Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science
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Study the Oxidative Stress Statues in Cardiovascular and Hypertension Diseases in Female Patients

A Thesis

Submitted to the College of Science/Al-Nahrain University as a Partial Fulfillment of the Requirements for the Degree of M.Sc. in Chemistry

By

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April 2015 **Jumada II 1436**

Dedication

To The Realest And Purest Love In The World

Mother

And To All My ***Family***

And to my country ***Iraq***

And to ***Iraqi soldiers****, those who far from as now but keeping us continue life*

Yasmeen

Acknowledgment

First of all I would like to thank our **God** for his merciful and continuous help through my life.

I am very thankful for chemistry department of college of science of al-Nahrain University for give me research part.

I thank Dr Alaa the supervisor for my study.

I am very grateful for friendly assistance and continuous kindly advices by Dr. Perry Habeeb in different stages along this study.

I also very thankful to staff of Ibn Al-Nafese for their help to gain and stored sample in my study.

I am very thankful to Dr. Wisam for his continuous advice through study.

I would like to thank Mr. Zaid N. Abbas for his big assistance in molecular study.

I would like to thank Dr. Rasha Saad for her facilitate to gain close access papers.

I am specially thankful to my friend Ala'a for her assistance and kind standing behind me along study.

Appreciable thanks are to those brave men on the fire borders, saving their citizens, families, and land, make us continue life, work and study, sacrificing their own souls, they deserve our success as a gift.

Supervisor Certification

I certify that this thesis entitled **" Study the Oxidative Stress Statues in Cardiovascular and Hypertension Diseases in Female Patients"** was prepared by **"Yasmeen Muhialdeen Hussein"** under my supervision at the College of Science/ Al-Nahrain University as a partial requirements for the Degree of Master of Science in Chemistry.

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Abbreviations

8-hydroxy-dGTP	8-hydroxy-Deoxyguanosine Triphosphate
8-OHdG	8-Hydroxydeoxyguanosine
8-oxodG	8-Oxo-2'-deoxyguanosine
Ang II	Angiotensin II
ATP	Adenosine Triphosphate
BBA	Binding Buffer
BCG	Bromocresol Green
BMI	Body Mass Index
C.V	Cardiovascular
CAD	**C**oronary Artery Disease
CAT	Catalase
CHD	Coronary Heart Disease
CLD	Cell Lysis Buffer
CVDs	Cardiovascular Diseases
dGTP	Deoxyguanosine Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
eNOS	Endothelial NO-Synthase
GPx	Glutathione Peroxidase
GSH	Glutathione
GSR	Glutathione Reductase
GSTM1	Glutathione S-Transferase Mu 1
GSTs	Glutathione-s-Transferases
GSTT1	Glutathione S-Transferase Theta-1

H_2O_2	Hydrogen Peroxide
HAS	Human Serum Albumin
HDL-C	High-Density Lipoprotein- Cholesterol
HRP	Horseradish Peroxidase
HT	Hypertension
LDL-C	low-density lipoprotein- Cholesterol
$LOO^{\cdot}$	Lipid Peroxy Radical
MDA	Malondialdehyde
MnSOD	Manganese Superoxide Dismutase
NADH	Nicotinamide Adenine Dinucleotide
NER	Nucleotide Excision Repair
NO	Nitric Oxide
$O_2^{\cdot}$	Superoxide
$OH^{\cdot}$	Hydroxyl Radical
ONOO	Peroxynitrite
PCR	Polymerase Chain Reaction
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
T_2DM	Type 2 Diabetes Mellitus
TBA	Thiobarbituric Acid
TG	Triglyceride
VLDL	Very low-density lipoprotein- Cholesterol

Committee Certfication

We, the examining committee certify that we have read this thesis entitled **" *Study the Oxidative Stress Statues in Cardiovascular and hypertension diseases in female patients"*** and examined the student "**Yasmeen Muhialdeen Hussein**" in its contents and that in our opinion, it is accepted for the Degree of Master of Science in Chemistry.

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Summary

Hypertension (HT) and cardiovascular diseases (CVD) share in that one of the main reasons for them is to increase the oxidative stress, which in turn increases the severity of the disease and exacerbation of symptoms. Reactive molecules produced from oxidative stress, in addition to causing tissue damage by oxidation of biomolecules like DNA, lipids, proteins and sugars; it is lead to the formation of mediators with potent inflammatory effect. The objective of this study was to investigate the some markers of oxidative stress in HT and CVD patients in addition to some biochemical parameters related to these diseases.

This study involved 84 female subjects aged between (30-65) year equally divided to three groups, first and second one belong to CVD and HT patients from Ibn Al-Nafese hospital, while the third one for apparently healthy 28 subjects considered as control group. For each subject in the three groups these markers and parameters were evaluated; malondialdehyde (MDA), 8-hydroxydeoxyguanosine (8-OHdG), albumin, uric acid, urea, creatinine. Also electrophoretic study on the DNA of patients and control group controlled by placental DNA was done to investigate the possible oxidative stress after Fenton reaction in vitro on it. And study the deletion of Glutathion S transferase mu (GSTM1) gene using polymerase chain reaction PCR.

The results were compared to control; Lipid profile show higher significant ($p<0.01$) levels in both CVD and HT compared to control group. There was a significantly higher ($p<0.01$) in MDA, and 8-OHdG levels in both CVD and HT patients, while serum albumin shows significantly ($p<0.01$) lower levels. Uric acid unexpectedly shows higher levels than control group with ($p<0.01$) although is antioxidant. The electrophoresis study for GSTM1 gene show a significant difference ($p<0.01$) in deletion of this gene. Further there is appositive correlation

in deletion of this gene and higher MDA and 8 OHdG and negative with albumin while no correlation with urea, creatinine and uric acid.

The elevation levels of oxidative stress markers may be due to oxidative damage of tissues that caused by these inflammatory diseases. It concludes there was a positive relation between oxidation results from these diseases and their developments and suggest increase need to intake of antioxidants as precaution in front of these disease.

Contents

Chapter One Introduction & Literature Review

Chapter Two Materials & Methods

Chapter Three Results & Discussion

List of Tables

List of Figures

1.1 The Arterial Structure

The arterial wall consists of three layers : First intima, is the most inner layer of the arterial wall, separated from the vascular lumen and circulating blood by only a single layer of endothelial cells. The endothelial cell residing at the critical interface between blood flow and the arterial wall, functions as a permeability barrier, forms a thrombo resistant surface, and tends to regulate vascular tone by producing nitric oxide, and endothelium. The intima is composed of two layers; a superficial proteoglycan-rich layer and a deeper musculoelastic layer. Often, these layers can only be recognized in areas of adaptive intimal thickening[(1)].

The second layer is the media which separated from the inner layer of the vessel wall, (the intima), by continuous elastic lamina, called internal elastic lamina, the intima of human epicardial coronary arteries is thickened, even without any signs of atherosclerosis, in response to changes in physiological factors such as increased tension or disturbance of shear stress in the vessel wall. In general, intimal thickening in coronary arteries is diffused, and eccentric intimal thickening typically only occurs at the branching points of the arteries, involving half of the vessel circumference[(2)]. This appears as atherosclerosis, including hypertension and cardiovascular diseases.

Adventitia is the third layer, It is outermost connective tissue covering of any organ, vessel, or other structure. It is also called the tunica adventitia because it is considered extraneous to the artery[(3,4)]. The arterial structure shown in (Fig. 1-1)

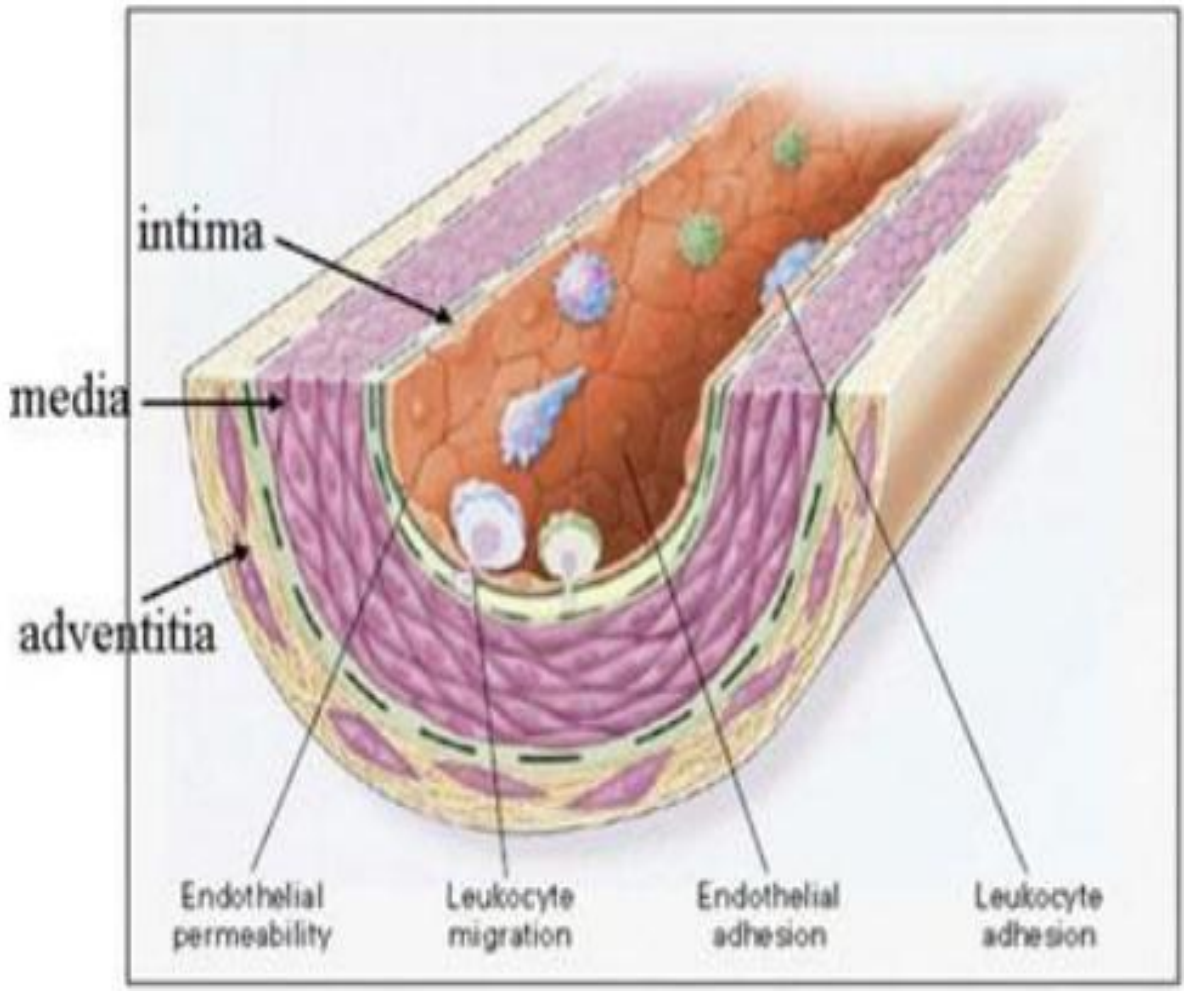


Figure (1-1):The arterial structure[5]

1.2. Cardiovascular Diseases

1.2.1 Definition

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels and they include:

- Coronary heart disease: disease of the blood vessels supplying the heart muscle.
- Rheumatic heart disease: damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria.
- Congenital heart disease: malformations of heart structure existing at birth.

