can bind. This kind of measurement is known as indirect measurement **(Mukkamalla., 2022).**

Transferrin is normal or low and serum [iron] is high in iron overload, indicating a high percentage saturation of total iron binding capacity (TIBC).In haemochromatosis, when saturation of the TIBC often climbs beyond 60% fairly early in the disorder, this impact is particularly noticeable **(Beckett, *et al.*, 2010).**

1.6.2. Iron transporter (divalent metal transporter 1 (DMT1)):

Also known as divalent cation transporter 1 (DCT1) and natural resistance-associated macrophage protein 2 (NRAMP 2) **(Kayaaltı, *et al*., 2015)**.

DMT1 is a protein that binds a number of different divalent metals, including as cadmium (Cd2+), copper (Cu2+), and zinc (Zn2+), although it is best recognized for transporting ferrous iron (Fe2+). Body iron reserves control DMT1 expression to preserve iron homeostasis. It performs H+ linked transport of divalent metal cations from the intestinal lumen into the cell on the apical membrane of enterocytes in the digestive tract. Due to the fact that DMT1 also transports Zn2+, Mn2+, and Cd2+, which might cause toxicity issues, it is not a 100% selective transporter **(Kwong & Niyogi, 2009).**

DMT1 transports iron from the gastrointestinal tract into the enterocytes that line the duodenum. Therefore, for absorption, iron must be in the divalent state. This may be accomplished by the duodenal cytochrome b, which is also found on the cell membranes of enterocytes, or by the reduction of Fe3+ to Fe2+ by vitamin C **(Kemna, *et al.*, 2008)**

This is owing to the poor selectivity for iron ions, which prevents it from distinguishing between other metal ions. Additionally, it results in a competition

amongst the metal ions for transportation, and iron ion concentrations are often much lower than those of other ions **(Gulcin & Alwasel, 2022)**.

DMT1 expression was discovered in the striatum, cerebellum, and thalamus of the brain. The ependymal and endothelial cell staining indicates that DMT1 plays a significant role in iron transfer into the brain **(Skjørringe, *et al*., 2015)**, (figure1.12) **(Yanatori & Kishi, 2019).**
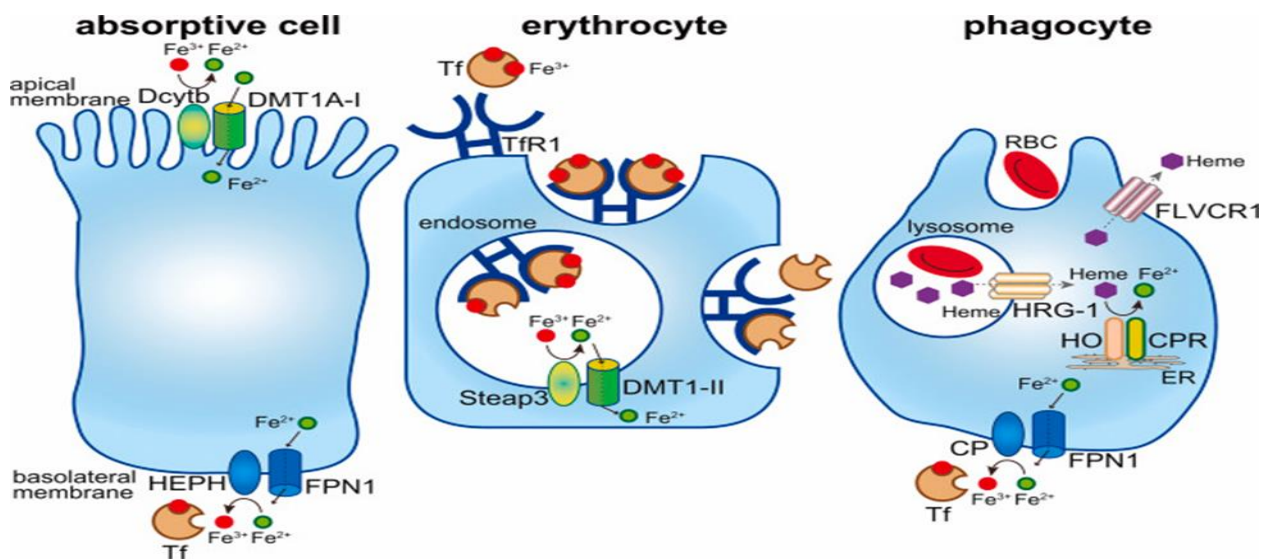
(Figure 1.12) DMT1 and iron transport (Yanatori & Kishi, 2019).

1.6.3. 5-lipoxygenase (5-LOX)

5-lipoxygenase (LOX) is an immune cell-expressed non-heme iron-containing dioxygenase that catalyzes the first two stages in the production of leukotrienes. It is generally recognized that iron-associated pro-inflammatory, illnesses such cancer, neurological diseases, and atherosclerosis are linked to 5-LOX activation in innate immune cells **(Dufrusine, *et al*., 2019).**

Since 5-LOX is expressed in neurons of the central nervous system, it may contribute to neurodegeneration. The enzymatic activity of 5-LOX may cause 5-

LOX-triggered cell death, however the 5-LOX protein may also have nonenzymatic effects **(Manev,** *et al***., 2000).**

The LOX-5 inhibitors help guard against neurotoxicity caused by β -amyloid. Lipoxygenase (LOX) expression is greater in the AD brain, and has been associated with higher levels of Aβ production and tau phosphorylation and levels of 5-LOX in PD models represent their role in brain disorders **(Javed,** *et al.***, 2022).**

1.6.4. Malondialdehyde (MDA)

The lipid peroxidation process in an organism is initiated by free radicals. One of the final products of polyunsaturated fatty acid peroxidation in cells is malondialdehyde (MDA). A rise in free radicals leads to an excess of MDA generation. Malondialdehyde levels are frequently used as indicators of oxidative stress for the patient **(Ito,** *et al.***, 2019).**

Unsaturated carbonyls, like as malondialdehyde (MDA), have a half-life that is frequently longer than that of free radicals, which allows them to spread from the sites of formation to other locations in vivo and cause further oxidative/carbonyl stressors. One of the most significant free radical damage intermediates is MDA **(Phaniendr,** *et al***., 2015).**

1.6.5. Lipid profile

One of the body's fattiest organs is the brain, has a particularly high concentration of lipids. Consequently, lipids account for 50–60% of the cell membrane in nerve cells **(Cermenati,** *et al***., 2015).**

After the age of 50, it has been observed that the concentrations of the majority of lipid species in the human brain drop **(Naudí,** *et al***., 2015)**.

The information at hand suggests that lipid homeostasis and metabolism are closely related to human brain aging. It is conceivable to hypothesize that regional changes in the lipid matrix of the human brain may be responsible for the pathophysiological processes involved in (AD) and (PD), given that the human brain performs a wide range of motor, sensory, regulatory, and cognitive functions that decline with advancing age **(Mesa-Herrera, *et al*., 2019).**

1.6.6.Complete Blood Count (CBC)

A blood examination used to examine general health and identify a variety of disorders, such as leukemia, anemia, and infections. A total blood count might reveal unexpected cell count increases or declines. Those modifications may indicate a health problem that need more examination.It is as follows: White blood cells combat infection, whereas red blood cells transport oxygen; Hematocrit, a measure of the quantity of red blood cells in the blood, and hemoglobin, the oxygen-carrying protein in red blood cells; Platelets, which assist in blood clotting. **(Ahmed, *et al*., 2020).**

1.7 Implications and contribution to the knowledge Gap

Current research on ferroptosis in neurological diseases mainly focuses on the critical pathways of ferroptosis. At the same time, ferroptosis was found to play a bidirectional regulation role in neurological diseases. Therefore, the specific regulatory mechanisms of ferroptosis in neurological diseases still need further exploration to provide new perspectives for applying ferroptosis in neurological diseases. Mainly, there is an urgent need for a specific ferroptosis biomarker in such cases.

Research on ferroptosis is still in the initial stage, and many obstructions remain to be resolved. It is essential to underlying the mechanism of the bidirectional regulation

of (p53 Tumor protein) in ferroptosis, which is still unclear. Iron has been proven to be critical in ferroptosis, which could catalyze lipid peroxidation through a Fenton reaction to mediate ferroptosis. However, other studies have also shown that other metal elements, such as copper, can participate in the redox reaction and participate in ferroptosis. Therefore, are there other metal ions involved in regulating cell ferroptosis. Is iron necessary for ferroptosis, or can other elements replace it.

Currently, known upstream genes involved in regulating iron metabolism, including FPN, and TFR1, can affect the occurrence of ferroptosis, but it is essential to specify the molecular mechanism, which is downstream iron metabolism.

A combination of multiple biomarkers may help detect ferroptosis cell death in time. The challenge remains how to transform primary research findings into clinical applications. Solving these challenges requires a further understanding of ferroptosis's molecular mechanisms and signal transduction. Ferroptosis regulates the inflammatory response, but the specific mechanism of ferroptosis promoting inflammation is currently unclear.

1.8 Aims of the Study

The current study aimed to:

1. Estimat the level of free Iron, storage Iron (Ferritin), Iron transporter (DMT1)، and Total Iron binding capacity(TIBC) in Alzheimer's and Parkinson's disease.
2. Examin of the oxidative status, Lipid، and CBC in Alzheimer's and Parkinson's disease.
3. Study the correlation of serum biomarkers with Alzheimer's disease and Parkinson's disease.

4. Investigated the diagnostic preferences of iron forms and CBC parameters in Alzheimer's and Parkinson's disease and control using ROC analysis.

Chapter Two

Materials

and

Methods

2. Materials and Methods

2.1. Study design

A case control study was conducted for the patients frome personal clinic (psychiatric clinic for Alzheimer's disease) and (Neurological clinic for Parkinson's disease) after being disgnosed by a specialist doctor.for the period of November /2022 to September /2023. a 80 samples were collected from 40 cases of neurodegenerative diseases(23 female and 17 male) . 24 Alzheimer's disease (AD)(15 female and 9 male),16 Parkinson's disease (PD) (8 female and 8 male) patients aged between (65-90) years, and 40 participants (34 female and 6 male) as a healthy control with age range (65-87).

2.2. Subjects:

2.2.1. Patients:

A Patients with Neurodegenerative diseases cases were selected from out patients' clinic after diagnosis of dementia and Parkinson's disease by specialist psychiatric and neurological consultant. A particular questionnaire form including descriptive information was designed and filled with each patient (Appendix). The questionnaire included name, age, sex, duration of disease, family history, chronic disease (diabetes,hypertension) and smoking.

2.2.2. Control group:

Control group of an apparently healthy 40 subjects (34 female and 6 male) healthy people between the age of (65-87). Blood samples were drawn from the volunteers, participants had no history of AD and PD.

2.3. Inclusion criteria:

Inclusion criteria is included Alzheimer's disease (AD) and Parkinson's disease (PD) from 65 years and above, after being disgnosed by a specialist doctor.

2.4. Exclusion criteria:

Exclusion criteria is included other neurological disorders,such as (Huntington's disease ,brain tumor, and subdural hematoma), Psychiatric disorders such as(primary affective disorders or major depression, alcoholism or other substance abuse). Reversible dementias and other medical disorders may reduce cognition, including overmedication; imparired pulmonary ,cardiac, renal or hepatic function ;anemia hypothroidism ;vitamin B12 or folat deficiency; malignancy.

2.5. Sample Collection:

5 mLs of blood sample were drown from patient by venipuncture using 5 mL disposable syringe. Each blood sample was partitioned to tow parts:

1. **3.5** mls of blood were left for 15 min at room temperature in gel tube. Serum was separated by centrifuging for 10 min at approximately 4000 xg. serum sample was collected by eppendroff and stored at -20°C to avoiding multiple freezing-thawing cycles. Serum samples were used to measure the levels of DMT1 , MDA, 5LOX , FreeIron , Ferritin ,TIBC ,and lipid profile (cholesterol, Low-density lipoprotein (LDL) , High-density lipoprotein (HDL) , Triglycerides).

2. **1.5** ml of blood was collected by Ethylenediaminetetraacetic acid (EDTA) anticoagulant tube. The samples were left on mixruller at room temperature to avoid clotting . The samples were used for determination of CBC.

2.6. Materials

2.6.1.Instruments and equipment: all instruments and equipment used throughout the study are listed in table 2.1.

Table (2.1) List of the equipment and instrumentsused in the current study

No	Laboratory equipment	Manufacturing company	Country
1	Micro Pipette 0.5-10μl, 10-100μl, 20- 200μl, 100-1000μl	Biobasic	Korea
2	Centrifuge	Fisher scientific	USA
3	Deep freezer refrigerator	Arcelik	Turkey
4	ELISA system	BioTek	USA
5	EDTA tubes	Mheco	China
6	Gel tube	Mheco	China
7	Sterile syringes	Sunshin medical	Korea
8	Timer with alarms	Junghans	Germany
9	Water bath	Memmert	Germany
10	Yellow tips (100μL)	JRL	Lebanon
11	Blue tips (1000 μL)	JRL	Lebanon
12	Eppendorf tubes 1.5ml an200Ml	ATACO	China
13	SIEMENS Autoanalyzer/ Dimension® Xpand® Plus	SIEMENS	Germany
14	Hitachi Cups	ArthAL-Rafidin	China
15	Micropipettes	Bioasic	Canada
16	Roller mixer	Mheco	China
17	Automated hematology analyzer Sysmex XP-300™ .	Mheco	China

2.6.2 List of kits:

Table(2.2) List of kits used in the current study

No.	Name of kit and reagent	Company	Country
1	Human MDA(Malondialdehyde) ELISA Kit	ELK Biotechnology	China
2	Human DMT1(Divalent Metal Transporter 1) ELISA Kit	ELK Biotechnology	China
3	Human 5-lipoxygenase (5-LO/LOX) ELISA kit	sunlong	China
4	Fe Reagent kit	dirui	China
5	Kit Ferritin	Maglumi	Germany
6	Total Iron-Binding Capacity Reagent kit(TIBC)	dirui	China
7	Total cholesterol kit	Randox	United Kingdom
8	Triglyceride kit	Randox	United Kingdom
9	HDL-cholesterol	Randox	United Kingdom
10	LDL- cholesterol	Randox	United Kingdom
11	Kit CBC	/	Jaban

2.7. Methods:

2.7.1. Enzyme-Linked Immunosorbent assay (ELISA) tests.

2.7.1.1.Human MDA(Malondialdehyde) ELISA Kit according to manufacture (ELK8428, 2022)

I. Principle: This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with Malondialdehyde(MDA) protein. Standards or samples are added to the appropriate

microtiter plate wells then with a biotin-conjugated antibody specific to Malondialdehyde(MDA). Next,Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of Malondialdehyde(MDA) in the samples is then determined by comparing the OD of the samples to the standard curve.

II. Kit components & Storage: Stored the kit at 4°C for 1 week. If the kit is not used up in 1 week, store the items separately according to the following conditions :

Tables(2.3) Kit components for MDA

Reagents	Quantity		Storage Condition
	48T	**96T**	
Pre-Coated Microplate	6 strips x 8 wells	12 strips x 8 wells	-20°C (6 months)
Standard (Lyophilized)	1 vial	2 vials	-20°C (6 months)
Biotinylated-Conjugate (100×)	30 μL	60 μL	-20°C (6 months)
Streptavidin-HRP (100×)	60 μL	120 μL	-20°C (6 months)
Standard/Sample Diluent Buffer	10 mL	20 mL	4°C
Biotinylated-Conjugate Diluent	5 mL	10 mL	4°C
HRP Diluent	6 mL	12 mL	4°C
Wash Buffer (25×)	10 mL	20 mL	4°C
TMB Substrate Solution	6 mL	10 mL	4°C (store in dark)
Stop Reagent	3 mL	6 mL	4°C
Plate Covers	1 piece	2 pieces	4°C

Reagent Preparation

1. Brought all kit components and samples to room temperature (18-25°C) before use.

2. If the kit will not be used up in 1 time, please only take out strips and reagents for present experiment, and save the remaining strips and reagents as specified.

3. Diluted the 25×Wash Buffer into 1×Wash Buffer with double distilled water.

4**. Standard Working Solution** - Centrifuge the Standard at $1000 \times g$ for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for about 10 minutes at room temperature, shaked gently (not to foam). The concentration of the standard in the stock solution is 1000 pg/mL. Please prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mixed each tube thoroughly before the next transfered, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL, 15.63 pg/mL, and the last EP tubes with Standard Diluent is the Blank as 0 pg/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as the Blank and do not pipette solution into it from the former tube.

| pg/mL | 1000 | 500 | 250 | 125 | 62.5 | 31.25 | 15.63 |
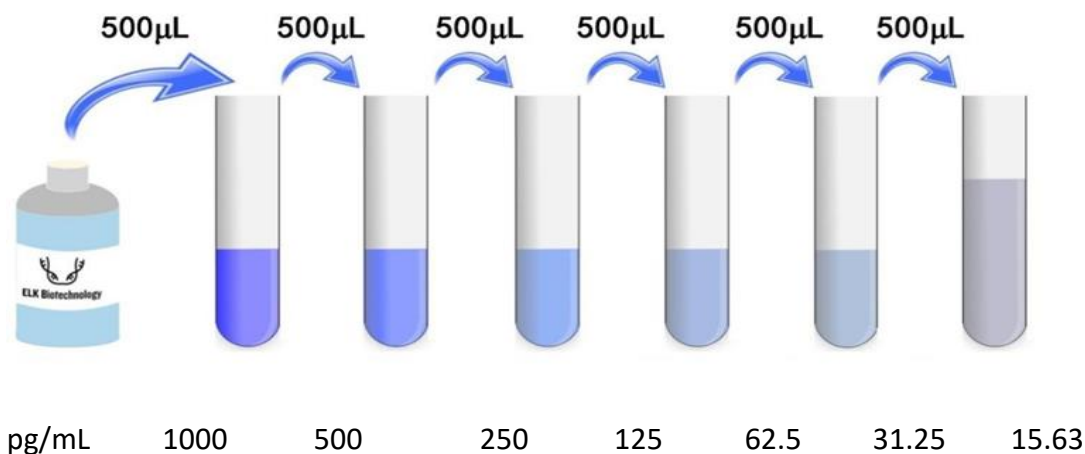
Figure(2.1)dultion of MDA kit

5. 1×Biotinylated-Conjugate and 1×Streptavidin-HRP Working Solution - Briefly spin or centrifuge the stock Biotinylated-Conjugate and Streptavidin-HRP before use. Dilute them to the working concentration 100-fold with Biotinylated-Conjugate Diluent and HRP Diluent, respectively. for example, 10 μL of Streptavidin-HRP with 990 μL of HRP Diluent.

6. TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Procedure:

1. Determined wells for Diluted Standard, Blank and Sample. Prepared 7 wells for Standard, 1 well for Blank. Add 50 μL of Standard Working Solution (please refer to Reagent Preparation) or Sample into each well (Blank is Standard Diluent). Then, add 50 μL of Biotinylated-Conjugate (1×) to each well immediately. Mix well, cover with the Plate Cover. Incubate for 1 hour at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2.Poured out the liquid of each well. Aspirate the solution and wash with 200 µL of 1×Wash Solution to each well and let it sit for 1-2 minutes. After the liquid has been decanted, completely remove the remaining liquid from all wells by snapping the plate onto absorbent paper. Totally wash 3 times. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and bloted it against clean paper towels to remove excess liquid. Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a void contaminat. (b) Paied attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Added 100 µL of Streptavidin-HRP Woking Solution (1×) to each well, cover the wells with the Plate Cover and incubate at 37°C for 60 minut.

4. Repeated the aspiration, wash process for total 5 times as conducted in step2.

5. Added 90 µL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement. Avoid placing the plate in direct light.

6. Added 50 µL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. The insertion order of the Stop Reagent should bethe same as that of the TMB Substrate Solution.

7. Wiped off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, rung the microplate reader and conduct measurement at 450 nm immediately.

Calculation of Results:

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between human MDA concentration in the sample and the assay signal intensity.

Average the duplicate readings for each standard, control, and samples. Create a standard curve with the Human MDA concentration on the y-axis and absorbance on the x-axis. Drawun the best fit straight line through the standard points and it can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor. using some plot software, for instance, curve expert.

2.7.1.2 Human DMT1(Divalent Metal Transporter 1) ELISA Kit according to manufacture (ELK4762, 2022).

Principle:

The test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Divalent Metal Transporter 1(DMT1). Standards or samples are added to the appropriate microtiter plate wells then with a biotin-conjugated antibody specific to Divalent Metal Transporter 1(DMT1). Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain Divalent Metal Transporter 1(DMT1), biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a

wavelength of 450nm ± 10nm. The concentration of Divalent Metal Transporter 1(DMT1) in the samples is then determined by comparing the OD of the samples to the standard curve.

Kit components & Storage: Stored the kit at 4°C for 1 week. If the kit is not used up in 1 week, store the items separately according to the following conditions after the kit is received.

Table(2.4) Kit components for DMT1

Reagents	Quantity		Storage Condition
	48T	96T	
Pre-Coated Microplate	6 strips x 8 wells	12 strips x 8 wells	-20°C (6 months)
Standard (Lyophilized)	1 vial	2 vials	-20°C (6 months)
Biotinylated Antibody (100×)	60 μL	120 μL	-20°C (6 months)
Streptavidin-HRP (100×)	60 μL	120 μL	-20°C (6 months)
Standard/Sample Diluent Buffer	10 mL	20 mL	4°C
Biotinylated Antibody Diluent	6 mL	12 mL	4°C
HRP Diluent	6 mL	12 mL	4°C
Wash Buffer (25×)	10 mL	20 mL	4°C
TMB Substrate Solution	6 mL	10 mL	4°C (store in dark)
Stop Reagent	3 mL	6 mL	4°C
Plate Covers	1 piece	2 pieces	4°C

Reagent Preparation:

1. Brought all kit components and samples to room temperature (18-25°C) before use.

2. If the kit will not be used up in 1 time, please only take out strips and reagents for present. experiment, and save the remaining strips and reagents as specified.

3. Diluted the 25×Wash Buffer into 1×Washed Buffer with double-distilled Water.

Standard Working Solution - Centrifuge the Standard at $1000 \times$ g for 1 minute. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the Standard in the stock solution is 10 ng/mL. Please prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.63 ng/mL, 0.32 ng/mL, 0.16 ng/mL, and the last EP tubes with Standard Diluent is the **Blank** as 0 ng/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment.

When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and does not pipette solution into it from the former tube.
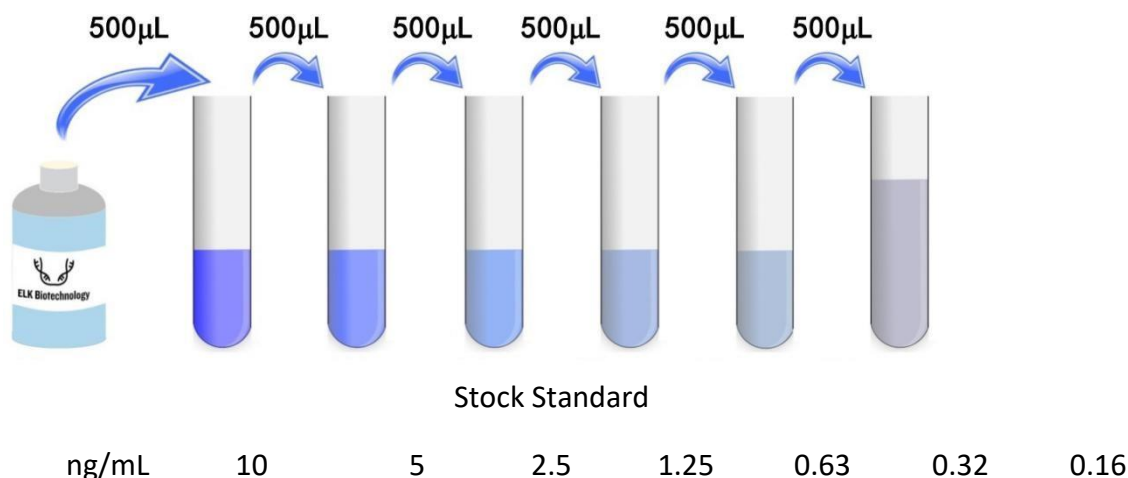
Figure(2.2) dilute DMT1 kit

5 .1×**Biotinylated Antibody and** 1×**Streptavidin-HRP -** Briefly spin or centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to the working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6.TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and **do not** dump the residual solution into the vial again

Procedure

1. Determined wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 100 μL each of Standard Working Solution (please refer to **Reagent Preparation**), or 100 μL of samples into the appropriate wells. Cover with the Plate Cover. Incubate for 80 minutes at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. Pour out the liquid of each well. Aspirate the solution and wash with 200 μL of 1×Wash Solution to each well and let it sit for 1-2 minutes. Remove the remaining

liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall of the wells to a void contamination.

(b) Paied attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Added 100 µL of Biotinylated Antibody Working Solution to each well, cover the wells with the Plate.

   Cover and incubate for 50 minutes at 37°C.

4. Repeated the aspiration,washed process for total 3 times as conducted in step 2.

Added 100 µL of Streptavidin-HRP Working Solution to each well, cover the wells with the plate sealer and incubate for 50 minutes at 37°C.

5. Repeated the aspiration, wash process for total 5 times as conducted in step 2.

6. Added 90 µL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for 20 minutes at 37°C (Don't exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution. Preheat the Microplate Reader for about 15 minutes before OD measurement.

   7. Added 50 µL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.

8. Wiped off any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Calculation of Results

Average the duplicate readings for each Standard, Control, and Samples and subtract the average zero Standard optical density. Construct a Standard curve with the Human DMT1 concentration on the y-axis and absorbance on the x-axis, and draw a best fit curve through the points on the graph. If samples have been diluted, the concentration read from the Standard curve must be multiplied by the dilution factor, using some plot software, for instance, curve expert.

2.7.1.3.Human 5-lipoxygenase(5-LO/LOX)ELISA Kit (LOX, 2022)

Principle

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antibody specific to 5-LO/LOX Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)-conjugated antibody specific for 5-LO/LOX is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain 5-LO/LOX and HRP conjugated 5-LO/LOX antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of 5-LO/LOX You can calculate the concentration of 5-LO/LOX in the samples by comparing the OD of the samples to the standard curve.

Tables(2.5)Materials provided with kit 5 LOX

No.	Materials provided with the kit	96 determinations	Storage
1	User manual	1	R.T.
2	Closure plate membrane	2	R.T.
3	Sealed bags	1	R.T.
4	Microelisa stripplate	1	2-8°C
5	Standard: 2700 pg/ml	0.5ml×1 bottle	2-8°C
6	Standard diluent	1.5ml×1 bottle	2-8°C
7	HRP-Conjugate reagent	6ml×1 bottle	2-8°C
8	Sample diluent	6ml×1 bottle	2-8°C
9	Chromogen Solution A	6ml×1 bottle	2-8°C
10	Chromogen Solution B	6ml×1 bottle	2-8°C
11	Stop Solution	6ml×1 bottle	2-8°C
12	Wash Solution	20ml (30X)×1bottle	2-8°C

Procedure

1. Dilution of Standards Diluted the standard by small tubes first, then pipette the volume of 50ul from each tube to microplate well,each tube use two wells, total ten wells.

Tables(2.6) Dilution of Standards for kit 5 LOX

1800 pg/ml	Standard No.1	300μl Original Standard + 150μl Standard diluents
1200 pg/ml	Standard No.2	300μl Standard No.1 + 150μl Standard diluents
600 pg/ml	Standard No.3	150μl Standard No.2 + 150μl Standard diluent
300 pg/ml	Standard No.4	150μl Standard No.3 + 150μl Standard diluent
150 pg/ml	Standard No.5	150μl Standard No.4 + 150μl Standard diluent
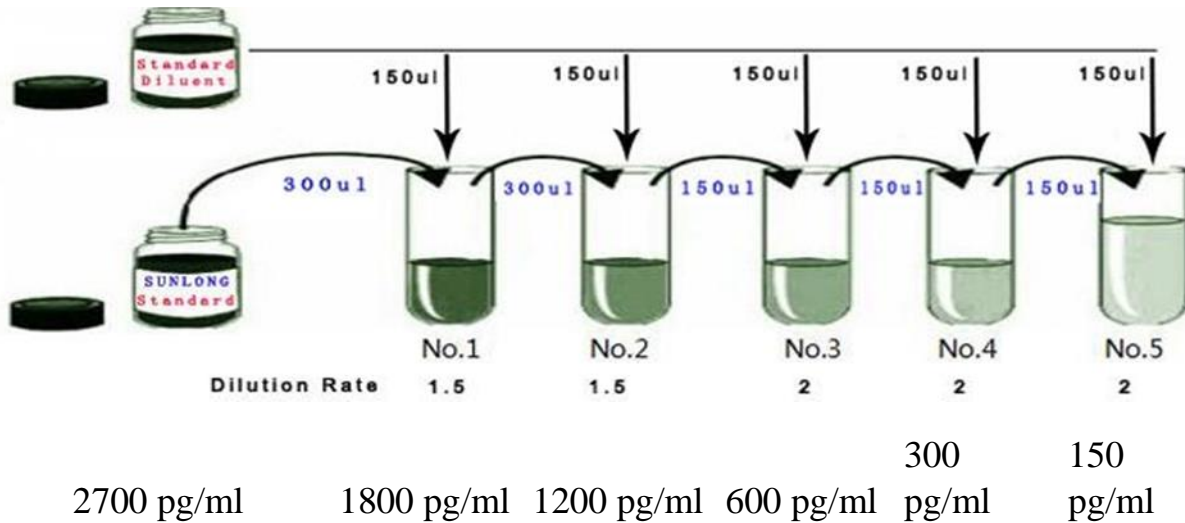
Figure(2.3) dultion 5LOX kit

2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40μl Sample dilution buffer and 10μl sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubation: incubate 30 min at 37°C after sealed with Closure plate membrane.