- Deep vein thrombosis and pulmonary embolism: blood clots in the leg veins, which can dislodge and move to the heart and lungs [6].

Cardiovascular diseases is the result of the accumulation of atheromatous plaques within the walls of the arteries that supply myocardium with oxygen and nutrients. After decades of progression, some atheromatous plaques may rupture and may thus severely restrict the flow of oxygen carrying blood to the myocardium. As a consequence, a heart attack can occur[7]

1.2.2 Symptoms

Often, there are no symptoms of the underlying disease of the blood vessels. A heart attack or stroke may be the first warning of underlying disease. Symptoms of a heart attack include[7,8].

- Pain or discomfort in the centre of the chest.
- Pain or discomfort in the arms, the left shoulder, elbows, jaw, or back.

In addition the person may experience difficulty in breathing or shortness of breath; feeling sick or vomiting; feeling light-headed or faint; breaking into a cold sweat; and becoming pale. Women are more likely to have shortness of breath, nausea, vomiting, and back or jaw pain.

The most common symptom of a stroke is sudden weakness of the face, arm, or leg, most often on one side of the body. Other symptoms include sudden onset of:

- Numbness of the face, arm, or leg, especially on one side of the body.
- Confusion, difficulty speaking or understanding speech.
- Difficulty seeing with one or both eyes.
- Difficulty walking, dizziness, loss of balance or coordination.

- Severe headache with no known cause.
- Fainting or unconsciousness [6,9,10].

The major risk factors for CVD including: high blood pressure (hypertension) smoking ,high blood cholesterol ,diabetes ,lack of exercise being overweight or obese ,a family history of heart disease and ethnic background [7] Growing evidence suggests that highly reactive oxygen derived free radicals reactive oxygen species (ROS) of endogenous or environmental origin play a cognitive role in the genesis and progression of various CVDs [8,9].

1.3 Hypertension:

1.3.1 Definition of Blood Pressure :

Blood pressure is a measure of the force that the circulating blood exerts on the walls of the main arteries. The pressure wave transmitted along the arteries with each heartbeat is easily felt as the pulse, the highest (systolic) pressure is created by the heart contracting and the lowest (diastolic) pressure is measured as the heart fills [11].

1.3.2 Definition of Hypertension :

Hypertension is defined as an average systolic blood pressure greater than or equal to140 mmHg or an average diastolic blood pressure of greater than or equal to 90 mmHg [12], Hypertension is a disease and blood pressure is a biomarker [13].

1.3.3 Symptoms of Hypertension

Hypertension is a disease which in its early stages is almost without obvious symptoms its identification is usually through screening. A proportion of people with high blood pressure report headaches (particularly at the back of

the head and in the morning), as well as lightheadedness, vertigo, tinnitus (buzzing or hissing in the ears), altered vision or fainting episodes[14].

1.4 Lipid profile

1.4.1 Difenition and Classification

Lipids play a critical role in almost all aspects of biological life, they are structural components in cells and are involved in metabolic and hormonal Pathways. Lipid metabolism is usefully based on the concept of lipoproteins, the form in which lipids circulate in plasma, large spherical complex molecule [15].

Chylomicrons contain a lipid core that composed of dietary lipid and lipid synthesized in the intestinal cells. Chylomicrons are the lipoprotein particles lowest in density and largest in size, and contain the highest percentage of lipid and the smallest percentage of protein[17].

Cholesterol is essential precursor of steroid hormones, bile acids and vitamin D, and is required structurally for cell membranes, strengthen cell membranes and make hormones [16], cholesterol risk levels are positively correlated to the development of atherosclerosis, a process initiated by deposition of excess insoluble sterols in the arterial wall. High cholesterol is a major risk factor for CVD and stroke [17]. The American Heart Association (AHA) has identified untreated total cholesterol <3.5-5 mmol/L (for children) and <5.18 mmol/L (for adults)[18]

Triglyceride (strictly triacylglycerol) is formed by reaction of two molecules of fatty acyl-CoA with glycerol 3-phosphate to form phosphotidic acid , this is dephosphorylated to a diacylglycerol and then acylated by a third molecule of fatty acyl-COA to yield a triacylglycerol[17] Triglyceride is presented in dietary fat ,and can be synthesized in the liver and adipose tissue

to provide a source of a stored energy, which can be mobilized when required. Levels of serum triglycerides have also been linked positively to lower levels of high-density lipoprotein- Cholesterol (HDL-C) and low-density lipoprotein-Cholesterol (LDL-C) particle size . Both these conditions have been linked to an elevated risk of developing diseases[16,17], The value of triglyceride is lower than 1.69 mmol/L [18]. After its absorption into the intestinal mucosal cell, cholesterol, together with triglycerides, phospholipids, and a number of specifi apoproteins, is assembled into a large lipoprotein a *chylomicron*

1.4.2 Lipoproteins

The major plasma lipids are not circulating free in the blood. They are bound to a specific protein, (Apoproteins) to form large spherical complex molecule called lipoprotein, which are transported through plasma[20]

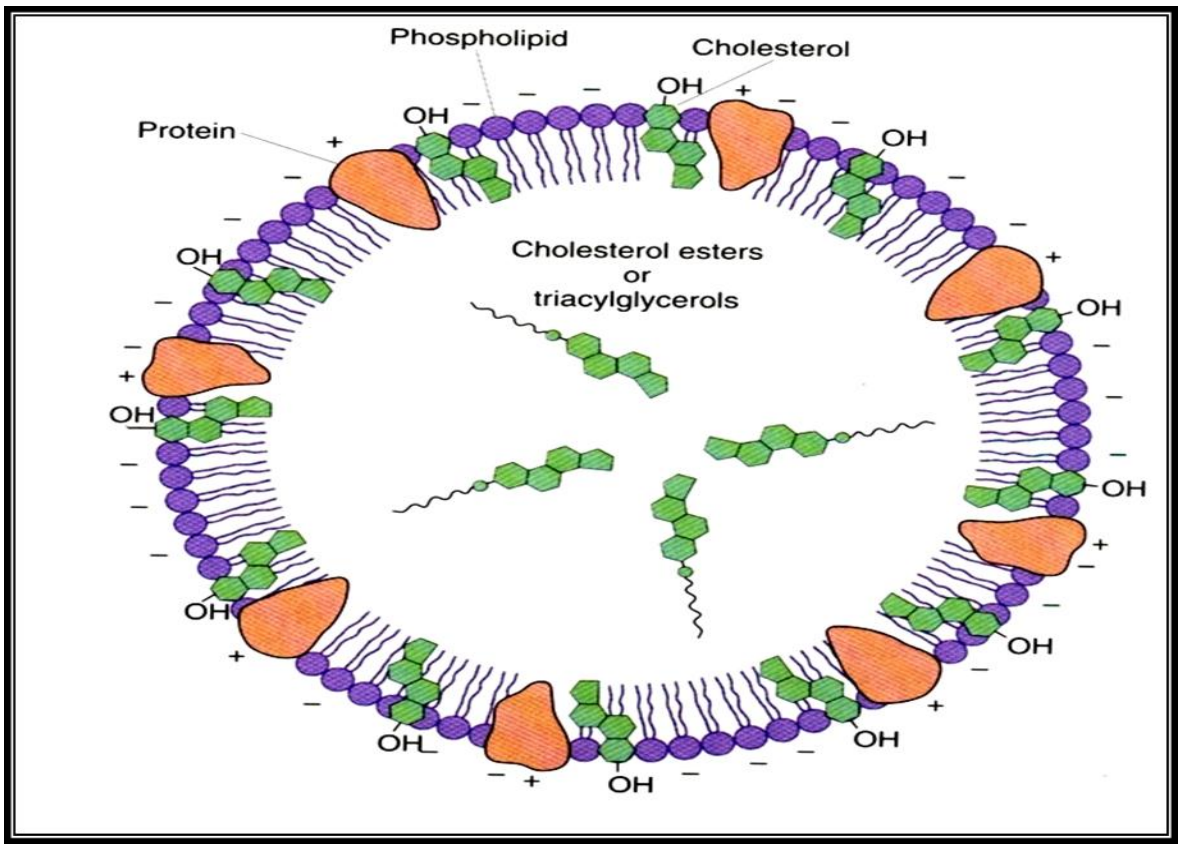


Figure 1-2: The structure of a plasma lipoprotein[21]

High-density lipoprotein- Cholesterol transport cholesterol to liver or steroidogeine organs such as adrenal , ovary and tests for synthesis of steroid hormone[22]. HDL promote expulsion of cholesterol from peripheral cells and , indirectly from the body thus; protecting against cardiovascular disease. Several studies of large populations have shown that the risk of developing manifestation of ischemic heart disease is inversely related to the serum concentration (HDL-cholesterol).[23,24,25], The recommended HDL level is above 1.0mmol/L (men) and above 1.2mmol/L (women)[18,26].

Low-density lipoprotein- Cholesterol LDL carry cholesterol to peripheral tissues and help regulate cholesterol levels in those tissues[16], making it available to the tissue cells for membrane or hormonal synthesis and for storage for later use. This type of cholesterol is considered harmful as it transports a large amount of cholesterol. The recommended LDL level is below 3.0mmol/L [17,18]

Cardiac diseases and Hypertension are known to be associated with alterations in lipid metabolism which gives rise to abnormalities in serum lipid and lipoprotein levels [12]

Standard lipoprotein analysis of measurement of total serum cholesterol, total triglyceride, high density lipoprotein cholesterol, and calculation of low-density lipoprotein cholesterol is medically necessary for prediction of risk for coronary artery disease

1.5 Oxidants:

1.5.1 Free Radicals :

Free radicals can be defined as molecules or molecular fragments having one or more unpaired electrons in atomic or molecular orbitals. Examples Nitric oxide ($NO^{\cdot}$), superoxide($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), lipid peroxy radical ($LOO^{\cdot}$). This unpaired electron(s) frequently gives a considerable degree of reactivity to the free radical[27]. Radicals derived from oxygen represent the most essential class of radical species generated in living systems[28].