4. Dilution: dilutedthe concentrated washing buffer with distilled water (30 times for 96T and 20 times for 48T).

5. Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

6. Added 50 μl HRP-Conjugate reagent to each well except the blank control well.

7. Incubation as described in Step 3.

8. Washing as described in Step 5.

9. Coloring: Added50 µl Chromogen Solution A and 50 µl Chromogen Solution B to each well, mix with gently shaking and incubate at 37°C for 15 minutes. Please avoid light during coloring.

10. Termination: added50 µl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.

11. Readed absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

Calculation

Known concentrations of Human 5-LO/LOX Standard and its corresponding reading OD is plotted on the log scale (x-axis) and the log scale (y-axis) respectively. The concentration of Human 5-LO/LOX in sample is determined by plotting the sample's O.D. on the Y-axis. The original concentration is calculated by multiplying the dilution factor.

2.7.2. Serum lipid profile assay

A- Determination of serum total cholesterol

Principle: Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase to cholest-4-ene-3one and hydrogen peroxide. The hydrogen peroxide combine with hydroxyl benzoic acid (HBA) and 4-aminoantipyrine to form a chromophore (quinonamine dyes) which is quantitated at wave length 500 nanometer and the color intensity is proportional to cholesterol concentration.Normal range is less than 200 mg/dL .

Procedures: 100 microliter of separated serum was placed in cuvate to be analyzed. the concentration of the total cholesterol is measured automatically by using SIEMENS Autoanalyzer/ Dimension® Xpand® Plus.

B. Triglyceride

Principle: Triglycerides are measured enzymatically in serum or plasma using a series of coupled reactions in which triglycerides are hydrolyzed to produce glycerol. Glycerol is then oxidized using glycerol oxidase, and H202, one of the reaction products, is measured as described above for cholesterol.absorbance is measured at 500 nm.Normal ranges is less than 150 mg/dL.

Procedures: 100 microliter of separated serum was placed in cuvate to be analyzed and the concentration of the total cholesterol is measured automatically by using SIEMENS Autoanalyzer/ Dimension® Xpand® Plus.

C. Determination of high-density lipoprotein cholesterol (HDL)

Principle:

The Ultra HDL assay is a homogeneous method for directly measuring HDL cholesterol concentrations in serum or plasma without the need for offline pretreatment or centrifugation steps.

The method uses a two-reagent format and depends on the properties of a unique detergent. In the first reagent, non-HDL unesterified cholesterol is subject to an enzyme reaction and the peroxide generated is consumed by a peroxidase reaction yielding a colorless product. The second reagent consists of a detergent (capable of solubilizing HDL cholesterol), cholesterol esterase (CE), and chromagenic coupler to develop color for the quantitative determination of HDL cholesterol . Normal range is 40-60 mg/dL.

Procedures

One handred microliters of separated serum were placed in cuvate to be analyzed and the concentration of the High-Density Lipoprotein cholesterol is measured automatically by using SIEMENS Autoanalyzer/ Dimension® Xpand® Plus.

D. Low-density lipoprotein cholesterol(LDL)

Principle:

The method uses two reagent format and depends on the properties of a unique detergent. This detergent, RI, solubilizes only the non-LDL particles. The cholesterol released is consumed by cholesterol esterase and cholesterol oxidase in a non-color-forming reaction. A second detergent, R2, solubilizes the remaining LDL particles and a chromogenic coupler allows form color formation. The enzyme reaction with LDL in the presence of the coupler reduces color that is proportional to the amount of LDL cholesterol present in the sample.

Normal range :(100-159 mg/dL).

Procedures 100 microliters of separated serum was placed in cuvate to be analyzed and the concentration of the low-density lipoprotein is measured automatically by using architect

2.7.3. Iron forms

A-Free Iron:

Test principle

Iron bound to transferrin is released in an acidic medium as ferric iron and is then reduced to ferrous iron in the presence of ascorbic acid. Ferrous iron forms a blue

complex with Ferene. The absorbance at 595 nm is directly proportional to the iron concentration.

Fe^{2+} + 3 Ferene → Ferrous Ferene (blue complex)
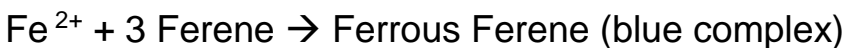
Reagent composition components

Reagent **1:** Acetate Buffer, pH 4.5   1 mol/L

Thiourea   120 mmol/L.

Reagent **2**:   Ascorbic Acid   240 mmol/L   Ferene   3 mmol/L Thiourea   120 mmol/L.

Reagent preparation:

Reagents are ready to use.

Calibration: The assay requires the use of an Iron Standard or Calibrator.

B-total iron binding capacity(TIBC):

  Principle: Ferric chloride saturating solution (Reagent 1) is mixed with the sample to bind all available apotransferrin binding sites with iron. Alumina adsorbent in the TIBC column removes excess iron from the serum mixture. The mixture is then analyzed for total iron using the Iron assay and the result is multiplied by the dilution factor of 3 to compensate for dilution of the serum by the saturating solution.

Reactive Ingredients	Concentration
Ferric Chloride	500 μg/dL
Citric Acid	1 mmol/L

Procedure

1. Ensureed pouches are at room temperature before opening. Remove the required number of columns from the foil pouches. Reseal the pouch by folding the open end several times.

2. Accurately pipet 300 μL of patient serum or control material and 600 μL of Saturating Solution into a clean, disposable plastic test tube properly labeled with patient identification.

3. Cap tightly and mix thoroughly by inversion five times.

4. Allowed to stand at room temperature for 5 to 10 minutes.

5. Poured the serum mixture into the column.

 NOTE: Do not use a column more than once. All columns and test tubes must be appropriately labeled to maintain proper sample identification.

6. Allowed at least 8 minutes for the serum mixture to pass through the column.

7. Removeed and discarded the columns, then assay the eluant within 15 minutes.

8. Runed the TIBC assay on the eluant using Iron reagent. Refer to the package insert for the Iron reagent.

For a detailed description of how to run an assay, refer to Section 5 of the instrument-specific operations manua.

Results:

The calculation for saturation level requires both the TIBC result and the serum iron result.

$$\%\,Saturation = \frac{Serum\ Iron}{TIBC} \times 100$$

C-Measurement of Ferritin:

Principle:

 Ferritin was a two-sandwich chemluminescence immunoassay. The sample and magnetic microbeads coated with anti-Ferritin monoclonal antibody were mixed thoroughly and incubated,then a wash cycle was done. After sedimentation in a magnetic field, the supernant was poured, and then another wash cycle was done. Subsequently, the starter 1+2 were added to initiate a chemiluminescent reaction. The light signal was measured by a photomultiplier as relative light unit (RLUs), which was proportional to the concentration of Ferritin present in the sample.

Prepration of the reagents: Resuspension of the magnetic microbeads took place automatically when the kit was loaded, the magnetic microbeads were ensured to be fully resuspended homogenous prior to use.

 Dilution: The automatic sample dilution was available after dilution settings were done in MAGLUMI series fully-auto chemiluminescence immunoassay analyzer user software.

 Calculation of results: The analyzer automatically calculated the Ferritin level in each sample by mean of a calibration curve which was created by a 2-point calibration master curve method. The result was expressed in ng/mL.

2.7.4. Determination of Complete blood count (CBC):

The measures of CBC were done by XP-300™ Automated hematology analyzer Sysmex.

Principle:

. detection method for WBC.

. detection method for RBC/PLT.

.Non-cyanide hemoglobin analysis method for HGB**.**

Parameters: WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, LYM%, MXD%, NEU%, LYM#, MXD#, NEU# RDW-SD, RDW-CV, PDW, and MPV.

2.8. Statistical Analysis:

Information from the questionnaire from all participants was entered into a data sheet and assigned a serial identifier number. Multiple entries were used to avoid errors. The data analysis for this work was generated using The statistical package for the social sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA), and the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Copyright (2013 – 2020). Descriptive statistics were performed on the participants' data of each group. Values were illustrated by n (%) for categorical. The distribution of the data was checked using the Shapiro-Wilk test as numerical means of assessing normality.

The association between the analyzed factors was estimated using odds ratios (ORs) and a 95% Confidence Interval Range which was calculated by a non-conditional logistic regression.

Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p-values <0.05 (two-side) were considered to be statistically significant.

The optimal threshold with high specificity and sensitivity for critical cases was detected using receiver operating characteristic (ROC) analysis. It was found out that all the values of P were two-sided, and a $P < 0.05$ was considered to be statistically significant.

Chapter Three

The Results

3. Results

3.1 Demographic and clinical characteristics

A total of 80 participated were included in this study, patient groups were divided into subgroups based on Age, gender, risk factors, family history, Duration of disease, medical history and Smoking status. The clinical demographic characteristics of the study groups were summarized in Table (3.1). The mean age of the cases was 75.65 years. The age range of participants was (47.5%) within **(65 - 73)** years old, (30%) of the patient were within **(74 -82)** year, while (22.5%) of the patient were within age range **(83-91).**

Also, the analysis of data was illustrated that about (42.5%) patients were having duration of disease less than one years, (20%) were having duration within (1-2) years, and 37.5% of the patients group were having duration more than three years.

Table 3.1: Descriptive of the demographic characteristics of the study population (N=80).

Variable	Groups	Patient N=40	Control N=40
Age Groups	65 - 73 Years	19	33
	74 -82 Years	12	3
	83 - 91 Years	9	4
Sex	Male	17	6
	Female	23	34
family history of diseases	Yes	18	0
	No	22	40
Duration of disease	Less than one years	17	0
	1 -2 Years	8	0
	More than three years	15	0
DM	Yes	21	12
	No	19	28
HT	Yes	35	20
	No	5	20
Smoking	Yes	12	0
	No	28	40

A	65 - 73 Years	74 -82 Years	83 - 91 Years
Patient	19	12	9
Control	33	3	4

B	Normal weight	Over weight	Obesity
Patient	7	14	19
Control	6	13	21
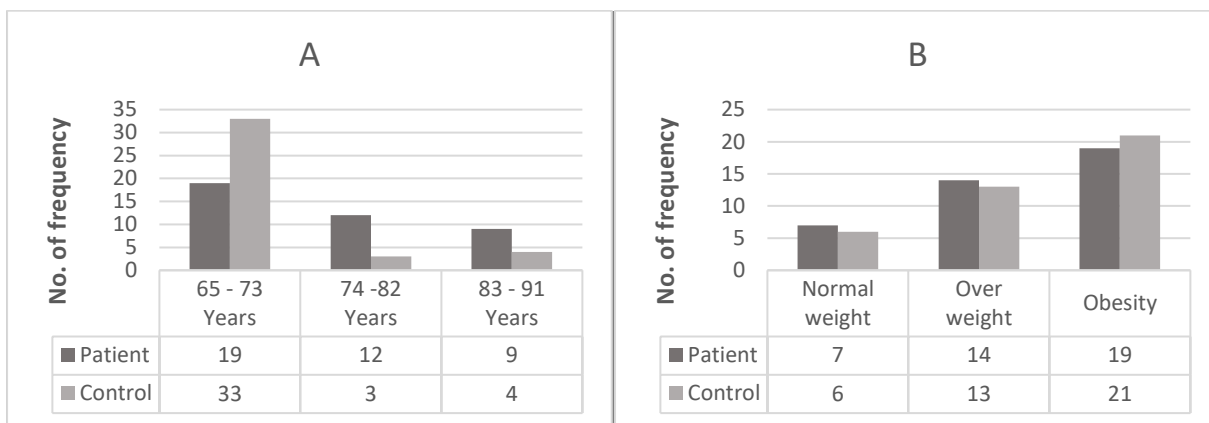
Figure 3.1: Baseline characteristics and Demographic Descriptive of the study population in patients compared to the control, the number of participants (n= 80): (A) Age group (B) BMI groups

Chronic disease

	DM Yes	DM No	HT Yes	HT No	Smoking Yes	Smoking No	Risk factors Yes	Risk factors No	family history Yes	family history No
Patient	21	19	35	5	12	28	1	39	18	22
Control	12	28	20	20	0	40	0	40	0	40
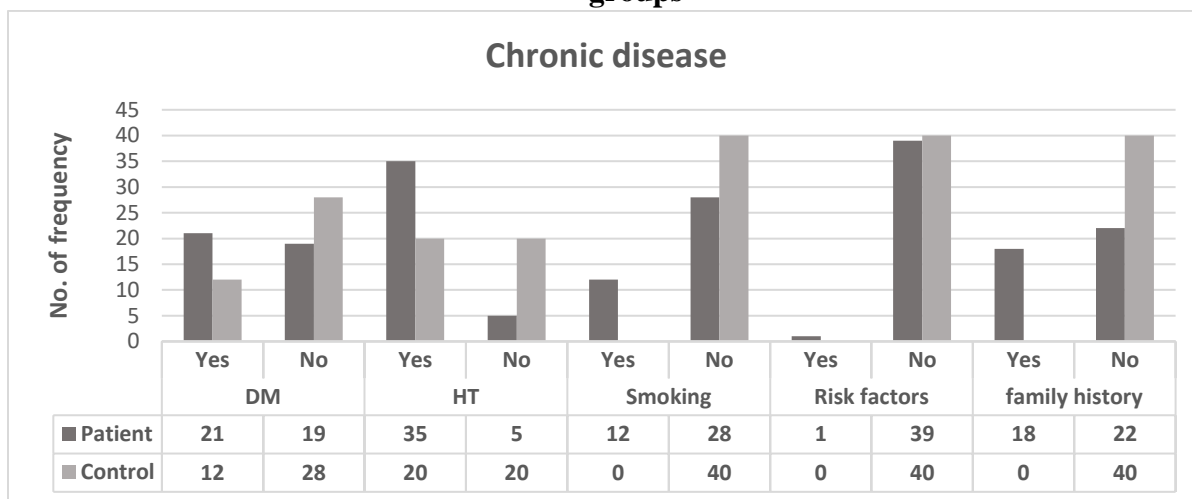
Figure 3.2 Descriptive of the study population in patients compared to the control group, the number of participants (n= 80) based on the history of Chronic disease.