1.5.2 Reactive Oxygen Species :

Although molecular oxygen (O_2) has two unpaired electrons in two different orbitals, it is not a free radical. Molecular oxygen, however, reacts rapidly with most other radicals, forming other free radicals that are more reactive[29].

Reactive oxygen species (ROS) is a group term used for a group of oxidants, which are either free radicals or molecular species capable of generating free radicals[30], may initiate from both exogenous and endogenous sources.

Exogenous sources consist of environmental agents, radiation, therapeutic agents, and tobacco smoke. Endogenous sources involved mitochondria, peroxisomes, and inflammatory cell activation as summarized in Figure (1-3).

In a perfect world, cells will use oxygen to produce adenine tri phosphate (ATP) and water without any toxic byproducts or activated (also active) oxygen

species. However, damage to mitochondria in pathophysiological conditions or through mitochondrial dysfunction, the electron transport mechanism in the mitochondrial respiratory chain is impaired. This leads to

reactive oxygen species formation (e.g., superoxide anion) from one-electron reduction of oxygen [29]

Hydrogen peroxide is not a free radical, but is a precursor of free radicals. UV radiation causes the hemolytic cleavage of the oxygen–oxygen bond to form hydroxyl radicals (·OH). Redox metal ions (Fe^{+2} or Fe^{+3} or Cu^{+}) react with hydrogen peroxide to produce hydroxyl radicals (the Fenton reaction) [29]

In physiological conditions, low levels of ROS play a protecting role in the organism, while elevated levels of ROS can cause damage to cell structures, nucleic acids, lipids and proteins or DNA damage [30,31]

Though the Fenton's reaction, these hydrogen peroxides react with metal iron or copper to form more highly reactive hydroxyl ions, $^{\cdot}OH^{-}$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + {}^{\cdot}OH$

Though Haber-Weiss reaction , catalyzed by metals :

$O_2^{\cdot} + H_2O_2 \longrightarrow O_2 + {}^{\cdot}OH + {}^{\cdot}OH$

H_2O_2 reacts with Cl^{-}, Br^{-}, I^{-} and is used by myloperoxidase to form more reactive hypochloric acid/hyperchlorite.[32]

$H_2O_2 + Cl \rightarrow HOCl + OH^{-}$

Formation of peroxynitrite is the primary reaction[31].

$O_2 + NO \rightarrow ONOO^{\cdot}$

The second order rate constant for the formation of the ·OH via the Fe(II)- mediated Fenton reaction is 76 L mol^{-1} s- , This indicates that there may be between 46 to 458 ·OH formed per second in a cell, indicating that even the lower rate of reaction is biologically significant [33].

Another immunologically relevant reactive oxygen species is hypochlorous acid (HOCl, or bleach). HOCl is formed from

myeloperoxidase/H_2O_2-dependent oxidation of chloride anion. HOCl has intermediate reactivity-more reactive than H_2O_2 but considerably less reactive than hydroxyl radicals[29].

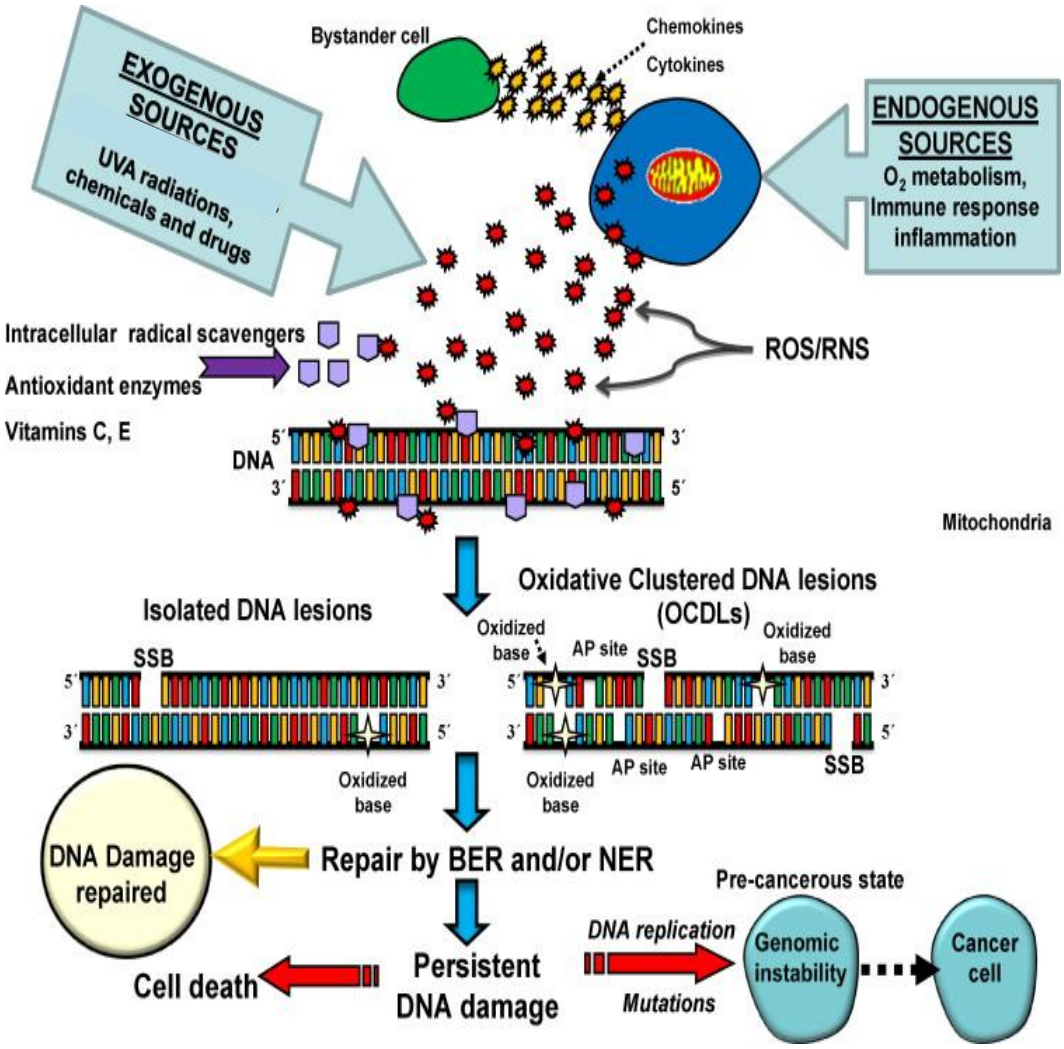


Figure (1-3) : Sources and Effects of Reactive Oxygen Species [34], AP: abasic site, BER: base excision repair, NER: nucleotide excision repair, ROS/RNS: final outcome is the production of reactive oxygen/nitrogen species, SSB: single strand breaks

1.6 Antioxidant Defense System:

The balance between the creation and removal of ROS is controlled by a variety of DNA repair enzymes and antioxidants[31,32]. The human body involved a complex antioxidant defense system which depends on the dietary

intake of antioxidants as well as the endogenous production of antioxidative compounds Antioxidants can be classified into a number of different groups:

1. Antioxidant enzymes: which scavenge free radicals. These enzymes make up a preventive type of antioxidative network superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR).

2. Antioxidative proteins :that avoid the formation of ROS include hemoglobin, transferrin, albumin, lactoferrin

3. Small-molecular-weight compounds: are able to respond directly to and eliminate free radicals and are therefore called radical-scavenging antioxidants ascorbic acid (vitamin C), tocopherols (vitamin E), glutathione (GSH), uric acid, selenium, bilirubin, glucose.

4. Ubiquinone (coenzyme Q-10)

5. Flavonoids [29,36].

1.7 Oxidative Stress

Oxidative stress may occur if the production of ROS exceeds the antioxidant capacity of the cell [29].

Although cells are equipped with an impressive stock of antioxidant enzymes as well as small antioxidant molecules, these agents may not be sufficient enough to normalize the redox status during oxidative stress[37]

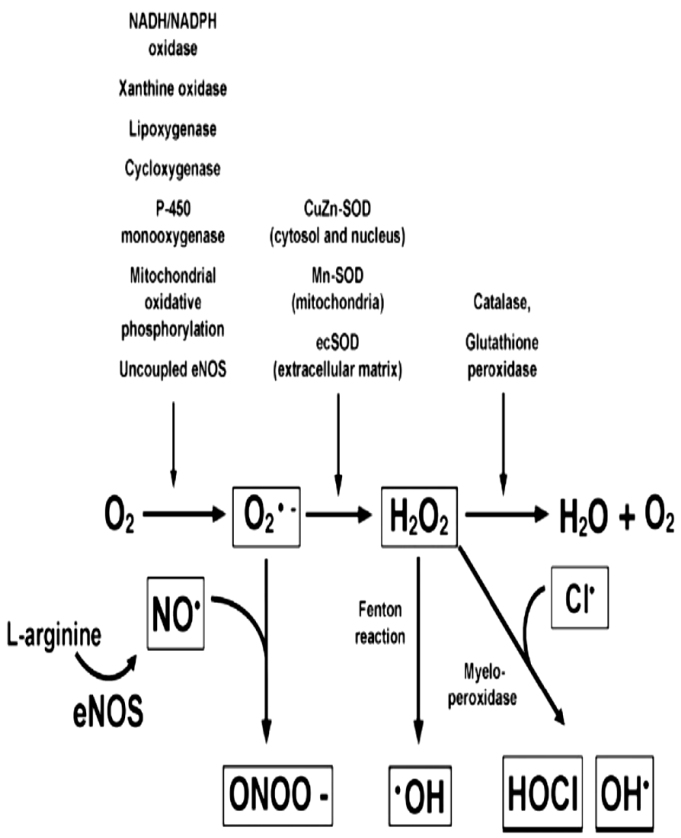


Figure (1-4) . Generation and metabolization of reactive oxygen . [38]

1.8 Oxidative stress and Arterial Diseases :

ROS has a key role in the pathogenesis of hypertension. The modulation of the vasomotor system includes ROS as mediators of vasoconstriction made by angiotensin II (AII), endothelin-1 and urotensin-II [(39)].

ROS are raise in hypertension in response to vessel stimulation by mechanical stretch or AII. Reaction of ROS with endothelium released NO inhibits vasodilatory or antisclerotic effects of NO and thus can exacerbate the disease [(40)].

(Ang II) levels are raised but ROS is actually decreased, perhaps due to of the accompanying increase in super oxide dismutase (SOD) expression[(41)]. This ability to increase antioxidant defenses may be sufficient to protect the vasculature from low levels of oxidant stress, allowing ROS to function as signaling molecules. However, when ROS production becomes overwhelming, compensatory mechanisms are inadequate and patho physiological consequence[(42)]. Endothelial cells are able to synthesize and secrete a large spectrum of antiatherosclerotic substances, the most characterized of which is nitric oxide (NO), a gas generated from the metabolism of L-arginine by constitutive endothelial NO synthase[(43)].

Consequently the bioavailability of NO, leading to vasoconstriction, platelet aggregation and adhesion of neutrophils to the endothelium. Excessive ROS production can cause oxidative damage to biological macromolecules such as DNA, lipids, carbohydrates and proteins [(44,45)].

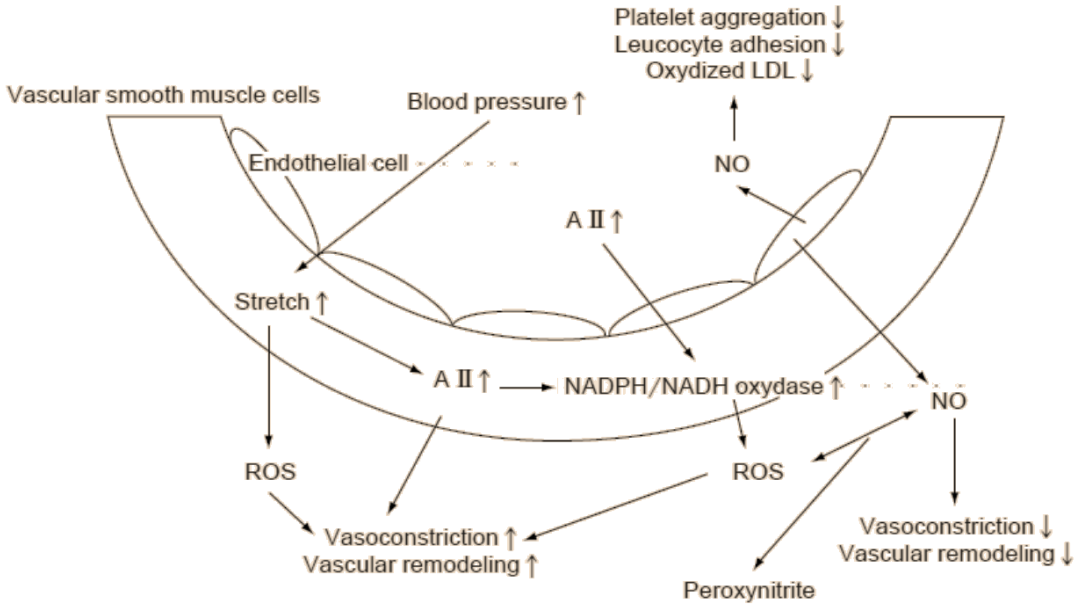


Figure (1-5) Reactive oxygen species in the arterial .(NO nitrogen oxide , AII angiotensin)[44].

1.9 Definition of Oxidative Stress Biomarkers:

A commonly used definition is which proposed in 2001 by the biomarkers definition working group by the national institute of health and food and drug administration (NIH/FDA) in the USA " a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention [46].

There are three types of biomarkers:

1) disease biomarkers-used to monitor and diagnose the progression of a disease.

2) drug efficacy/toxicity biomarkers- used to monitor the efficacy or toxicity of treatment regime

3) pharmacodynamics markers for monitoring pharmacological response [47].

1.10 Oxidative Stress Biomarker

1.10.1 Malondialdehyde (MDA)

A. Definition:

Malondialdehyde (MDA) is an end-product of the radical-initiated oxidative decomposition of polyunsaturated fatty acids; therefore, it is frequently used as a biomarker of oxidative stress [(48)]. It is highly cytotoxic because of its ability to bind proteins or nucleic acids very quickly [(49)]. Its chemical structure is below.

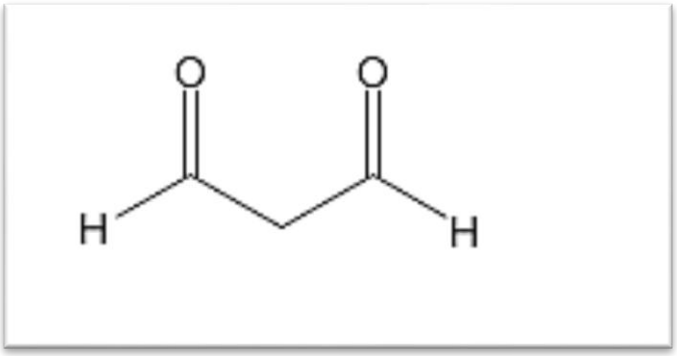


Figure (1-6): Chemical structure of Malondialdehyde [(50)]

Malondialdehyde has been shown to be produced by reaction of hydroxyl radicals with deoxyribose moieties [(51)]. High reactivity of this molecule mainly based on its electrophilicity production it strongly reactive toward nucleophiles, such as basic amino acid residues (i.e., lysine and histidine). This reactivity is not only based on MDA's aldehydic nature but is also influenced by its 1,3-dialdehydic structure making it possible to form mesomerically stabilized Schiff base[(52)]

B. Reactivity and toxicity

In its physiological state, at natural pH, MDA is of low chemical reactivity. Nevertheless, this molecule is able to interact with nucleic acid bases to form several different adducts [(50)]. This adduct possesses a blocked Watson-Crick base pairing region that has been shown to be mutagenic[(53)]. Also it is react with protein such as collagen. In addition to that the MDA

product can cause the cross-linkage of membrane elements by affecting the ion exchange from cell membranes[54] , which gives way to aftermaths including a change in ion permeability and enzyme activity[55]. Therefore MDA is able to impair several physiological mechanisms of human body through its ability to react with molecules such as DNA and proteins; therefore it's useful to consider this molecule as something more than a lipid peroxidation product [50].

Clinical data have indicated that MDA-type epitopes are prominent and prevalent and are important in cardiovascular disease, (Bartoli), (Seema L.) and (Khalid Al-Fartosi) found higher MDA is correlated with chronic cardiac disease. [56-58]

1.10.2 8-Hydroxydeoxyguanosine (8-OHdG)

A. Definition

8-Hydroxydeoxyguanosine (8-OHdG) is an oxidized nucleoside that is excreted in the bodily fluids with DNA repair. 8-OHdG is a most common stable product of oxidative DNA damage following enzymatic cleavage after ROS induced 8-hydroxylation of guanine base on mitochondrial and nuclear DNA [59]. It is can be recognized at an increase level in all bodily fluids and tissues, where an inflammatory process exists [60], and is considered a measure of DNA oxidation in response to free radicals [61].

The synthesis of (8-OHdG) is the main DNA modification induced by (ROS) and may be responsible for DNA base mutations. It has been demonstrated that oxidative DNA adducts accumulate and are only repaired through enzyme pathways, causing in further DNA damage [62].

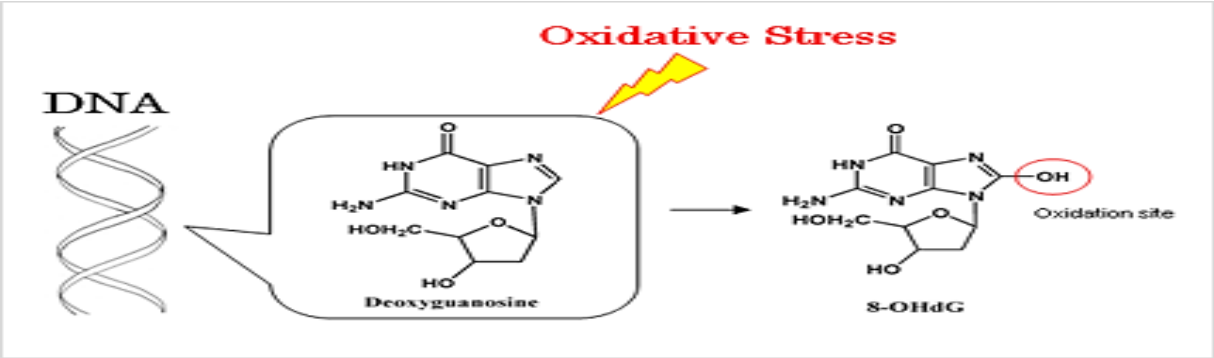


Figure (1-7): Producing 8-OHdG. [64]

B. Generation and Metabolism of 8-hydroxydeoxyguanosine

When DNA is attacked by oxidative stress such as ROS, ultraviolet light, or genotoxic agents, guanine is easily oxidized into 8-oxo-7,8-dihydroguanine(8-oxo-Gua). The existence of this oxidized guanine in genomic DNA can cause transversion mutation such as G-T or G-A binding, accumulation of which can lead to detrimental consequences [65].

8-OHdG, a nucleoside form of 8-oxoGua, is generated from either damaged oligomer which contains 8-oxo-Gua by NER or from cytoplasmic oxidized nucleotides like 8-hydroxy-deoxyguanosine triphosphate (8-hydroxy-dGTP).[66]

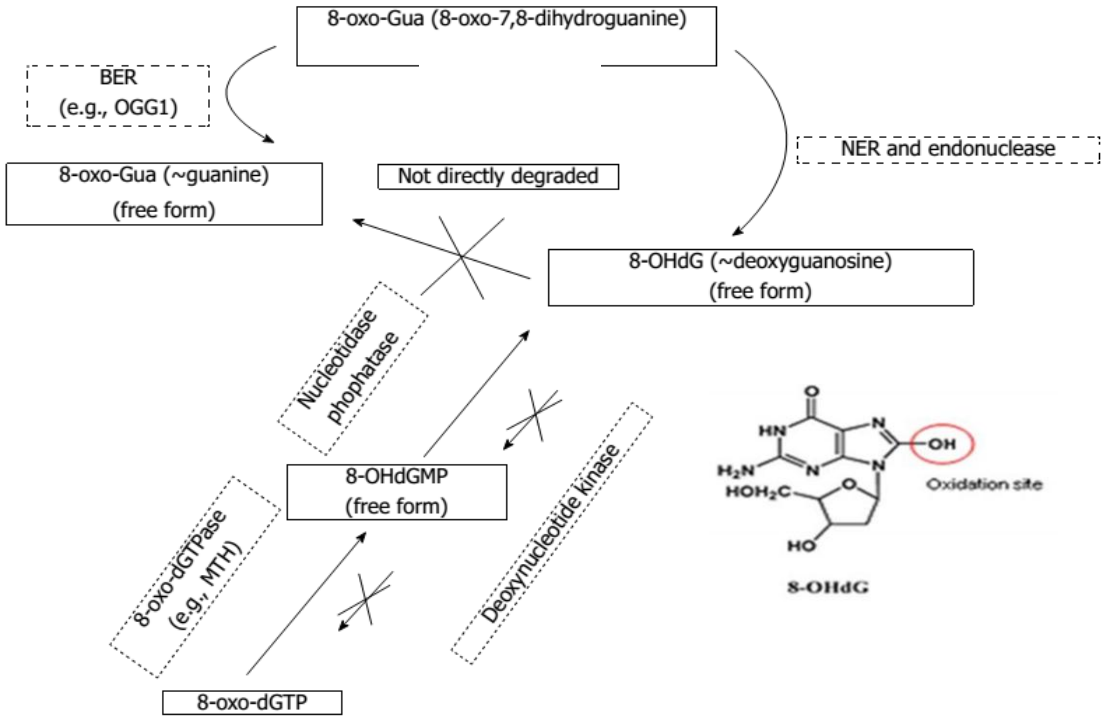


Figure (1-8) Generation and metabolism of 8-hydroxydeoxyguanosine [65].