	Male	Female
Patient	17	23
Control	6	34
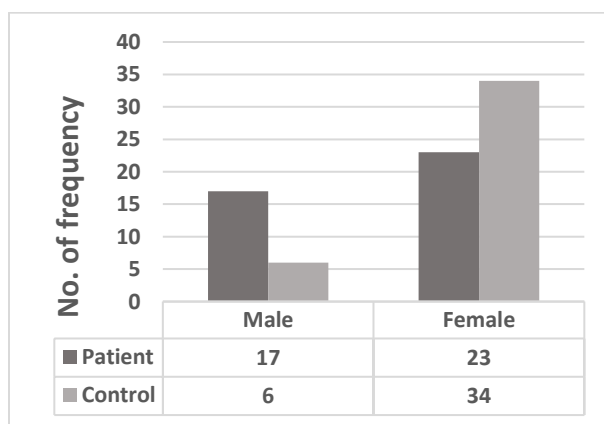
Figure 3.3 Descriptive of the study population in patients compared to the control group, the number of participants (n= 80): based on gender
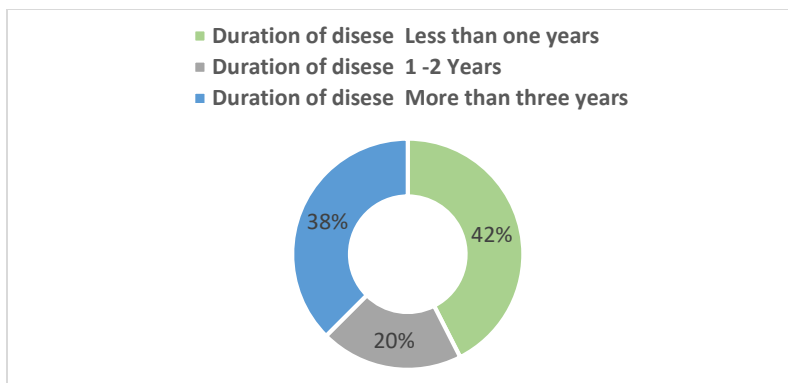
Figure 3.4: Descriptive of the study population in patients compared to the control group, the number of participants (n= 80): based on the duration of disease.

Table 3.2: Difference between mean levels of biochemical in the neurodegenerative disease with patients and control groups.

Biomarkers	Patient		Control		
	Median(Min-Max)	Mean±SD	Median(Min-Max)	Mean±SD	
Biomarkers					
TIBC	329.5(211-491)	(335.53±64.76)	63(45-99)	(63.12±12.16)	
5LOX	189(76.99-302.42)	(182.20±54.93)	237.36(169.05-322.3)	(242.02±47.13)	
MDA	1146.4(174.98-14)	(913.03±417.62)	758.74(191.33-1313.54)	(723.93±372.31)	
DMT1	0.89(0.03-3.04)	(0.95±0.66)	1.11(0.08-2.761)	(1.28±0.74)	
Free Iron	65.5(28-107)	(66.26±20.28)	20(8-56)	(21.25±8.20)	
Ferritin	127.58(24.45-472.3)	(159.77±116.39)	68.72(27.7-203)	(78.18±43.42)	
Lipid profile					
Cholesterol	173.5(110-271)	(177.66±42.61)	187.5(91-310)	(184.89±55.81)	
TG	115(50-524)	(129.60±80.47)	109(50-279)	(126.17±61.03)	
HDL	40(21-94)	(40.45±12.06)	34(11-74)	(36.32±14.31)	
LDL	154.5(78.8-247.6)	(154.60±46.40)	169.3(69-270)	(165.21±54.89)	
CBC					
WBC	7.08(3.9-12.1)	(7.49±2.09)	7.2(4.2-15)	(7.34±2.20)	
LYM	2.2(0.4-12)	(2.50±1.79)	2.35(1.1-3.6)	(2.37±0.49)	
RBC	4.56(3.56-5.35)	(4.50±0.46)	4.39(3.40-4.92)	(4.38±0.38)	
HGB	13(10.3-16.4)	(12.90±1.20)	12.4(9.1-14.4)	(12.23±1.29)	
RDW	47.35(15.8-75.5)	(50.05±9.59)	53(37.7-64.6)	(52.92±6.15)	
PLT	222(62-460)	(235.00±60.41)	260.5(161-441)	(268.35±61.31)	
PDW%	36(11.6-48)	(29.99±13.78)	42(38.7-46.4)	(41.84±1.77)	
PCT	0.21(0.04-0.30)	(0.21±0.05)	0.23(0.14-0.37)	(0.24±0.04)	
P-LCR	24.2(8.1-40.6)	(24.54±7.66)	20.6(12.0-36.1)	(21.37±5.71)	

3.2 Difference between the level of biomarkers in the patient and control groups

Generally, patients with the neurodegenerative disease were shown an increasing range level of TIBC, MDA, Free Iron, and Ferritin when compared to the healthy control groups, while the range level of 5LOX and DMT1 was decreased compared to healthy control.

Results were indicating a significant difference in TIBC, MDA, Free Iron, and Ferritin levels among groups, The means and standard deviations were presented in (table 3.2). The mean levels of TIBC, MDA, Free Iron, and Ferritin in patients were (335.53 ± 64.76) & (913.03 ± 417.62) which were significantly higher than for the Control (63.13 ± 12.16) & (723.93 ± 372.31), ($p \leq 0.001$), as shown in figure (3.5).

	TIBC	5LOX	MDA
Patient	335.53	190.69	913.03
Control	63.13	242.02	723.93

	Patient	Control
Free Iron	69.1	21.25
Ferritin	190.49	78.18

	Patient	Control
DMT1	0.95	1.28
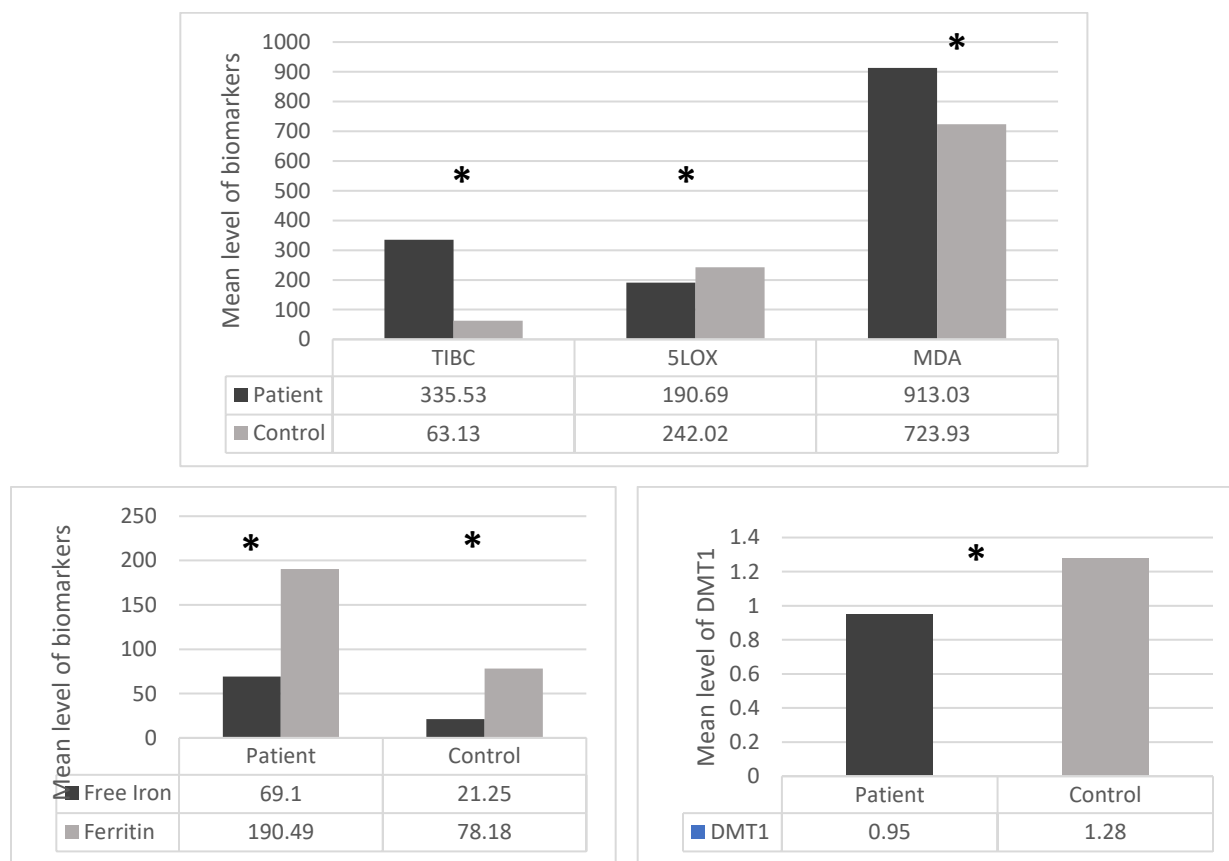
Figure 3.5: Mean Levels of biomarkers in neurodegenerative disease compared to the control group (T-test was *: significant at p ≤ 0.05, NS= Non-significant)

Serum concentrations of cholesterol, triglycerides, and apolipoprotein B (ApoB)-containing lipoproteins including very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) is all normal in patients groups. Results indicated an insignificant difference (P > 0.05) of all Lipid profiles in the neurodegenerative disease group compared to the control as shown in figure 3.6.

	Chol	TG	LDL	HDL
Patient	177.66	129.6	154.6	40.45
Control	184.89	126.17	165.21	36.32
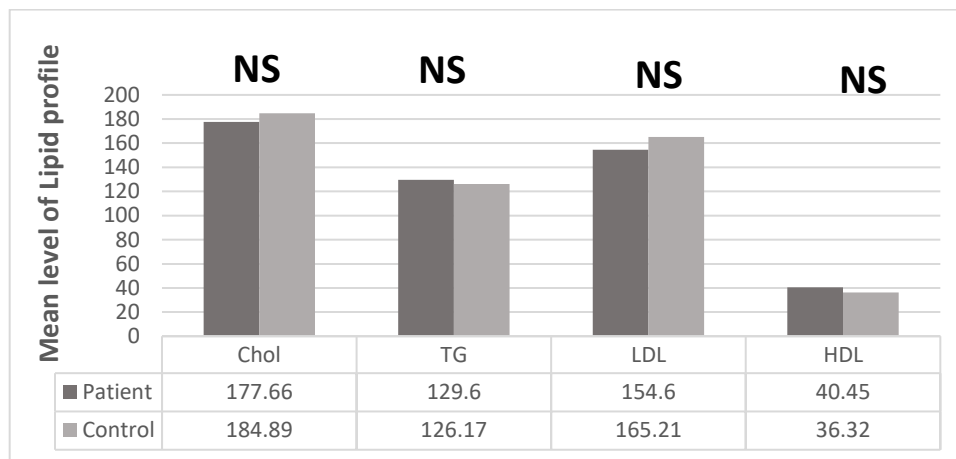
Figure 3.6: Mean level of Lipid profile in neurodegenerative disease group compared to control: (T-test was *: significant at p ≤ 0.05, NS= Non-significant)

Table 3.3: Examination of the level of CBC in NDDs compared to control

Biomarkers	Patient N=40	Control N=40	P value
WBC	7.49±2.09	7.34±2.20	0.757[NS]
LYM	2.50±1.79	2.37±0.49	0.647[NS]
RBC	4.50±0.46	4.38±0.38	0.183[NS]
HGB	12.90±1.20	12.23±1.29	**0.018[S]**
RDW	50.05±9.59	52.92±6.15	0.119[NS]
PLT	235.00±60.41	268.35±61.31	**0.017[S]**
PDW%	29.99±13.78	41.84±1.77	**<0.001[S]**
PCT	0.21±0.05	0.24±0.04	**0.012[S]**
P-LCR	24.54±7.66	21.37±5.71	**0.039[S]**
T-test was *: significant at p ≤ 0.05 **N: number of cases; SD: standard deviation; S: significant; NS= Non-significant.**			

3.3 Difference between the level of biomarkers in the NDDs and control groups

In figure 3.7, the mean level of serum biomarkers was also examined. A statistically significant difference was found among TIBC levels in patient groups (p= <0.001). Post hoc testing using LSD adjustment showed that the mean level of TIBC in the Parkinson's disease group was (370.94 ±62.74) which is significantly higher than in other groups.

A statistically significant difference was found among mean of 5LOX of neurodegenerative disease and control groups (p= <0.001). Post hoc testing using LSD adjustment showed that the mean 5LOX for the control group (242.02±47.13) is significantly higher than that of other two groups. A statistically significant difference was also found among mean of Free Iron and Ferritin for the neurodegenerative disease and control groups (p= <0.001). Post hoc testing using LSD adjustment showed that the mean of Free Iron and Ferritin for the Parkinson's disease group and Alzheimer's disease (74.40±16.37) and (201.26±129.01) respectively is significantly higher than that of other two groups.

No significant difference was found between other mean of parameters and duration of treatment groups, (p > 0.05).

	Alzheimer's disease	Parkinson's disease	Control
■ TIBC	311.92	370.94	63.13

	Alzheimer's disease	Parkinson's disease	Control
■ 5LOX	182.34	181.99	242.02
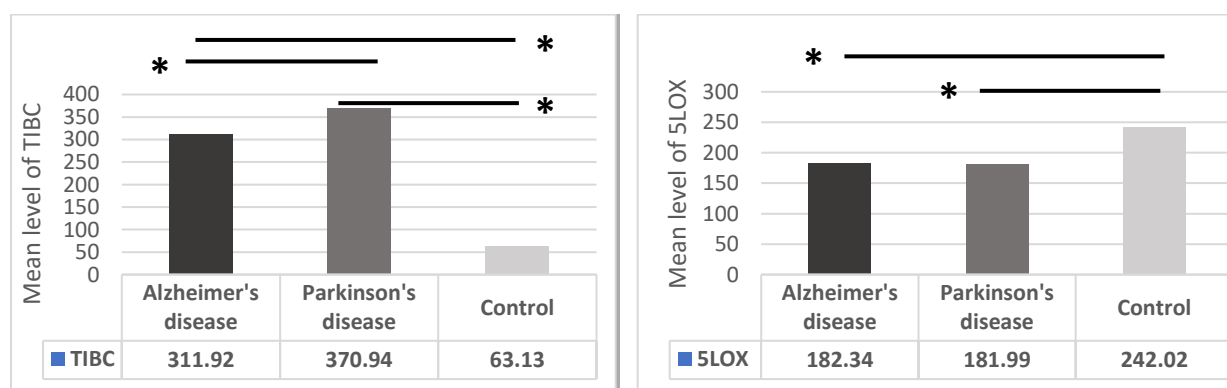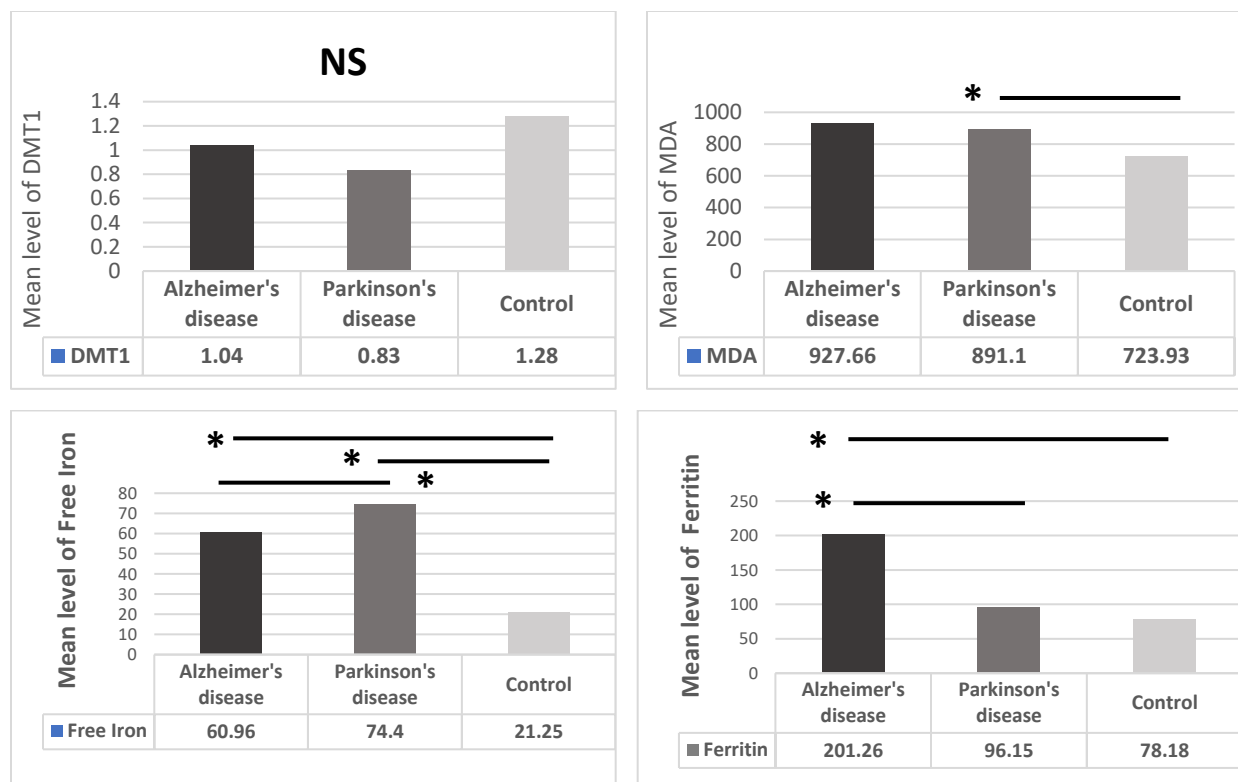
Figure 3.7: Difference between mean levels of biochemical in neurodegenerative disease between three groups two cases and one control(ANOVA-test was *: significant at p ≤ 0.05, NS= Non-significant)