8-OHdG has been reported to be strongly associated with diabetes mellitus, cancer, gastrointestinal diseases , 8-oxodG has also been found to be increased in atherosclerosis plaques and have been found to correlate in patients with end-stage renal disease [61, 66-70].

1.10.3 Glutathione S-Transferases mu gene:

Glutathione S-transferase (GST) is a multigene family of enzymes that detoxify reactive electrophiles, products of oxidative stress, and known or suspected carcinogenic compounds through conjugation with reduced glutathione (GSH) [71].

Glutathione-s-transferases (GSTs) are one of the most important supergene family of isoenzymes known to catalyze the detoxification of reactive electrophilic compounds, such as carcinogens, therapeutic drugs, environmental toxins, and products of oxidative stress, chiefly by conjugation

with soluble glutathione [72]. In addition, GSTs are able to modulate the induction of other enzymes and proteins which are important in cellular functions, such as DNA repair, and are therefore important in maintaining genomic integrity [73].

At present, eight distinct classes of the soluble cytoplasmic mammalian glutathione S-transferases have been known : alpha, kappa, mu, omega, pi, sigma, theta and zeta. The genes encoding the mu class of enzymes are organized in a gene cluster and are known to be highly polymorphic [73,74].

Figure(1-9): Genomic Location of the Human MU –Class Glutathione S-Transferase (www.genecard.org) [75]

Genetic polymorphisms of these GST enzymes are in the form of gene deletions, yielding no transcription or translation of the respective proteins. Decreased functionality of detoxifying and antioxidant enzymes may pose a greater risk of toxic insult potentially caused by lowered control of reactive oxygen species leading to chronic oxidative stress [(76)], the oxidative stress might be the necessary link between GSTM1 activity and arterial diseases. It is worth noting that deleted polymorphisms in the GST genes may also influence the susceptibility to coronary artery disease by modulating the detoxification of genotoxic atherogen [(73)]. Due to genetic variation, higher amounts of ROS reaching tumor cells and exaggerating cytotoxic activity may increase survival. Since radiotherapy exerts part of its antineoplastic effect via the generation of oxidative stress, the presence of this gene will prolong survival in patients who received radiotherapy [(76)].

The GSTM1 gene was studied as marker for oxidative stress, Massimo found correlation of null GSTM1 and increase exposure to ozone [(77)]. In cardiac patient null GSTM1 associated with endothelial injury and dysfunction and observed association between asthma risk and the *GSTM1* deletion [(78,79)].

1.11 Antioxidant Molecules

1.11.1 Albumin

Albumin is a non-glycosylated protein of 66 kDa ; its normal plasma concentration is between 35 and 50 g/l constituting up to 60% of total plasma proteins. Its half-life is 20 days in normal conditions [(80)]. The human serum albumin (HSA) structure consists of a single-chain polypeptide of 585 amino acid residues and approximately 67% alpha-helix and no beta-sheet . The antioxidant properties of HSA related to its structure.

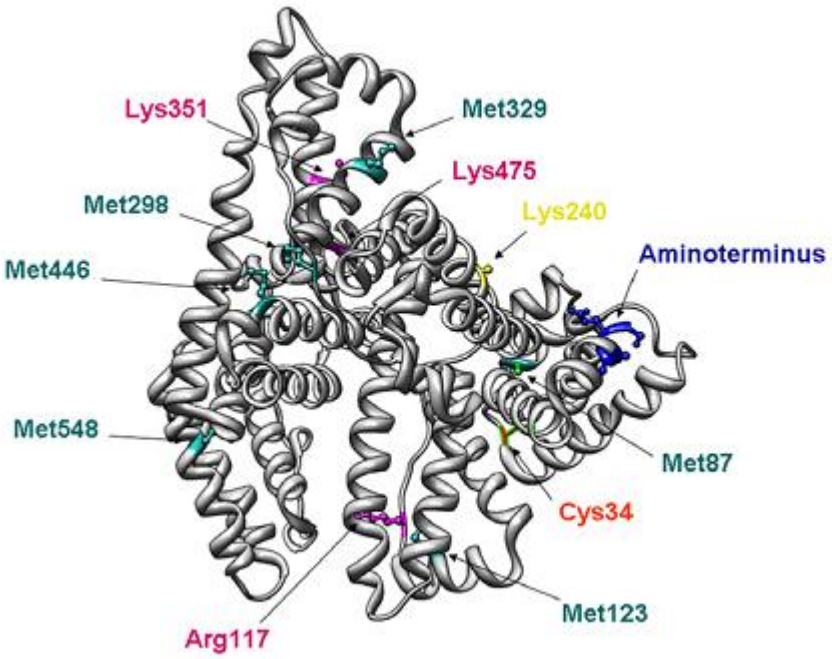


Figure (1-10) Structure of Albumin [81]

(HSA) shows antioxidant capability related to ligand-binding capacities HSA is well known for binding a large variety of molecules, including fatty acids, drugs, hormones, and metal ions [80].The main ligands of HSA implied in direct or indirect antioxidant functions of the protein are transition metal ions (copper and iron essentially) [81]. Free redox-active transition metal ions like (Cu(II) and Fe(II)) can potentially be extremely pro-oxidant. Indeed, through the Fenton reaction, they can interact with hydrogen peroxide (H_2O_2) catalyzing the production of aggressive ROS [29].

The radical-trapping properties, upon secretion in physiological conditions, one-third of the HSA pool exists as disulfides mixed with cysteine, homocysteine, or glutathione (GSH) (HSA-S-S-R) and two-thirds of the HSA molecules exist in a reduced form with a free thiol in the Cys-34 residue (HSA-SH), named as human mercaptalbumin [81,84]. This redox thiol group, in connection with the high concentration of HSA in the circulation, accounts for 80% thiols in plasma, constituting the major extracellular source of reactive free thiol [85]. Working as a free radical scavenger, the Cys34 residue is able to

trap multiple ROS and RNS, such as hydrogen peroxide (H_2O_2), peroxynitrite (ONOO-) or superoxide [81].

It also function antioxidant from its ability to bind bilirubin and homocysteine because it contains one high affinity site (Lys240) for bilirubin[86]. The resulting HAS bound bilirubin acts as an inhibitor of lipid peroxidation and thus represents an indirect antioxidant property of HAS[81,86].

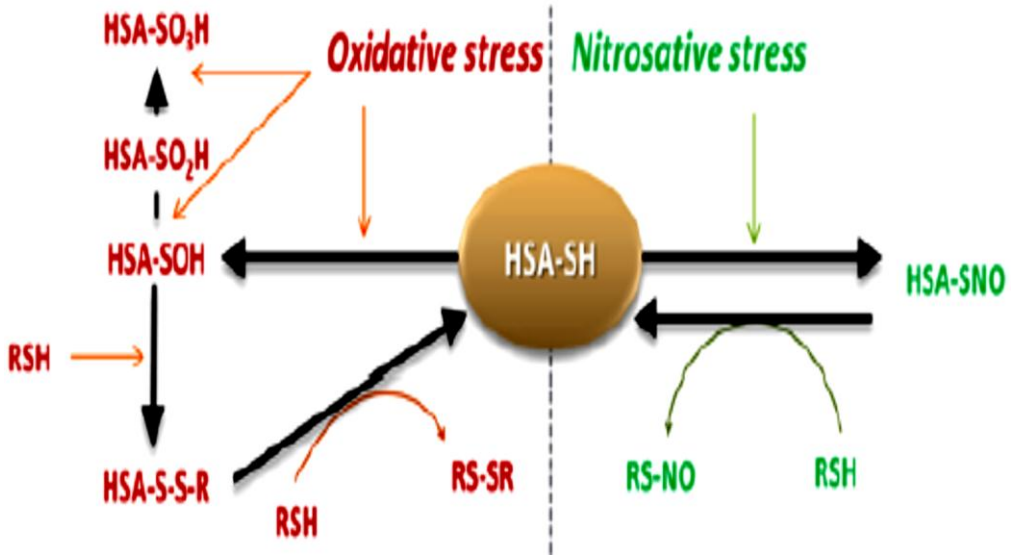


Figure (1-11) :Overview of steps leading to Cys-34 oxidation and thiolation (highlighted in red). [86]

1.11.2 Uric Acid

Uric acid (UA) is the end of purine catabolism and is excreted in urine of humans. It is a weak organic acid with a pK of 5.75, and exists mainly as monosodium urate (MSU) at physiological pH [87]. Uric acid structure is shown figure 1-12

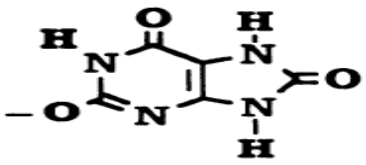


Figure (1-12) Urate anion structure[30]

Uric acid synthesis can promote oxidative stress directly through the activity of xanthine oxidoreductase. This enzyme is synthesized as xanthine dehydrogenase, which can be converted to xanthine oxidase by calcium-dependent proteolysis or modification of cysteine residues [30]. In doing so, the enzyme loses its ability to bind NADH by alterations in its catalytic site and, instead, transfers electrons from O_2, thereby generating O_2^{-}[30,87].Subsequently, uric acid was recognized to be a powerful antioxidant that scavenges singlet oxygen, oxygen radicals, and peroxynitrite and chelates transition metals. Urate thus accounts for approximately half of the antioxidant capacity of human plasma, and its antioxidant properties are as powerful as that of ascorbic acid[87]. The formation of uric acid as shown in figure (1-13) Many enzymes are involved in the conversion of the two purine nucleic acids, adenine and guanine, to uric acid [88]