3.4 Difference between the level of biomarkers in NDDs for duration of the disease

In the figure (3.8), the mean level of serum biomarkers was also examined based on the duration of disease in neurodegenerative cases.

A statistically significant difference was found among the mean of TIBC duration of disease groups (p= 0.044). Post hoc testing using LSD adjustment showed that the mean TBIC for the (More than 3 years) group (353.67±67.25) is significantly higher than that of the other two groups, A statistically significant difference was found among the mean of Ferritin duration of disease groups (p= 0.014). Post hoc testing using LSD adjustment showed that the mean Ferritin for the (1-2 years) group

(331.36 ± 289.28) is significantly higher than that of the other two groups. No significant difference was found between other mean of parameters and duration of treatment groups, ($p > 0.05$).
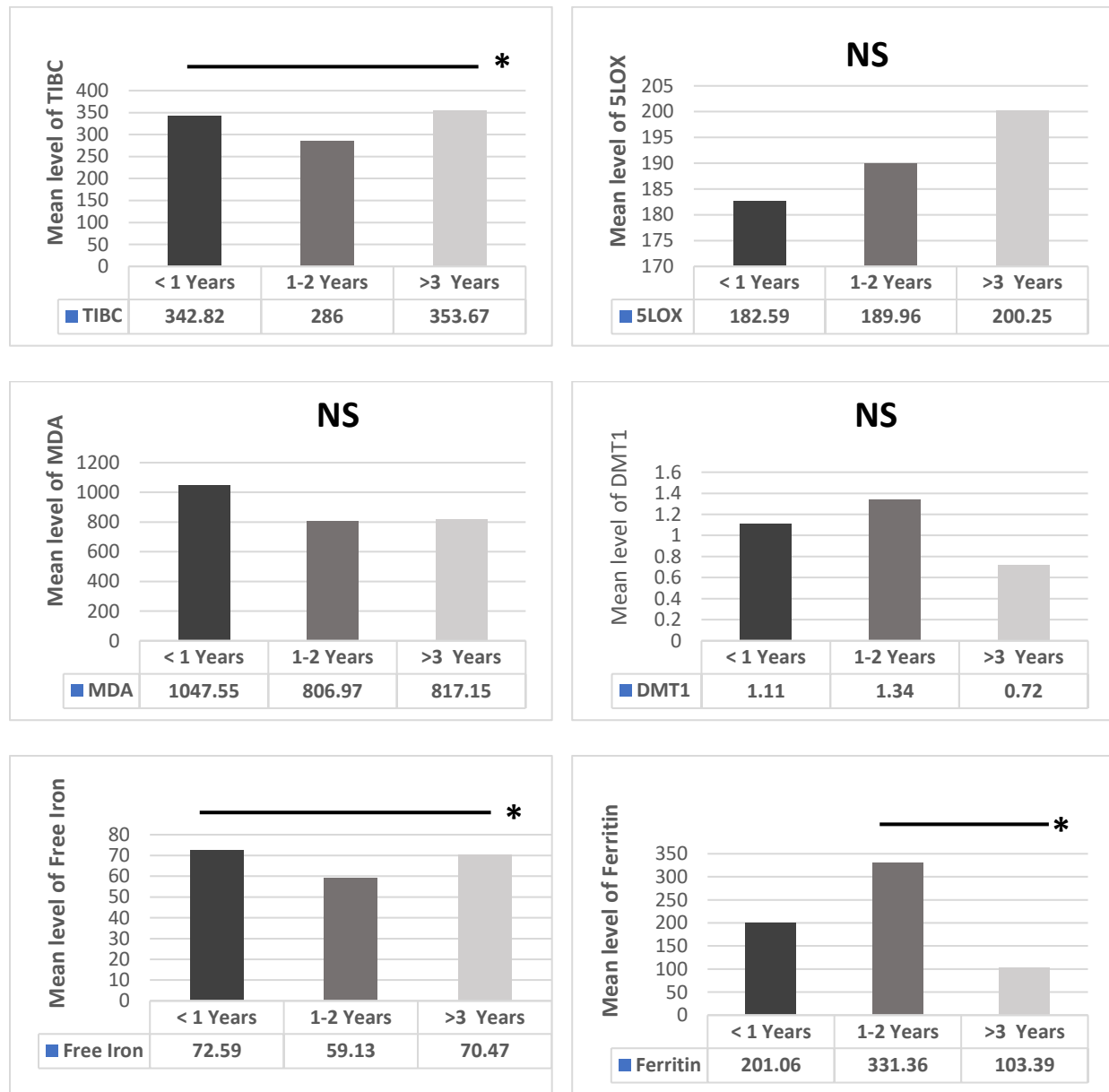
Figure 3.8: Mean difference of biochemical parameters between duration of neurodegenerative groups(ANOVA-test was *: significant at p ≤ 0.05, NS= Non significant) .

3.5 Difference between the level of biomarkers with Age in the patient and control groups

In figure (3.9), the mean level of biochemical in the Patients and control groups according to the Age groups was examined. In the range of age groups (**65 - 73**) years, the level of TBIC, free Iron, ferritin, MDT, and 5LOX increased significantly in the patients group compared to control, P value <0.05.

In the range of age groups (**74 -82**) years, TBIC, DMA, and free Iron were increased significantly in the patients group compared to the control, P-value <0.05, the mean level was 341.24±62.42, 329.07±89.19and 64.67±19.56 respectively in the patient. While, in the range of age groups (**83 - 91**) years, the level of TBIC and Iron increased significantly in the patients group compared to the control, with P-value <0.05, the mean level was 339.67±71.08, and 64.44±21.40 respectively in the patient.

Mean level of TIBC	65 - 73 Years	74 -82 Years	83 - 91 Years
	*	*	*
Patients	329.21	341.24	339.67
control	62.94	58	68.5

Mean level of 5LOX	65 - 73 Years	74 -82 Years	83 - 91 Years
	*	NS	NS
Patients	192.76	172.17	162.42
control	247.45	217.35	215.67

Mean level of MDA	65 - 73 Years	74 -82 Years	83 - 91 Years
	NS	*	NS
Patients	960.25	870.31	753.85
control	739.21	329.07	893.99

Mean level of DMT1	65 - 73 Years	74 -82 Years	83 - 91 Years
	*	NS	NS
Patients	0.86	1.04	0.95
control	1.29	1.06	1.33

Figure 3.9: Mean difference of biochemical parameters between neurodegenerative groups and control based on the Age of participants (ANOVA-test was *: significant at p ≤ 0.05, NS= Non-significant).

3.6 Difference between the level of biomarkers with gender in the patient and control groups

Table (3.4) illustrates the mean level of the biochemical in the Patients and control groups according to gender. Results were shown that the levels of TIBC, 5LOX, MDA, DMT1, Free Iron, and Ferritin were increased markedly in the patients group in both male and female groups compared to the control, p values were <0.001.

Table 3.4: The effect of gender on the biochemical parameters according to the Patients and control groups.

Biomarker	Male			Female		
	Patients N=16	control N=6	P value	Patients N=16	control N=8	P value
TIBC	337.00±75.69	69.50±23.27	**<0.001**	334.43±57.14	62.00±9.17	**<0.001**
5LOX	169.96±53.72	227.40±19.9	**0.02**	190.72±55.32	244.60±50.20	**<0.001**
MDA	822.39±390.5	633.35±247	0.337	980.03±432.71	739.91±362.9	**0.027**
DMT1	0.85±0.63	1.11±0.63	0.406	1.02±0.69	1.31±0.76	0.156
Free Iron	69.94±19.88	25.00±7.82	**<0.001**	63.59±20.61	20.59±8.20	**<0.001**
Ferritin	167.23±103.4	80.14±27.03	**0.006**	154.34±127.08	77.83±46.00	**0.012**

3.7: Study the association of biomarkers with patients' groups

Multinominal logistic regression was performed to analyze the associating of the TBIC, 5LOX, MDA, DMT1, Free Iron and Ferritin with the neurodegenerative disease. It was found that all the biomarkers showed a highly significant differences in neurodegenerative disease   and represented as a risk factors factor (OR 1.291; 95% CI: (1.275-1.308) , OR: 1.045; 95% CI: (0.965-1.050), OR: 1.003; 95% CI: (1.000-1.005), OR: 1.228; 95% CI: (1.102-1.368) and OR: 1.019; 95% CI: (1.010-1.029)) respectively for Alzheimer's and (OR 1.313; 95% CI: (1.313-1.313) , OR: 1.038; 95% CI: (0.963-1.040), OR: 1.003; 95% CI: (1.000-1.010), OR: 1.273; 95% CI:(1.136-1.426) and OR: 1.006; 95% CI:(0.995-1.017)) respectively for Parkinson's.

Table 3.5: The multinomial logistic regression of neurodegenerative disease with levels of biomarkers

Variable	Groups	OR (Lower – upper)	P value
TIBC	**Control**	1[a]	-
	Alzheimer's disease	1.291 (1.275-1.308)	**<0.001[S]**
	Parkinson's disease	1.313 (1.313-1.313)	**<0.001[S]**
5LOX	**Control**	1[a]	-
	Alzheimer's disease	1.045 (0.965-1.050)	**<0.001[S]**
	Parkinson's disease	1.038 (0.963-1.040)	**<0.001[S]**
MDA	**Control**	1[a]	-
	Alzheimer's disease	1.003 (1.000-1.005)	**0.05[S]**
	Parkinson's disease	1.003 (1.000-1.010)	**0.013[S]**
DMT1	**Control**	1[a]	-
	Alzheimer's disease	0.618(0.290-1.318)	0.213[NS]
	Parkinson's disease	0.368(0.142-0.953)	0.788[NS]
Free Iron	**Control**	1[a]	-
	Alzheimer's disease	1.228(1.102-1.368)	**<0.001[S]**
	Parkinson's disease	1.273(1.136-1.426)	**<0.001[S]**
Ferritin	**Control**	1[a]	-
	Alzheimer's disease	1.019 (1.010-1.029)	
	Parkinson's disease	1.006 (0.995-1.017)	**0.008[S]**
p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant			
1[a] : reference category is Control			

3.8: Receiver Operating Characteristic Analysis

ROC curve and AUC analysis for the MDA for neurodegenerative compared to control group

  Results of the receiver operating curve (ROC) curve and AUC analysis for the MDA, Iron, and Ferritin besides as possible diagnostic parameters. Free Iron level showed good diagnostic performance for predication the neurodegenerative disease compared to the control group; data are presented in table (3.6).

  For MDA levels: (sensitivity = 47.5%, specificity = 92.5%) at a level = 1172.974, Iron levels: (sensitivity = 79.2%, specificity = 97.5%) at a level = 36.5 and the Iron levels: (sensitivity = 55.6%, specificity = 90%) at a level = 110. Accordingly, the distribution of patients using   MDA cut-off values was presented in table (3.7).

  The p-values of the AUC were <0.05 and statistically significant. Youden's J statistics of the parameters in figure (3.10) confirm these results.

Table 3.6: AUC, optimal threshold, Sensitivity and specificity of proposed marker obtained by the ROC curves neurodegenerative disease patients

Test Result Variable(s)	MDA	Iron	Ferritin
AUP	67%	99%	71%
Sensitivity %	47.5%	79.2%	55.6%
Specificity %	92.5%	97.5%	90%
Youden index	0.4	0.947	0.456
Cut-off points	1172.974	36.5	110
CI (95%)	(0.550-0.792)	(0.586-0.828)	(0.586-0.828)
PPV	762%	88%	89.2%
NPV	92%	90.2%	90.2%
Accuracy	79.2%	80%	69.2%
P value	0.008[S]	<0.001[S]	0.002[S]

Figure 3.10: Receiver operating characteristics (ROC) curve analysis of MDA, Iron and Ferritin levels in the patient.

ROC curve and AUC analysis for the for TBIC, MDA, Iron and Ferritin for Alzheimer's disease

ROC curve and AUC analysis for the TBIC and MDA for Patients compared to the control group were performed. Results of the receiver operating curve (ROC) curve and AUC analysis for the diagnostic parameters were shown that TBIC and free Iron have a good performance for the prediction of Alzheimer's disease patients, data are presented in figures (3.11) and table (3.7).

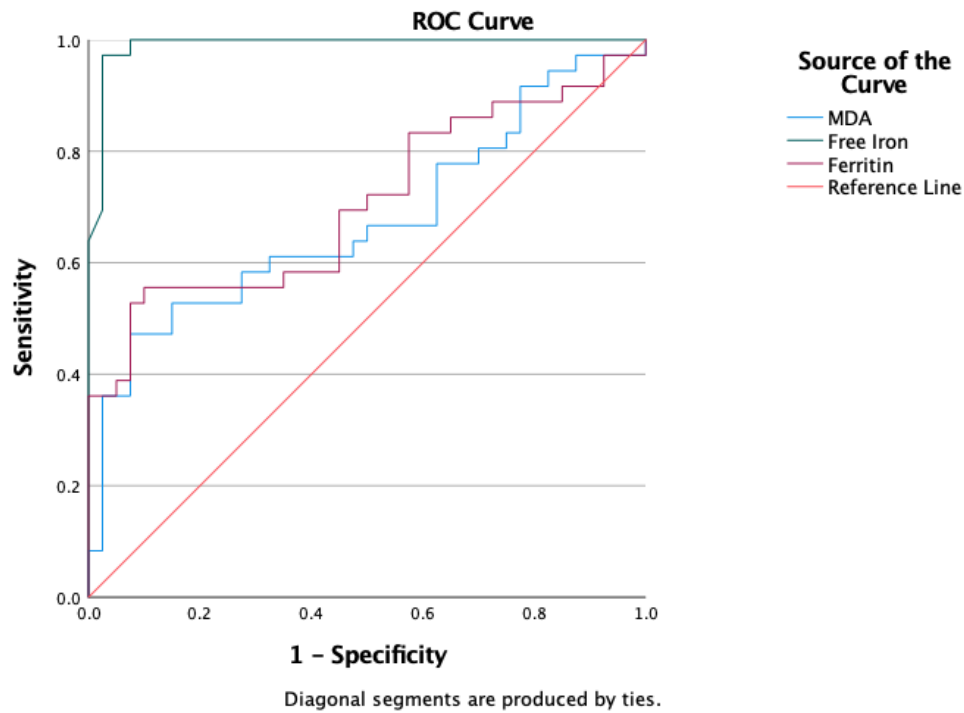For TBIC levels: (sensitivity = 98%, specificity 73.3%) at a level = 221, MDA levels (sensitivity 46%, specificity 84%) at a level = 1193.13, For Iron levels: (sensitivity = 79.2%, specificity 97.5%) at a level = 36.5, Ferritin levels (sensitivity 55.6%, specificity 90%) at a level = 110, the p-values of the AUC were <0.001 and

highly statistically significant. results confirmation of the Sensitivity & Specificity were confirmed using Youden's J statistics to the parameters.

Table 3.7: Receiver operating characteristic curve showing sensitivity and specificity of TIBC, MDA, Iron, and Ferritin in patients compared to Alzheimer's disease.

Test Result Variable(s)	TIBC	MDA	Iron	Ferritin
AUP	77.8%	64.5%	81%	76%
Sensitivity %	98%	46%	98%	60%
Specificity %	73.2%	84%	70%	98%
Youden index	0.732	0.297	0.685	0.591
Cut-off	221	1193.13	31.5	211
CI (95%)	(0.675-0.880)	(0.502-0.788)	(0.720-0.907)	(0.610-0.912)
PPV	84%	93%	94%	84%
NPV	84%	74%	74%	69%
Accuracy	84%	84%	85%	77%
P value	<0.001[S]	0.041[S]	<0.001[S]	<0.001[S]

Figure 3.11: Receiver operating characteristics (ROC) curve analysis of TIBC, MDA, Iron and Ferritin levels in Alzheimer's disease,

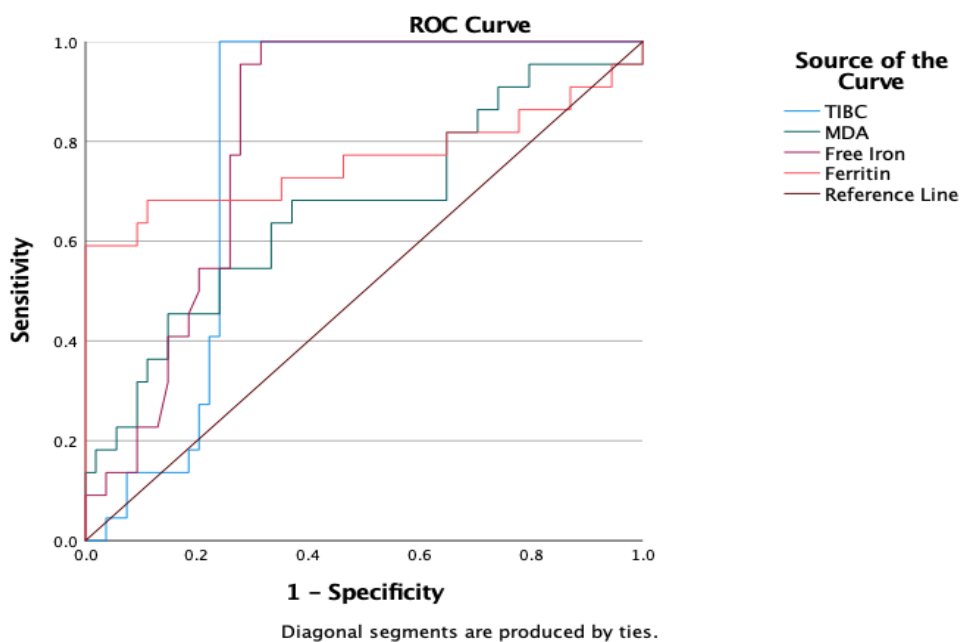**ROC curve and AUC analysis for the TBIC, MDA, Iron and Ferritin for Alzheimer's disease**

  Results of the receiver operating curve (ROC) curve and AUC analysis for the diagnostic parameters showed that TBIC and free iron have a good performance for prediction Parkinson's disease patients, data are presented in figures (3.12) and table (3.8).

  For TBIC levels: (sensitivity = 93.8%, specificity 84.4%) at a level = 308, MDA levels (sensitivity 50%, specificity 71%) at a level = 1172.974, For Iron levels: (sensitivity = 79.2%, specificity 97.5%) at a level = 36.5, Ferritin levels (sensitivity 55.6%, specificity 90%) at a level = 110. The p-values of the AUC were <0.001 and highly statistically significant. Results confirmation of the Sensitivity & Specificity were confirmed using Youden's J statistics to the parameters.

Table 3.8: Receiver operating characteristic curve showing sensitivity and specificity of TIBC, MDA, Iron and Ferritin in patients compared to Parkinson's disease

Test Result Variable(s)	TIBC	MDA	Iron	Ferritin
AUP	91.7%	57.7%	88%	50%
Sensitivity %	93.8%	50%	93%	92.9%
Specificity %	84.4%	71%	79%	30%
Youden index	0.782	0.281	0.597	0.219
Cut-off points	308	1172.974	55.5	55.3
CI (95%)	(0.852±0.982)	(0.413±0.741)	(0.810-0.958)	(0.345-0.627)
PPV	84%	84%	88%	90%
NPV	78%	84%	84%	74%
Accuracy	70%	84%	74%	84%
P value	<0.001[S]	0.342[NS]	<0.001[S]	0.872[NS]
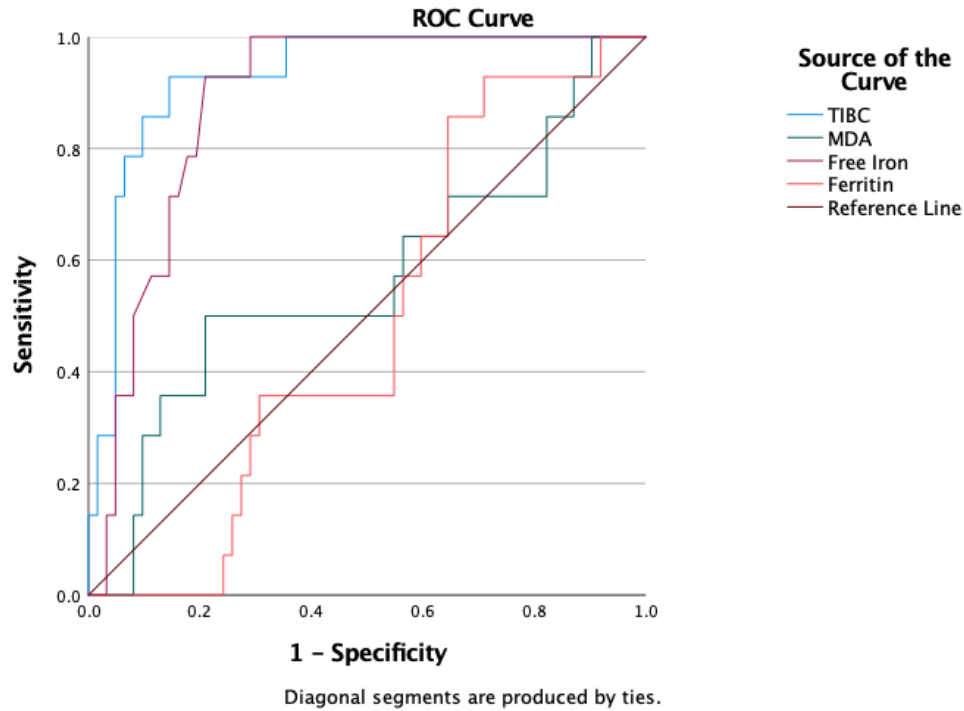
Figure 3.12: Receiver operating characteristics (ROC) curve analysis of TIBC, MDA, Iron and Ferritin levels in Parkinson's disease.

ROC curve and AUC analysis for the CBC parameters (HGB and P-LCR)

Results of the receiver operating curve (ROC) curve and AUC analysis for the HGB and P-LCR as a possible diagnostic parameter was also performed. Both markers were shown a good diagnostic performance for neurodegenerative disease compared to control group; data are presented in figure(3.13). and table (3.9).

For HGB levels: (sensitivity = 55%, specificity = 70%) at a level = 12.85, P-LCR levels: (sensitivity = 40%, specificity = 82.5%) at a level = 26.5. Accordingly, the distribution of patients using   HGB cut-off values was presented in table (3.9).

The p-values of the AUC were <0.05 and statistically significant. Youden's J statistics of the parameters in figure (3.13) confirm these results.

Table 3.9: AUC, optimal threshold, Sensitivity, and specificity of proposed marker obtained by the ROC curves neurodegenerative disease patients

Test Result Variable(s)	HGB	P-LCR
AUP	63.7%	61.9%
Sensitivity %	55%	40%
Specificity %	70%	82.5%
Youden index	0.25	0.225
Cut-off points	12.85	26.5
CI (95%)	(0.515-0.758)	(0.495-0.742)
PPV	67%	55%
NPV	54%	84%
Accuracy	66%	70%
P value	<0.06[NS]	0.210[NS]
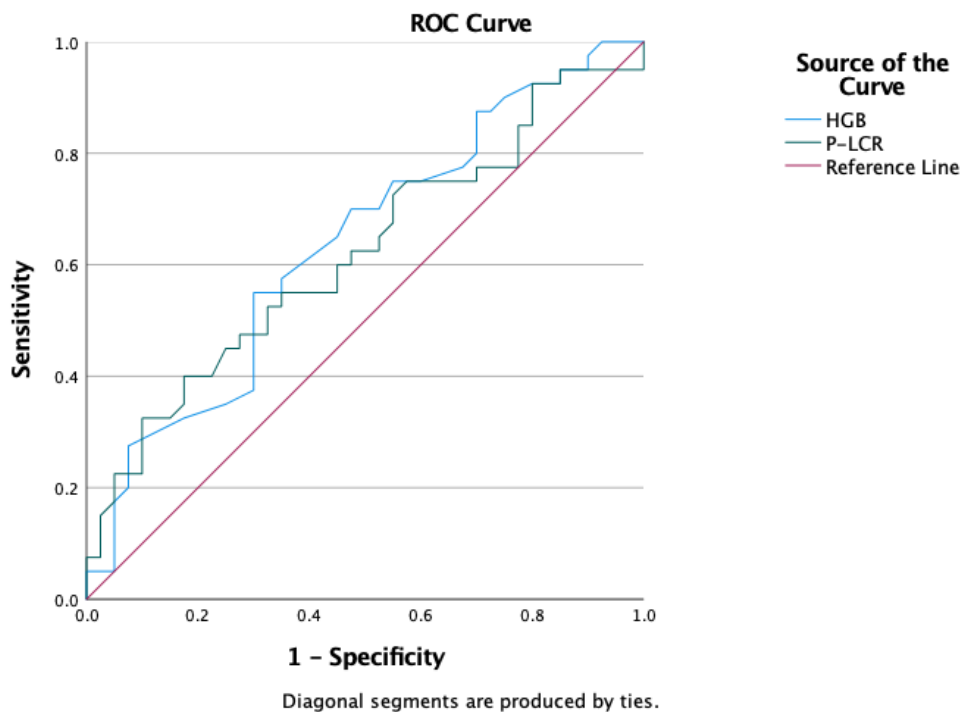
Figure 3.13: Receiver operating characteristics (ROC) curve analysis of HGB and P-LCR levels in patient ,

ROC curve and AUC analysis for the HGB and PDW% for Alzheimer's disease

PDW%showed high sensitivity for the prediction of Alzheimer's disease patients, while HGB showed high Specificity, data are presented in figures (3.14) and table (3.10).

For HGB levels: (sensitivity 33.3%, specificity 91%) at a level = 13.65, PDW% levels (sensitivity 99%, specificity 30%) at a level = 21.95. The p-values of the AUC were <0.001 and highly statistically significant. Results confirmation of the Sensitivity & Specificity were confirmed using Youden's J statistics to the parameters.

Table 3.10: Receiver operating characteristic curve showing sensitivity and specificity of HGB and PDW% in patients compared to Alzheimer's disease

Test Result Variable(s)	HGB	P-LCR
AUP	0.63	0.642
Sensitivity %	33.3%	99%
Specificity %	91%	30%
Youden index	0.244	0.286
Cut-off points	13.65	21.95
CI (95%)	(0.493-0.768)	(0.278-0.544)
PPV	77%	56.4%
NPV	55.22%	56%
Accuracy	58.75%	56.25%
P value	<0.06[NS]	0.210[NS]

Figure 3.14: Receiver operating characteristics (ROC) curve analysis of HGB and PDW% levels in Alzheimer's disease,

ROC curve and AUC analysis for the HGB and P-LCR for Parkinson's disease

In group of Parkinson's disease, HGB showed high sensitivity for prediction such cases, while P-LCR showed a high Specificity toward patients group, data are presented in figures (3.15) and table (3.11).

For HGB levels: (sensitivity 87.5%, specificity 30%) at a level = 11.85, P-LCR levels (sensitivity 62.5%, specificity 89.1%) at a level = 28. The p-values of the AUC were <0.001 and highly statistically significant. Results confirmation of the Sensitivity & Specificity were confirmed using Youden's J statistics to the parameters.