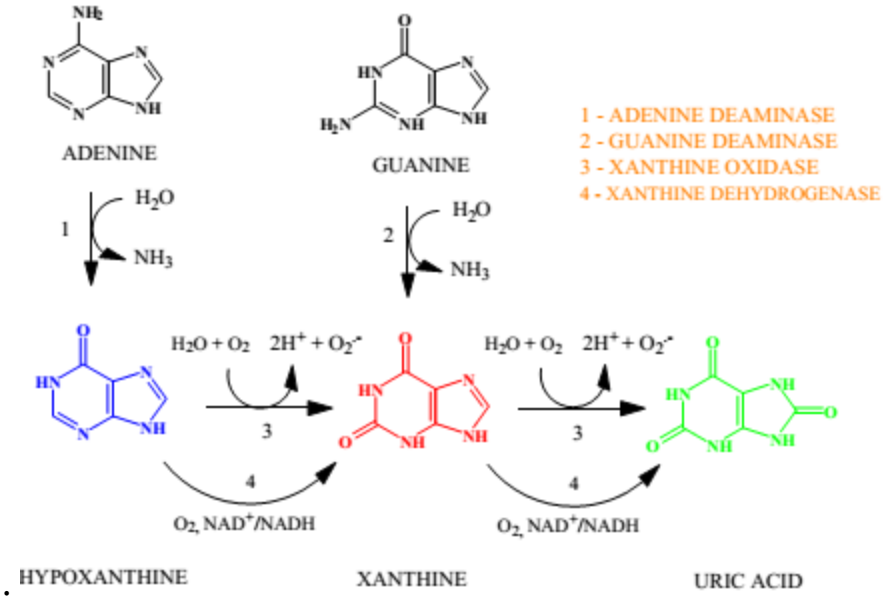


Figure (1-13) The Conversion Of Purines into Uric Acid [88]

1.11.3 Creatinine and Urea

Creatinine is the cyclic anhydride of creatine which is an end product of the dissociation of phosphocreatine [89]. The kidneys, liver and pancreas are sites of production of creatine which are enzymatically modulated. The initial reaction involves transmidation of arginine and glycine to form guanidinoacetic acid. This is then followed with methylation of acid occurs with s-anosylmethionine as the methyl donor [90]. Creatinine is then transported to other organs such as brain and muscles where it is phosphorylated to phosphocreatinine, a high-energy compound. The interconversion of phosphocreatinine to creatinine is a particular feature of the metabolic process of muscle contraction [90,91].

In human subjects, Creatinine reacts non-enzymatically with •OH to form creatol (CTL: 5-hydroxycreatinine) and demethylcreatinin (DMC) in a one to one ratio, and CTL partially decomposes to methylguanidine (MG) as shown in figure.(1-1).

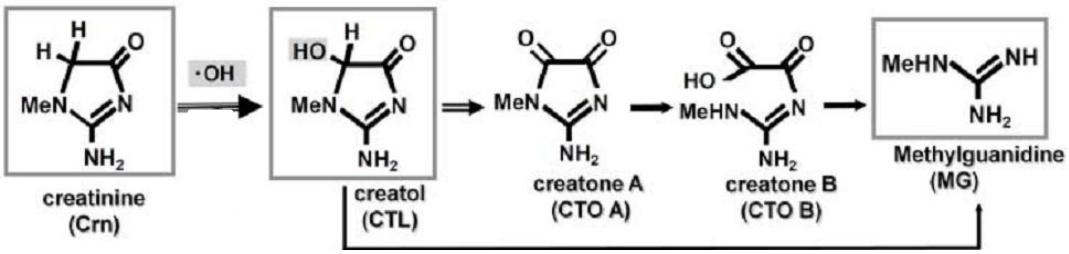


Figure. (1-14) Metabolic pathway of creatinine [91]

Urea is formed as a result of the catabolism of protein and amino acid and is usually predominantly cleared from the body by the kidneys. Synthesis from amino acid and nitrogen derived enzymes of the urea cycle [92]. Certain exogenous factors are known to affect urea concentration which includes high protein intake, increased protein catabolism, reabsorption of blood proteins dehydration and post cortisol treatment[93]. Decrease in renal blood flow results in decrease in globular filtration rate this leads to decrease in distal

tubular flow rate which leads to increase in urea reabsorption and decreased secretion which may be the reason for elevated serum urea concentration [17,93]. In available references I cannot find a path for urea as antioxidant

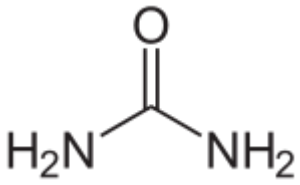


Figure (1-15) Structure of urea[94]

Aim of Study

1. Investigate oxidative stress effects using malondialdehyde and 8-OHdG as markers and albumin, uric acid, urea, creatinin as possible effective endogenous antioxidants, in serum of hypertensive and cardiovascular diseases patients.
2. Investigate possible associations among markers and parameters of oxidative stress in hypertension and cardiovascular diseases.
3. Investigate the possible effect of oxidative stress on glutathione-S- transferase m1 gene that leading to weaken of defense system of the body .

2.1 Subjects

The subject collected for this study include 56 female patients of two of atherosclerosis diseases namely hypertensive (28) and cardiovascular diseases(28) aged between 30–65 years referred to Ibn Alnafese hospital during the period from November 2013 to March 2014. The diagnosis of disease was made by physician with exclusion of presence of other diseases known to be associated with elevated oxidative stress (cancer, diabetes, arthritis, or cystic fibrosis and vitamin supplements taken in the last 4 weeks).

For comparative purpose, age matching group of 28 healthy female devoid of conditions like diabetes mellitus, epilepsy, psychiatric disorders or history of any drug intake are selected as control group.

A systematic questionnaire for the various etiological factors of relevant medical disorders (e.g. age, diabetes, blood pressure, varicocele, trauma, smoking) was administered (Appendix)

2.2 Materials

2.2.1 Chemicals and biological materials

The chemicals and biological materials used in this work are listed in table 2-1 below:

Table (2-1) : Chemicals and biological materials used in the study

Chemicals	***Suppliers***
Agarose	Promega(USA)
Albumin kit	Biosystem (France)
Cholesterol kit	Randox (United Kingdom)
Creatinine kit	Randox (United Kingdom)
DNA extraction kit	Promega (USA)

DNA ladder Marker (100bP)	Promega(USA)
Ethidium bromide	Promega(USA)
GoTaq Green Master Mix	Promega (USA)
GSTM1 Primers	Alpha DNA (Canada)
HDL – Cholesterol kit	Randox (United Kingdom)
Loading dye	Promega(USA)
Nuclease Free Water	Promega (USA)
Na-EDTA, $FeCl_2$	BDH (England)
Triglyceride kit	Randox (United Kingdom)
Thiobarbituric acid, Na_2HPO_4, NaH_2PO_4.	Fluka (Germany)
Trichloroacetic acid	Fluka (Germany)
TBE buffer	Promega(USA)
Urea kit	Biomerieux (France)
Uric Acid Kit	Spinreact (Spain)

2.2.2 Instruments

Table (2-2) Apparatus and Equipment used in this study

Name of instrument	***Company***
Centrifuge	Hettich (Germany)
Deep freeze	Froilabo (France)
Electronic sensitive Balance	DENVER (USA)
(Enzyme-Linked Immunosorbent Assay) Elisa	Asys (Australia)
Gel Documentation system	BioRad(USA)
Gel electrophoresis system	Major Science (Japan)

Incubator	BDH. (United Kingdom)
Microcenterifuge	Eppendrof (Germany)
Micropipette (Automatic)	Dragon (China)
Microwave	LG (Thailand)
Nano drop	Thermo (USA)
Refrigerator	Samsung (Thailand)
Rotisserie Shaker	Kolb (Germany) Karl
Thermocycler	BioRad (USA)
UV- visible spectrophotometer	Aple (Japan)
Water bath	JEIO TECH (Korea)
Vortex	Humatwist-Human (Germany)

2.3 Methods

2.3.1 Blood Sampling

Blood sampling was performed at 9.00 – 11.00 a.m. in the fasting state. A 10mL of venous blood was obtained. Two and half milliliter of these blood samples were added into EDTA tubes and used for DNA extraction and remained blood was allowed to clot for at least 10-15 min. at room temperature, centrifuged for (10) min. at (4000 rpm). Serum was divided into several parts by using sterilized eppindrof tubes, some for measuring the biochemical parameters and the other part was stored at -40 °C until the time of MDA and 8-OHdG assay.

2.3.2 Determination of Body Mass Index (BMI)

Height was measured to the nearest 0.5 cm and weight to the nearest 0.1 Kg. Body mass index is calculated by dividing subjects weight (Kg) by their height (m^2). BMI is calculated as:

$$BMI = \frac{\text{Mass (Kg)}}{[\text{Height (m)}]^2}$$

According to [WHO;and Fost] a BMI of (<16-18.5)indicates to underweight while a person with ~ 15-16 sever underweight and <15 is very sever underweight [95], 18.5-24.9 indicates a person of normal weight . A person with a BMI of 25 - 29.9 is overweight , while person with BMI $\geq$ 30-35 is moderately obese, $\geq$ 35-40 severity obese and $\geq$ 40 is very severity [96].

2.3.3 Determination of Hemoglobin (Hb)

A-Principle:

The Cell-Dyn 1700 System counts, sizes and classifies blood cells by impedance and focused flow methods which incorporates electrical resistance and electronic sizing principles. The Cell-Dyn 1700 system utilizes a spectrophotometric method for hemoglobin concentration, which is measured optically by absorbance at 540 nm [97]

B-Procedure:

Cell-dyn 1700 was needed approximately 30 µL from the whole blood Venous to complete the assay. The tube of the blood samples was presented under the needle and the start cycle trigger was pressed and then the results were taken from the Cell-dyn 1700.

2.3.4 Lipid Profile

2.3.4.1 Determination of Serum Cholesterol

Serum cholesterol level was measured by enzymatic end point method supplied by Giesse Diagnostic. This was determined by totally enzymatic method as supplied by Randox Company.

A-Principle:

The colorimetric measurement of cholesterol is performed using the commercially available kits for total cholesterol estimation which based on .In the presence of cholesterol esterase, the cholesterol esters in the sample are hydrolyzed to cholesterol and free fatty acid. The cholesterol produced is oxidized by cholesterol oxidase to Cholesterol-3-one and hydrogen peroxide is then detected by a chromogenic oxygen acceptor , phenol aminopyrine, in the presence of peroxidase [98].

Cholesterol ester $\xrightarrow{\text{Cholesterol esterase}}$ Cholesterol +fatty acid

Cholesterol $+O_2$ $\xrightarrow{\text{Cholesterol oxidase}}$ Cholesterol-3-one $+H_2O_2$

$2H_2O_2$ + phenol+4-aminoantipyrine $\xrightarrow{\text{Peroxidase}}$ quinonmine + $4H_2O$

The red quinonimine formed is proportional to the amount of cholesterol present in the sample

B- Solutions:

❖ **Reagent 1**

4-Aminonitropyrine (0.3 mmol/L)

Phenol (6 mmol/L)

Peroxidase (≥ 0.5 U/mL)

Cholesterol esterase (≥ 0.1 U/mL)

Pipes Buffer (80 mmol/L ; pH 6.8)

- **Standard Cholesterol** (5.1 mmol/L)

C-Procedures:

Three tubes were labeled as blank, sample and standard firstly. Ten µls of the serum were added to sample tube. followed by adding 1ml of reagent1.