Table 3.11: Receiver operating characteristic curve showing sensitivity and specificity of HGB and P-LCR in patients compared to Parkinson's disease

Test Result Variable(s)	HGB	P-LCR
AUP	54.2%	80.2%
Sensitivity %	87.5%	62.5%
Specificity %	30%	89.1%
Youden index	0.156	0.516
Cut-off points	11.85	28
CI (95%)	(0.395-0.690)	(0.674-0.931)
PPV	64%	75%
NPV	55%	88%
Accuracy	74%	84%
P value	0.601[NS]	**<0.001[S]**

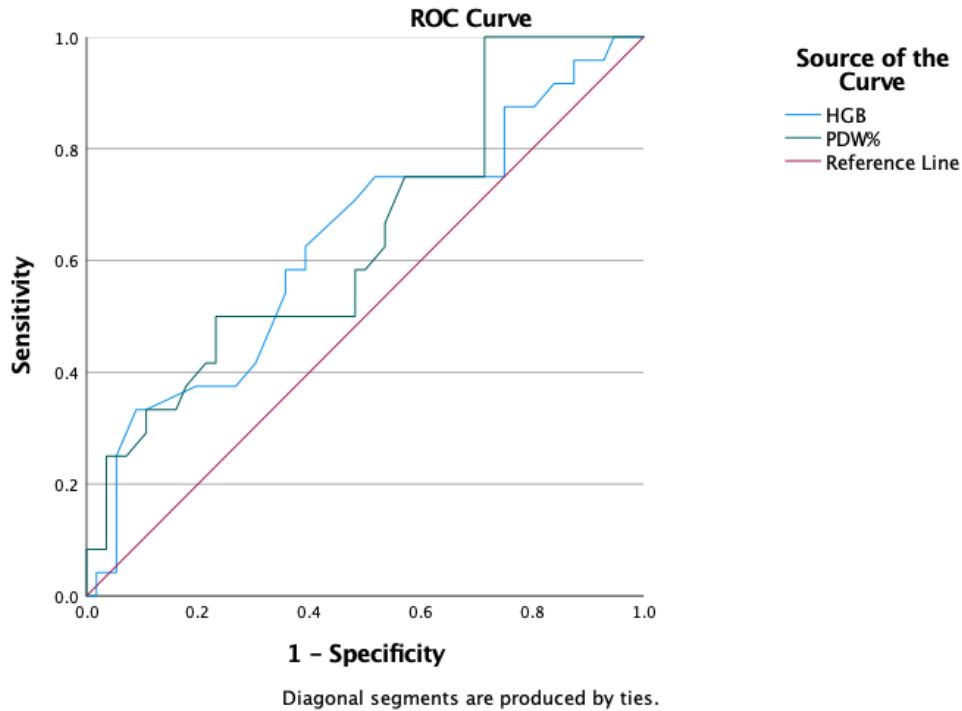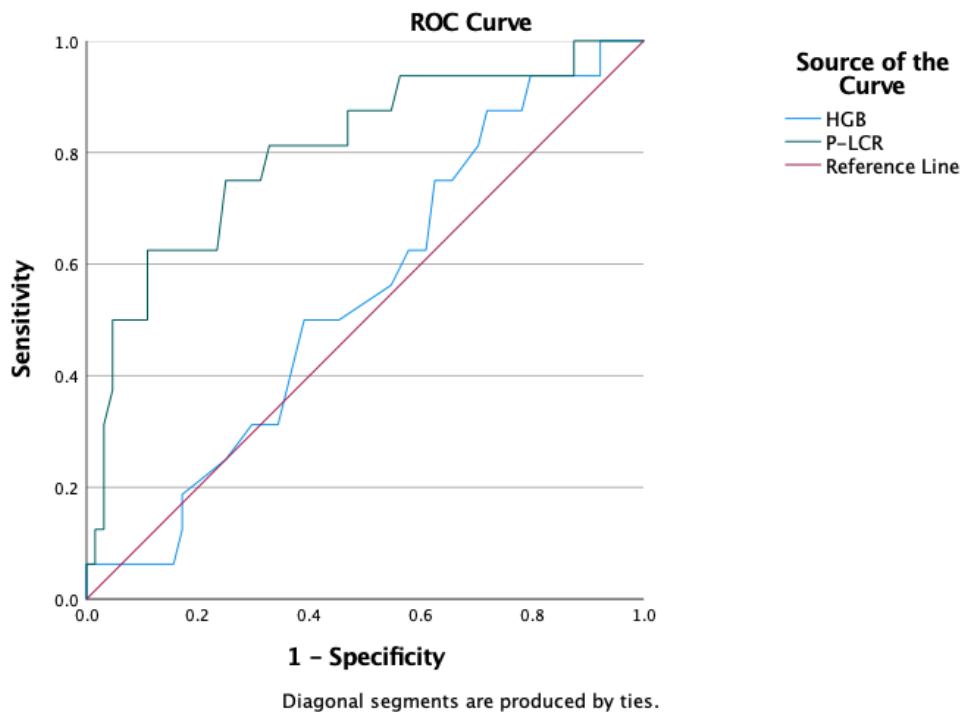**Figure 3.15: Receiver operating characteristics (ROC) curve analysis of HGB and P-LCR levels in Parkinson's disease,**

Chapter Four

Discussion

4. Discussion

Examination the level of Free Iron in Alzheimer's disease (AD), Parkinson's disease (PD) groups compared to control

In the current study, the level of **Free Iron** was increased in patient group when compared with control group and these results were agreed with many studies like **(Chen, *et al*., 2019)**.

Iron accumulation in the brain is believed to contribute to NDDs , including PD and AD . While an imbalance in brain iron status may cause free radical generation and oxidative damage, the possibility that such iron may be insoluble and unavailable for cellular use must also be considered **(Haacke EM, 2009)**.

Patients with AD have more severe iron deposition in the brain as compared to those of the same age who are healthy **(Liu, *et al*., 2018)**.

The normal physiological functions of the brain will be harmed if iron excess also has a neurotoxic impact. With aging, the brain's iron content steadily rises. It was interesting to see that AD patients' brains had much more iron than controls **(Du, *et al*., 2018),** these results were agreed with our result.

PD and iron deposition in the SNc are tightly connected. Iron buildup in the SNc and SNr is associated to the state of PD patients **(Chen, *et al*., 2019).** Parkinson's disease (PD) and other NDDs have been connected to the pathophysiology of iron-induced oxidative stress. When there is too much iron present, Fenton's reaction might result in the production of ROS **(Gaasch, 2007)**. It can alter the structural and functional characteristics of proteins and damage DNA **(Melis, *et al*., 2013)** . and promote polyunsaturated fatty acid peroxidation, which leads to alterations in cell membrane structure and functional loss **(Catalá, 2009).**

Although the exact cause of total iron buildup in the substantia nigra of Parkinson's disease patients is unknown, a number of potential causes have been put forth

increased blood-brain barrier permeability or blood-brain barrier dysfunction **(Kortekaas, *et al.*, 2005)**.

Increased blood-brain barrier permeability, inflammation, redistribution of iron inside the brain, and changes in iron homoeostasis are some of the potential causes of higher total iron concentrations with age **(Farrall & Wardlaw, 2009)**. The iron homoeostatic system may be compromised by aging processes **(Killilea, *et al.*, 2004)**.

Examination the level of ferritin in Alzheimer's disease (AD), Parkinson's disease (PD) groups compared to control

In this study, the level of **ferritin** was increased in patient when compared with control group and these results were agreed with many studies like**(Goozee, *et al.*, 2017).**

Iron is made accessible for crucial cellular functions by intracellular ferritin, which also protects lipids, DNA, and proteins from the iron's potentially damaging effects. Intracellular ferritin is crucial for maintaining iron homeostasis and is involved in a wide range of physiological and pathologic processes. In clinical practice, changes in serum ferritin are often observed and frequently signify disturbances in iron homeostasis or metabolism .Ferritin is primarily used as a serum marker of total body iron reserves in clinical medicine **(Koulanouzidis, *et al.*, 2009).**

Neurodegenerative disorders, hematophagocytic syndrome, and other potentially fatal illnesses are all intimately linked to ferritin **(Knovich, *et al.*, 2009).**

Alzheimer's disease is associated with ferritin, an important protein in the body that stores iron. Increased ferritin levels in the CSF have been associated with decreased cognitive function and a higher risk of mild cognitive impairment developing into (AD) **(Ayton, *et al.*, 2015)**.According to the study, ferritin has also been shown to have the potential to contribute to a blood biomarker panel for preclinical AD **(Goozee, *et al.*, 2017).**

Two proteins, transferrin receptor TfR and ferritin Ft, which are present in the majority of bodily cells, play a key role in the control and management of iron at the cellular level. The quantity of iron required is related to the number of membrane TfR and sequestered is proportional to the cytoplasmic level of Ft by the cell via modulating the degree of expression of these two proteins .The coordinated regulation of TfR and Ft by cellular iron occurs in the majority of cell types at the post-transcriptional level and is carried out by cytoplasmic RNA binding proteins, also referred to as the iron regulatory proteins IRPs **(Hentze & Kühn, 1996).**

Examination the level of TIBC in Alzheimer's disease (AD), Parkinson's disease (PD) groups compared to control

In the current study, the level of TIBCwas increased in patient group when compared with control group and these results were agreed with studies . Due to an imbalance in the iron, a redox active transition metal, is thought to play a role in the pathogenesis of AD by producing an excessive amount of free radicals while under oxidative stress **(Halliwell, 2006)**. The plasma levels of iron forms were evaluated in this investigation. The findings showed that the level of TIBC was dramatically rising.

Examination the level of DMT1 in Alzheimer's disease (AD), Parkinson's disease (PD) groups compared to control

In oure study, the level of DMT1was decreased in patient group when compared with control group and these results were agreed with studies.

Iron builds up in the central nervous system as a result of the changed expressions of DMT1, ferroportin, and hepcidin during PD **(Pieracci & Barie, 2005)** . In times of inflammation, astrocytes stimulate the blood flow of iron into the brain, causing neuronal iron to accumulate through the activities of microglia and astrocytes, leading to neurocytotoxicity and neurodegeneration **(Xiong, *et al*., 2016)**.

Although the exact cause of total iron buildup in the substantia nigra of Parkinson's disease patients is unknown, a number of potential causes have been put forth increased blood-brain barrier permeability or blood-brain barrier dysfunction **(Kortekaas, *et al.*, 2005)**. increased lactoferrin receptors in neurons and microvessels; elevated pro-inflammatory state; elevated DMT1 expression in dopamine neurons **(Salazar, *et al*., 2008)**· Mutations in genes related to iron transport and binding, as well as altered iron transport through transferrin-TFR type 2 **(Guerreiro, *et al*., 2006).** The change in DMT levels might be the result of a potential association between Parkinson's disease and single nucleotide polymorphisms in DMT1. This suggests that altered metal transport (especially of iron forms) may be intimately linked to the development of neurodegenerative diseases. It has been shown that age-dependent increases in brain metal concentration are associated with the course of certain neurodegenerative disorders **(Markesbery, *et al*., 1984).**

Contradicting our findings that low DMT1 expression is associated with gene enrichment of mitochondrial function is the fact that iron is essential for mitochondrial energy metabolism in oxidative phosphorylation as electron carriers. The correlation between up-regulated mitochondrial respiration and reduced DMT1 may be caused by the presence of other metals because of DMT1 binds a number of different divalent metals, including as cadmium (Cd^{2+}), copper (Cu^{2+}), and zinc (Zn^{2+}) **(Hokia, *et al*., 2019)**

DMT1 has a role in the function of transferrin, the main iron transporter that allows iron to enter mammalian cells. elevated DMT levels led to increased iron consumption and worsening iron accumulation in PD **(Zhong, *et al*., 2020)**

Examination the level of MDA in Alzheimer's disease (AD), Parkinson's disease (PD) groups compared to control

In this study, the level of MDA was increased in patient group when compared with control group and these results were agreed with studies.

MDA, a measure of oxidative stress and particularly lipid peroxidation, may be stated to be the molecular biomarker that is most useful in the early stages of AD development in all instances **(Guven,** *et al***., 2019).** MDA generated by free radicals impacting lipids was found to be considerably greater in the AD group **(Kheradmand,** *et al***., 2018).**

Some lipid peroxidation products were tested in human samples as possible biomarkers for AD. The most often examined biomarker in blood samples is MDA. In general, serum levels were greater in AD patients than in healthy subjects **(Shinto,** *et al***., 2014).**

Oxidative stress is a complex process that involve number of cellular signaling molecules in the form of proteins, enzymes, free radicals, etc. which either increase or decrease at the time of cellular damage **(Birben,** *et al.***, 2012**). This was in line with recent findings from prior research that showed PD patients have higher levels of oxidative stress than control groups **(Khan & Athar, 2018)**

Examination the level of 5LOX in Alzheimer's disease (AD), Parkinson's disease (PD) groups compared to control

In this study, the level of 5LOX was decreased in patient group when compared with control group and these results were agreed with studies

Previous research showed that 5LOX expression rises in the cortex and hippocampus of aged brain, two areas that are known to be more prone to neurodegeneration. This first finding served as the rationale for the concept that

5LOX and neurodegenerative disorders, which have aging as their primary risk factor, have a biological connection. . In agreement with that, hippocampi from AD patients had more 5-LOX immunoreactivity than those from controls **(Ikonomovic, *et al.*, 2008)**.

Growing evidence suggests that 5-LOX plays a role in the pathogenesis of (AD) and other aging-related neurodegenerative disorders. aging increases the expression and activity of 5-LOX in specific regions of the brain through epigenetic regulation **(Joshi, *et al*., 2014)**.

It has been shown that intracellular immunoreactivity of 5-LOX is elevated in the hippocampus of AD patients as compared to healthy age-matched controls in human brain post-mortem examination **(Ikonomovic, *et al*., 2008)**.

Double-labeling study has shown that the a plaques, NFTs, and vascular system are closely related to 5-LOX immunoreactivity. It's interesting to note that peripheral blood mononuclear cells (PBMCs) from late-onset AD (LOAD) patients exhibit higher levels of 5-LOX mRNA, protein, and activity. Additionally, the DNA methylation at the ALOX5 promoter has been observed to be decreased **(Chu & Praticò, 2016).**

It appears that deeper comprehension of LOX's function in the pathophysiology of AD might lead to the creation of far more potent disease-modifying strategies based on LOX inhibitors **(Czapski, *et al.*, 2016).**

Examination the level & Lipied profile of in Alzheimer's disease (AD), Parkinson's disease (PD) groups compared to control

In oure study, the level of Lipied profile was decreased in patient group when compared with control group and these results were agreed with studies.

Lipid and lipoprotein metabolism are altered in neurodegenerative disease, as shown in table 3.2 and figure 3.6. Although the specific pathophysiology of AD is still unknown, several studies have discovered a connection between cholesterol homeostasis and the disease. We are aware that the brain contains a lot of cholesterol. Because the blood barrier keeps the brain and periphery widely apart and prevents the entry of peripheral cholesterol, the brain and periphery have two different units of cholesterol **(Agarwal & Khan, 2020)**.

According to several research, total cholestrol TC is the single factor that is linked to AD. However, the theory is still not apparent. According to a research by He et al., the only change in TC levels seen in AD were elevated LDL, HDL, and triglycerides levels **(Qian,** *et al.***, 2016)**.

Low serum concentrations of TC and LDL can be related to a high prevalence of PD **(Xiaoyan,** *et al.***, 2015)**.The current findings demonstrated that participants with PD had significantly lower blood concentrations of TG, LDL, and TC than did control subjects. These results were consistent with earlier research; case-control studies on PD patients revealed that the blood levels of TC and LDL were much lower in those with PD than in the healthy group. According to this assertion, a higher risk of PD may be associated with decreased lipid fractions **(Cereda,** *et al.***, 2012).**

Parkinson's disease (PD) and other neurodegenerative diseases have been connected to the pathophysiology of iron-induced oxidative stress. When there is too much iron present, Fenton's reaction might result in the production of ROS **(Gaasch, 2007)**. It can alter the structural and functional characteristics of proteins and damage DNA (**Melis,** *et al.***, 2013**) . and promote polyunsaturated fatty acid peroxidation, which leads to alterations in cell membrane structure and functional loss (**Catalá, 2009)**

Neurodegenerative diseases including Alzheimer's disease and Parkinson's disease have been shown to have deregulated lipid homeostasis and particular lipid classes. Furthermore, recent research has demonstrated that the composition of membrane microdomains known as lipid rafts may vary in connection to neuronal dysfunction. Lipid rafts are essential components of cellular signaling systems. The aberrant protein distribution and aggregation, toxic cell signaling, and other neuropathological processes associated with these disorders may all be correlated with lipid change in these signaling platforms **(Mesa-Herrera, *et al.*, 2019)**.

The structure and function of the adult human brain do not degrade in a uniform manner, since the organ seems to choose susceptible neuronal groups concurrently with damage **(Wang, *et al.*, 2010)** . The loss of dendrites, the decrease and morphological variations of spine density, and changes in the chemical profile of synapses are among the very minor changes that contribute to the greatest functional impairment associated with normal brain aging **(Mattson & Magnus, 2006)**.

Rouser & Yamamoto reported finding evidence between lipid composition of the human brain to aging **(Rouser & Yamamoto, 1968)**. They discovered that the brain's lipid matrix suffers significantly throughout the course of a person's life. Total lipid levels were observed to rise during the first two decades of life, then gradually decline as persons aged and entered adulthood. also noticed a curvilinear decline of lipid levels in human brains with aging.

According to the research, one of the lipids in the brain that ages the fastest is cholesterol. It is commonly acknowledged that cholesterol production increases significantly throughout brain development but decreases steadily and slowly during maturity **(Dietschy & Turley, 2004)** .The majority of the information available indicates that the levels of cholesterol have gradually decreased in a range of brain

areas, including the human cortex, hippocampus, and cerebellum, synaptosomes, and cultured hippocampal cells (**Martín,** *et al***., 2010)**. In addition, low cholesterol makes hippocampus glia in primary culture more susceptible to excitotoxicity brought on by glutamate **(Chou,** *et al***., 2003).**

 It has been shown that the aging-related changes in cholesterol in neuronal membranes affect the fluidity of the cell membranes, increasing their stiffness and physico-chemical characteristics **(Vanmierlo,** *et al***., 2011).** As a result, the membrane structural defects brought on by cholesterol may have an impact on the actions of membrane-related proteins, changing signal transduction reactions. This effect may be especially significant in lipid rafts, which are important locations for signaling proteins to assemble into signalosomes, changing various transduction pathways that impact neuronal function (**Marin,** *et al.***, 2017)**.

    Due to a significant loss of neurons, AD brains show cortical atrophy (between 8 and 15% smaller than healthy brains) **(Castellani,** *et al***., 2010)** .Additionally, the AD brain experiences metabolic changes, including as differences in calcium homeostasis and modifications in the metabolism of phospholipids and cholesterol, which may eventually impair mitochondrial function . Calcium dysregulation has been shown to occur before and underlie other AD-related changes such oxidative stress, tau aberrant hyperphosphorylation, and synaptic plasticity deficits. Deterioration of ganglioside metabolism has also been linked to lipid changes seen in neuronal membranes during AD **(Zha,** *et al***., 2004) .**

  According to the above list of concepts, lipid profiles in AD patients show a reduction in ganglioside content in many brain regions linked to the disease's pathophysiology. Indeed, substantial differences between AD patients and healthy

participants of a comparable age have been found in gangliosides, sulfatides, and cerebrosides **(Brinton, 2013).**

  The increasing loss of dopaminergic neurons, on the other hand, is a hallmark of Parkinson's disease (PD) **(Broen, *et al.*, 2016).**

Furthermore, local lipid homeostasis may be a factor in the hazardous nucleation and aggregation of α-synuclein at the plasma membrane **(Eriksson, *et a*l., 2017)**.According to this line of reasoning, elevated cholesterol levels and the oxidized byproducts of cholesterol stimulate the buildup of α-synuclein **(Cheng, *et al.*, 2008)**.

  Previous research has demonstrated that certain oxysterols can control the transcription of the α-synuclein gene as well as other genes linked to neurodegeneration and neuroinflammation **(Barceló-Coblijn, *et al*., 2007)**. Sphingolipid metabolism may potentially have a role in the pathological development of PD, according to several reports. As a result, increased sphingomyelinase activity has been seen in PD brains, which promotes an increase in ceramide levels and apoptosis.All of these findings point to the involvement of many aging and PD pathology-related variables in the effect of lipid profile alterations in nerve cell membran.

Examination the level of Platelet in Alzheimer's disease (AD), Parkinson's disease (PD) groups compared to control

In the current study, the level of Platelet was increased in patient group when compared with control group.

  It is interesting that no research have looked at the connection between platelet characteristics and neurodegenerative disease. Additionally, there are no studies comparing patients and controls using platelet characteristics such P-LCR. Platelets have been proven to have high quantities of and expression of several

neurotransmitters and their receptors in addition to neurochemicals **(Camacho & Dimsdale, 2000).**

In many mental and neurological illnesses, platelet serotonin serves as a vital research tool since it has a variety of membrane receptors and various physiological interactions **(Skop,** *et al***., 1994)**.

Because platelets and serotonergic neurons both produce related enzymes and receptors, changes in central serotonin levels frequently mirror changes in platelet serotonin levels **(Horstman,** *et al***., 2010).**

In addition, the quantity of serotonin transporters on platelets primarily controls the plasma levels of 5-hydroxytryptamine receptors (5HT) **(Lesch,** *et al***., 1993)**. Numerous NDDs have had their platelet monoamine oxidase (MAO) activity measured **(Mimica,** *et al***., 2008).** and mental health conditions. Human platelets express the three main glutamate transporters and exhibit particular sodium-dependent glutamate uptake activities in a number of central nervous system (CNS) illnesses **(Begni,** *et al***., 2005)**.

Behari et al., **(Behari & Shrivastava, 2013)** reported a connection between platelets and apoptosis. Apoptosis protein activating factor-1, caspases, proapoptotic and antiapoptotic proteins, and others are expressed by platelets **(Leytin,** *et al***., 2009)**.

The depolarization of the mitochondrial inner membrane, the release of cytochrome, the expression of proapoptotic and antiapoptotic Bcl-2 family proteins, the exposure of phosphatidyl serine, platelet shrinkage, fragmentation to microparticles, blebbing, and filopod extrusion are all signs of apoptosis in human platelets **(White,** *et al***., 2012)** . As a crucial aspect of apoptosis, all these may be efficiently examined in platelets **(Seghatchian & Krailadsiri, 2001).**

As a result, multiple platelet apoptosis indicators point to yet another possible platelet function in the pathophysiology of several NDDs. Physiological stress and chemical stimuli have the natural inclination to cause platelets to clump together when

they become activated. Aggregation and shape change are caused by an increase in cytosolic calcium, enzyme activation, enhanced mobility, and exposure of platelet membrane components **(Perrotta, *et al*., 2003).**

A useful technique in the investigation of cardiovascular and cerebral vascular illnesses is the evaluation of platelet aggregation as a platelet function test. According to prior research, platelet activation may serve as a useful predictive biomarker for cognitive deterioration in NDDs like AD **(Stellos, *et al*., 2010).** Aggregation and activation of platelets has also been seen in NDDs like ALS, MS, PD **(Lim, *et al*., 2009) .**

Due to all of these factors, measuring the platelet aggregation pattern and activation may be a useful investigative technique for a variety of NDDs.

Chapter Five

Conclusions

&

Recommendations

5. Conclusions and Recommendations

Conclusions

From all data and correlations of different variables in the present study, it could be concluded that: -

❖ Patients with Neurodegenerative disease (NDDs) showed an increasing range level of the TIBC, MDA, Free Iron and Ferritin when comparing to the healthy control groups, while the range level of 5LOX and DMT1 decreased compared to healthy control.

❖ The serum concentrations of cholesterol, triglycerides and apolipoprotein B (ApoB)-containing lipoproteins (including very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) are all normal in patients group

❖ Results of the receiver operating curve (ROC) and AUC analysis for the iron forms as possible diagnostic parameters showed that Free Iron and TIBC have a good diagnostic performance for predication neurodegenerative disease compared to control group.

❖ Results of the receiver operating curve (ROC) curve and AUC analysis for the HGB and P-LCR as diagnostic parameters showed that HGB and P-LCR have a good performance for prediction **Alzheimer's disease** and **Parkinson's disease**.

❖ People over the age of 65 who have family history diseas of AD and PD should undergo periodic iron forme test to avoid cell death in the brain.

❖ **Recommendation**

   The current research project has the following recommendations for studies**:**

- Researchers might be highlighting the potential role of lactoferrin receptors in neurons of neurodegenerative cases.

- It might be a good idea to focus on the platelet and their aggregation products such as platelet monoamine oxidase (MAO) activity and compared their level in mental health conditions.

- Since it has been reported a connection between platelets and apoptosis,it would be worth looking for the correlation between the neurodegenerative disease and Apoptosis proteins such as activating factor-1, caspases, proapoptotic and antiapoptotic proteins.

Chapter Six

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Appendices

Appendices

Questioner:

Name: NO. of phone:

Gender:

Weight: height: BMI:

Name of disease: Duration of disease:

Risk factor: family history:

Chronic disease: DM: HT: other:

Smoking: active: passive:

parameter	Result
Free iron	
freetin	
TIBC	
MDA	
5LOX	
DMT1	
Lipid profile	
CBC	

الملخص

الأمراض التنكسية العصبية(NDDs)هي حالات مرضية مرتبطة بالعمر موصوفة بموت الخلايا العصبية غير المنضبط مما يؤدي إلى تنكس تدريجي في وظائف الدماغ. تؤثر NDDs على ملايين الأشخاص في جميع أنحاء العالم. .تتعلق هذه الأمراض بآليات موت الخلايا المعقدة والمسارات المتعددة ، والتي تشمل التراكم المفرط لبيروكسيد الحديد والدهون في مناطق مختلفة من الدماغ. تشمل الأمراض غير السارية الأكثر شيوعا مرض الزهايمر (AD)، ومرض باركنسون (PD)، ومرض البريون ، والتصلب الجانبي الضموري (ALS)، ومرض هنتنغتون(HD)، وضمور العضلات الشوكي ، وترنح العمود الفقري المخيخي.

Ferroptosisهو نوع من موت الخلايا المبرمج يعتمد على الحديد ويتميز بتراكم بيروكسيدات الدهون ، وهو متميز وراثيا وكيميائيا عن الأشكال الأخرى لموت الخلايا المنظم مثل موت الخلايا المبرمج. يبدأ داء الحديديات بفشل الدفاعات المضادة للأكسدة المعتمدة على الجلوتاثيون ، مما يؤدي إلى اكسدة الدهون وموت الخلايا في نهاية المطاف.

هدفت هذه الدراسة إلى تقييم دور الحديد الحر ، ونقله مثل ناقل المعادن ثنائي التكافؤ 1 (DMT1) ، وقدرة ربط الحديد الكلي (TIBC) و (5LOX) lipoxygenase -5 ، و Malondialdehyde (MDA) ومستوى الفيريتين في مرض الزهايمر والشلل الرعاشي.

نوع الدراسة هو مراقبة الحالة تم جمع 80 عينة من 40 حالة (24) مرض الزهايمر من (عيادة الطب النفسي) و(16) مرض باركنسون (من عيادة الأمراض العصبية) الذين تتراوح أعمارهم بين (90-65) سنة و 40 مشاركا كعنصر تحكم صحي مع الفئة العمرية (87-65).

تم قياس مستويات مصل DMT1 و MDA و 5LOX باستخدام تقنية المقايسة المناعية المرتبطة بالإنزيم (ELISA)وتم إجراء مستويات مصل الاختبارات البيوكيميائية (ملف الدهون ، الحديد الحر ، الفيريتين و TIBC باستخدام محلل الكيمياء السريرية ، وتم إجراء تعداد الدم الكامل بواسطة محلل أمراض الدم الآلي XP-300™ Sysmex.

أشارت النتائج إلى أن المرضى الذين يعانون من مرض تنكسي عصبي أظهروا زيادة كبيرة في مستوى قياس TIBC و MDA والحديد الحر والفيريتين عند مقارنتهم بمجموعات التحكم الصحية كانت قيمة (p< 0.05)،

في حين انخفض مستوى النطاق 5LOX و DMT1 مقارنة بالسيطرة الصحية وأظهر5LOX فقط اختلافات ذات دلالة إحصائية كانت قيمة ($p < 0.05$)تم إجراء منحنى ROC وتحليل AUC للمؤشرات الحيوية. زيادة مستوى الحديد لاقى أداء جيدا للتنبؤ بالأمراض التنكسية العصبية ، وكان TIBC أفضل تنبؤ لحالات مرض الزهايمر ومرض باركنسون. وخلصت الدراسة إلى أن أشكال الحديد ارتبطت بنجاح في تشخيص الأمراض التنكسية العصبية. من المحتمل أن يلعب التماثل المعيب في الحديد ونشاط الأكسدة والاختزال دورا في علم الأمراض العصبية لمرض الزهايمر ومرض باركنسون.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الطب

فرع الكيمياء والكيمياء الحياتية

دراسة سمية الحديد،والتحلل التأكسدي للدهون وموت الخلايا نوع Ferroptosis لدى مرضى الزهايمر والشلل الرعاشي

رسالة ماجستير

إلى مجلس كلية الطب/ فرع **الكيمياء والكيمياء الحياتية** / جامعة كربلاء كجزء من متطلبات نيل درجة الماجستيرفي الكيمياء السريرية

من قبل

زهراء ناجي عبد الرزاق

بكالوريوس علوم كيمياء – كلية علوم البنات جامعة بابل 2012

إشراف

<table>
<tr><td>الأستاذ المساعد الدكتور</td><td>الأستاذ المساعد الدكتور</td></tr>
<tr><td>أثير حميد الغانمي</td><td>رنا مجيد حميد</td></tr>
<tr><td>دكتوراه كيمياء النانو</td><td>دكتوراه كيمياء حياتية</td></tr>
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