In standard tube 5.1 mmol/L of standard were added, then added 1ml of reagent 1. Finally, 10 µl of ddH_2O were mixed with 1 ml of reaction reagent in blank tube. Reaction mixtures were mixed well, incubated for 5 min. at 37°C. Samples and standard were read at 500 nm against blank.

D- Calculation:

$$\text{Cholesterol (mmol/L)} = \frac{A\ \text{Sample}}{A\ \text{Standard}} \times \text{Standard Concentration(5.1 mmol/L)}$$

2.3.4.2 Determination of Serum Triglyceride (TG)

Measuring of serum triglyceride concentration was done by colorimetric method (with deprotinization) using kit provided by Randox ,laboratory Ltd-United Kingdom.

A-Principle:

The TG were determined after hydrolysis with lipase enzyme into fatty acids and glycerol. The resultant glycerol is then phosphorelated in the presence of ATP and glycerokinase to give hydrogen peroxide that react in presence of peroxidase with 4-aminoantipyrine and parachlorophenol to give colored chromophene quinoneimine as in the following equation [(99)]:

$TG + 3H_2O \xrightarrow{\text{Lipase}}$ glycerol + fatty acids

Glycerol + ATP $\xrightarrow{\text{Glycerol kinase}}$ glycerol-3-phosphate +ADP

Glycerol-3-phosphate $\xrightarrow{\text{Glycerol-3-phosphate oxidase}}$ dihydroxy acetone phosphate + H_2O_2

$2H_2O_2$ +parachlorophenol +4-amino antipyrine $\xrightarrow{\text{Peroxidase}}$ Quinoneimine $+4H_2O$

The intensity of the coloration (Quinoneimine) measured is proportional to the TG content of the sample.

B. Solutions:

- **Reagent 1** (buffer):

  Pipes (50mmol/L ; pH 7.5)

  p-chlorophenol (2mmol/L)

- **Reagent 2 (**Enzymes**):**

  Lipoprtien lipase (LPL)(150000 U/L)

  Glycerolkinase(GK) (500 U/L)

  Glycerol-3-oxidase(GPO) (2500 U/L)

  Peroxidase (POD) (440U/L)

  4-Aminophenazone(4-AP) (0.1mmol/L)

  ATP (0.1mmol/L)

- **Working reagent**: was prepared by dissolving reagent 2 reagent 1, then was capped and was mixed gently to dissolve contents .

C-Procedures:

One ml of working reagent was placed in to blank , standard, and sample tubes then 10 µl of standard was placed on the standard tube and 10 µl of sample was placed in the sample tube. Tubes were mixed and incubated for 5 mins at the room temperature. Absorbance was read for the samples and standard against the blank at 505nm.

D- Calculation:

Triglyceride (mmol/L) = $\frac{\text{A Sample}}{\text{A Standard}}$ x standard concentration (2.2 mmol/L)

2.3.4.3 Determination of Serum High-Density Lipoprotein-Cholesterol (HDL-Cholesterol)

A. Principle:

The chylomicrons and lipoproteins of VLDL and LDL contained in the sample are precipitated by the addition of phosphotungistic acid in the presence of magnesium ions.

The supernatant obtained after centrifugation contains HDL. From which the cholesterol can be determined using the cholesterol enzymatic reagent and by following the same method for total cholesterol estimation [100].

B. Solutions:

❖ **Reagent (1)**:

Phosphotungstic acid (0.55 mmol / L)

Magnesium chlorid (25 mmol /L)

pH 6.2

❖ **Cholesterol Enzymatic working solution:**

Cholesterol oxidase (300 U / L)

Peroxidase (1250 U / L)

Cholesterol esterase (300 U / L)

4 – Amino phenazone (0.4 mmol / L)

❖ **Reagent (2) standard**

Cholesterol (50 mmol / L)

C-Procedures:

In test tube 0.5 µls of the serum and 0.5 µls of reagent 1 were added and mixed well, followed by centrifugation at 3000 rpm for 10 minutes then the supernatant obtained after centrifugation contained HDL-cholesterol from which the cholesterol can be determined using the cholesterol enzymatic reagent and by following the same method for total cholesterol estimation .

D- Calculation:

$$\text{HDL- Cholesterol} = \frac{\text{A Sample}}{\text{A Standard}} \times \text{Standard Concentration}(50\ \text{mmol / L}) \times 2^{*}$$

2^{*} =Factor of dilution

2.3.4.4 Determination of Serum Low-Density Lipoprotein-Cholesterol (LDL-Cholesterol)

Low-density lipoprotein-cholesterol was estimated by using formula of friedwald [101]

Principle:

LDL-cholesterol is very difficult to isolate and measure .Hence, LDL level is most usually derived by the **Friedwalds formula** as follows

LDL- cholesterol = Total cholesterol – [HDL- cholesterol + TG/5]

2.3.4.5 Determination of Serum Very Low-Density Lipoprotein-Cholesterol (LDL- Cholesterol)

Very low-density lipoprotein- cholesterol was estimated by using formula of Friedwald [101]

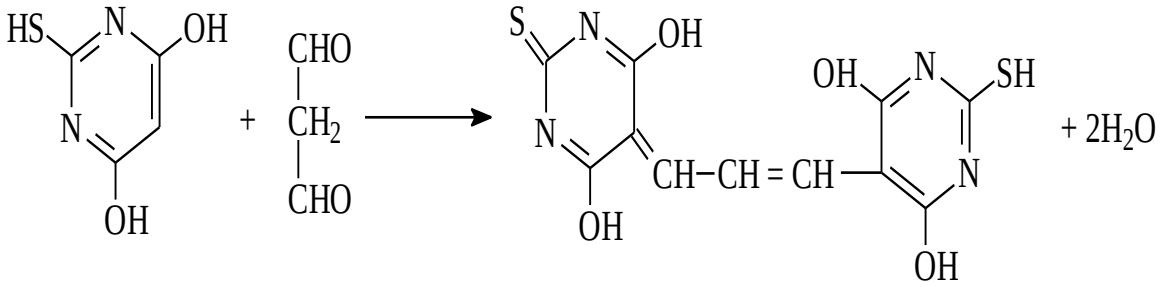
[VLDL-Cholesterol] = TG/5

2.3.5 Determination of Serum Malondialdehyde Level (MDA)

A-Principle:

Lipid peroxidation end products, particularly malondialdehyde (MDA) react with thiobarbituric acid under acidic conditions and heating to give a pink color that is measured spectrophotometrically at 532 nm [102].

The molar extinction coefficient of MDA is $1.56 \times 10^5 M^{-1} cm^{-1}$.



TBA **MDA** **Product (red color)**

B-Solution:

17.5% w/v trichloroacetic acid (TCA)

0.6% w/v 2-thiobarbituric acid (TBA)

70% w/v TCA

C-Procedure:

Sequentially 0.25 ml of serum , 0.5 ml 17.5% TCA and 0.5 ml 0.6% TBA were added to sample tube and mix well; sample tubes were boiled for 30 mins. After cooling, 1 ml of 70% TCA is added with mixing; allow standing at room temperature for 20 minutes. Clear supernatant was separated after centrifuge at 3000 rpm for 15 minutes and read at 532 nm against blank

Blank was prepared by adding 0.25 ml distilled water instead of test serum and processed similarly.

D- Calculation:

$$\text{Serum MDA}(\mu\mu\text{mol/}) = \frac{\text{Absorbance of sample}}{1.56\times10^{5}\ \text{cm}^{-1}\ \text{mol}^{-1}} \times \text{Dilution factor} \times 10^{6}$$

Dilution factor = 9.

2.3.6 Determination of Serum 8-Hydroxy-Desoxyguanosine(8-OHdG)

A-principle:

This assay employs the competitive inhibition enzyme immunoassay technique. An antibody specific to 8-OHdG has been pre-coated onto a microplate. standards or samples are added to the appropriate microtiter plate wells with HRP-conjugated 8-OHdG and incubated. A competitive inhibition reaction is launched between 8-OHdG (Standards or samples) and HRP-conjugated 8-OHdG with the pre-coated antibody specific for 8-OHdG. The more amount of 8-OHdG in samples, the less antibody bound by HRP-conjugated 8-OHdG. Then the substrate solutions are added to the wells, respectively. And the color develops in opposite to the amount of 8-OHdG in the sample. The color development is stopped and the intensity of the color is measured.

B- solution:

Standards: (2, 8, 40, 200 and 800) ng/mL

HRP-conjugate

Wash Buffer

Substrate A

Substrate B

Stop Solution

C- Procedure:

8-Hydroxy-desoxyguanosine measured in serum sampled were conducted according to manufacture protocol CUSABIO/China as the quantitative determination of endogenic human 8-OHdG concentration (Test Kit No.CSB-E10140h). 50µL of Standard or Sample was added per well then 50µl of HRP-conjugate were added to each well (not to Blank well), Mixed well and then incubated for 1 hour at 37°C.

Wells were wished with wash buffer three times. Then 50µL of Substrate A and Substrate B were added to each well, mixed well and incubated for 15 minutes at 37°C.

The reaction was stopped by added 50µL of Stop Solution. The concentration were determined by absorption at 450 nm using microplate reader.

D-Calculation:

Standard curve created by reducing the data using computer software and then by the same program calculation the concentration of samples.

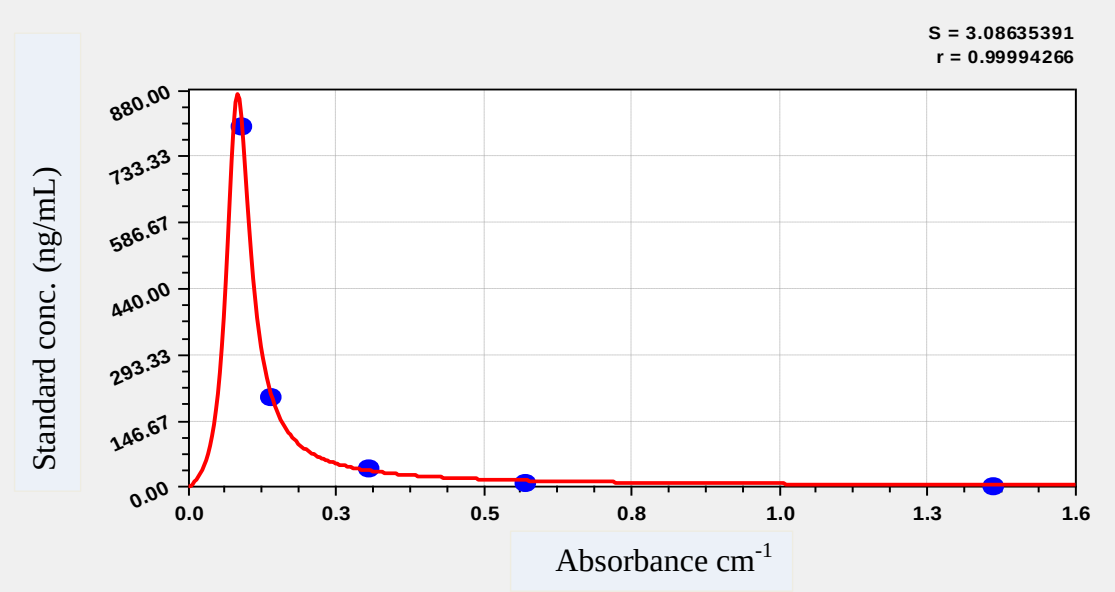


Figure (2-1) Standard curve for 8OHdG

2.3.7 Determination of Albumin

A. Principle

The method is based on the specific binding of bromocresol green (BCG), an anionic dye, and the protein at acid pH with the resulting shift in the absorption wavelength of the complex. The intensity of the color formed is proportional to the concentration of albumin in the sample[103] .

$$\text{BCG + Albumin} \xrightarrow{\text{pH 4.3}} \text{BCG-albumin complex}$$

B –Solution:

Reagent 1:

Succinate buffer (75mmol/L; pH 4.2)

bromocresol green(BCG) 0.12 mmol/L

Tensioactive 2 g/L (w/v)

Albumin standard: Bovine serum albumin (5 g/dL (50g/L)).

C- Procedure

Two tubes were labeled as blank, sample and standard firstly. 10 µL of the serum and of 2mL of BCG were added to sample tube.

In standard tube 10 µL of standard were added, , then 2ml of BCG were added, mixed and let to stand for 1 minute at room temperature. Read the absorbance (A) of the samples and the standard at 630 nm against the reagent blank(BCG).

2.3.8 Determination of Serum Uric Acid

Serum uric acid level was measured by enzymatic method supplied by Giesse Diagnostics[104] .

A-Principle:

Uric acid is oxidized by uricase to allantoin and hydrogen peroxide.The released hydrogen peroxide together with 3-hydroxy-2,4,6-triiodobenzoic acid and 4-amino antipyrine ,in the presence of peroxidase ,form a red dye compound. The intensity of the red color produced is directly proportional to the uric acid quantity in serum.

$$\text{Uric acid} + O_2 + H_2O \xrightarrow{\text{Uricase}} H_20_2 + \text{allantoin}$$

$$2H_2O_2 + [\text{3-hydroxy-2,4,6-triiodobenzoic acid}] + \text{4-amino- antipyrine} \xrightarrow{\text{Peroxidase}} H_2O + \text{quinonimine}$$

B-Solution:

- **Reagent 1** (Buffer solution)

phosphate buffer (50 mmol/L; pH 7.4)

2-4dichlorophenol sulfonate (DCPS) (4 mmol/L)

- **Reagent 2** (vial of enzymes)

Uricase (70 U/L)

Peroxidase(660 U/L)

- **Reagent 3**

Standard Uric acid (60 mg/L)357umol/L

- **Working reagent** was prepared by dissolved reagent 2 in reagent 1 .

C- Procedure:

Two tubes were labeled as sample and standard . 25 µLs of the serum and standard were added to labeled tubes respectively then 1ml of working reagent was added.

All tubes were mixed well and incubated for 5 minute at 37 ^{o}C . Sample and standard were read at 510 nm against blank(Working reagent).

2.3.9 Determination of Serum Urea

Blood urea level was measured by enzymatic colorimetric (Berthelot modified method) method supplied by Biomerieux company.

A-Principle

Urease catalyses the conversion of urea to ammonia. In a modified Berthelot reaction ,the ammonium ions react with a mixture of salicylate, hypochlorite and nitroprusside to yield a blue-green (Indophenol).The absorbance of this dye is proportional to the concentration of urea in the sample: [(105)].

Urea $+H_2O \xrightarrow{\text{Urease}} 2NH_3+CO_2$

NH_3 +salicylate +hypochloritic $\xrightarrow{\text{Nitroprusside}}$ 2,2-dicarboxy indophenol

B- Solution:

- **Reagent (1)** Standared Urea 8.3 mg/dL
- **Reagent (2)** (Enzyme) Urease 350 KU/L
- **Reagent (3)** Color reagent:

Phosphate buffer (50 mmol/L ; pH 8)

Sodium Salisylate (62 mmol/L)

Sodium Nitroprusside (3.35 mmol/L)

EDTA(1 mmol/L)

- **Reagent (4)**

Alkaline reagent

Sodium Hydroxide NaOH (0.5 mmol/L)

Sodium Hypocloride NaClO (24.8 mmol)

C-Procedures:

Three tubes were labeled as blank, sample and standard firstly. 10 µLs of the serum were added to sample tube. Followed by adding 1mL of reagent 1.

In standard tube 10 µLs of standard were added, then diluted with 1mL of reagent 1.

In blank tube 1ml of reagent 1 was added. All tubes were mix well and incubated for 5 minute at 37 ^{o}C. Finally, 1mL of reagent 4 were added to all tubes, mixed well and incubated for 5 minute at 37 ^{o}C .Sample and standard were read at 590 nm against blank.

D-Calculation:

$$\text{Urea (mmol/L)} = \frac{\text{A Sample}}{\text{A Standard}} \times \text{Standard Concentration(8.3 mg/dL)}$$

2.3.10 Determination of Serum Creatinine

A-Principle

Creatinine in alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the creatinine concentration [106].

B- Solution:

- **Reagent1:** Picric acid 25 mmol/L
- **Reagent 2:** Alkaline buffer
- **Phosphate buffer *(300* mmol/L ; pH 12.7)**
- **SDS (2.0 g/L (w/v))**

Creatinine standard 177µmol/L (2 mg/dL)

Working reagent: Mix 1 volume of reagent1 and 1 volume of reagent 2.

C-Procedures:

Two tubes were labeled as sample and standard. 100 µls of the serum , standard were added to labeled tubes. followed by adding 1ml of reagent 1 to each tube.

In blank tube 1ml of reagent 1 mixture was added. Then mix well and the absorbance A_1 was read after 30 seconds of the standard and sample. Exactly 2 minutes later, absorbance A_2 of standard and sample was read all against blank.

D-Calculation:

$\Delta A_{sample} = A_2 - A_1$

Creatinine (mmol/L) = (Δ A sample / Δ A standard) * standard concentration(2.03 mmol/L)

2.3.11 Molecular Biological Studies:

2.3.11.1 DNA Extraction

Principal:

The ReliaPrep™ gDNA Miniprep System provides a fast, simple technique for preparation of purified and intact DNA from mammalian. Samples are processed using a binding column in a micro centrifuge tube. The genomic DNA isolated is of high-quality and can be used in common applications such as Agarose gel analysis, restriction enzyme digestion and PCR analysis [(107)]

- **Procedure of DNA Extraction from Blood**

DNA Extraction from blood samples were conducted according to manufacture protocol (Promega).200 µl of blood were added to tube containing the Protenase K solution and mixed.Blood cells were lysised by adding cell Lysis Buffer (CLD)to mixture and vorted properly. After incubation at 56°C for 10 minutes.

The mixture was then adjusted to high salt concentration binding Buffer (BBA) and applied to silica-based column. Column were wished with wash solution by

centrifugation and then DNA eluted from the column with Nuclease-Free water. The DNA concentration war determined by absorption at 260 nm using Nanodrope.

- **Procedure of DNA Extraction from Placenta**

Fresh tissue placenta specimens were fixed by normal saline for 2 weeks then washed several times with phosphate buffer saline pH=7.5, tissues were crushed and homogenized in the same solution and the DNA was extracted according to manufacture protocol (Promega)[108]

2.3.11.2 Fenton Reaction in Vitro on Human DNA on placental and healthy female DNA samples

Reagent:

$(NH_4)_2Fe(SO_4)_2.6H_2O$
EDTA
H_2O_2 (0.3%)
sodium ascorbate
sodium acetate
thiourea
phosphate buffer

The Fe II EDTA solution was prepared by mixing equal volumes of 0.2 mM $(NH_4)_2Fe(SO_4)_2.6H_2O$ and 0.4 mM EDTA.

Procedure:

In eppendorf tube added 10 µl of each Fe^{+2}EDTA solution, DNA(80 ng/ mL) , phosphate buffer , H_2O_2 (0.3%) and sodium ascorbate and premixed on the inside wall of an Eppendorf tube and then immediately added to the DNA solution and allowed to react for 2 min**.** Reactions were stopped by addition of 100µ L of a stop solution containing 10 mM thiourea, 30 mM EDTA, and 0.6 mM sodium acetate.

These conditions were determined to result in no more than one strand break per DNA molecule [109].

2.3.11.3 GSTM1 Deletions Analysis by PCR

Principal:

The GSTM1 deletion detection system consists of two primer pair using in three PCR reactions which provide a simple and rapid method for the detection of specific region that deleted in GSTM1 locus. This system is designed to detect famous and candidate deletion occurring in GSTM1 region. The primers have been combined into two reactions PCR. This makes it possible to determine the presence or absence of GSTM1, null allele and microdeletions in GSTM1 gene performing two independent PCR amplifications.

This Part of research has been done in Biotechnology department / Molecular Biology Lab/ University of Al-Nahrain

Primers

PCR reaction was performed using 4 deletion-specific primers for GSTM1 which are (GSTM1 del. Up-F, GSTM1 del. Up-R, GSTM1 del. Do-F and GSTM1 del. Do-R). These primers were designed from non-coding sequence flanking the gene.

Lyophilized primers were dissolved in a nuclease free water to give a primary concentration of (100 µM) (as stock solution). For working solution, 10µl of stock was dilute with 90µl of nuclease free water to get (10 µM) as a final concentration. The sequences of these primers were explained in (Table: 2-3):

Table2-3: Primer Sequence used for GSTM1 deletion detections.

Primer	**Size**	**Forward**
GSTM1 del. Up-F	**200 bp**	CGTTAGGATCTGGCTGGTGT
GSTM1 del. Up-R		GGGGCTGCACTCAGTAAGAC
GSTM1 del. Do-F	**179 bp**	CCTGGATGTCCCATTCATTC
GSTM1 del. Do-R		AGATTGGGTCCTGGAGACCT

❖ **Screening Procedure**

According to the (Masood and Kayani)[110], the PCR conditions and preparing the deletion specific primers in three set was accomplished; thus, the following sets were adopted.

❖ **Procedure of Sets A and B**

In eppindrof tubes added 12.5 µL from Green Taq Master mixture, 1µL GSTM1 del. Up-F ,1 µL GSTM1 del. Up-R , 8.5 µL D.W and 2 µL DNA sample.

1. **Set A**: In this set 2 primers were used (GSTM1 del. Up-F and GSTM1 del. Up-R). This set was corresponded to an upstream promoter region.

Table 2-4: PCR program for mixture A.

No.	Steps	Temperature	Time	No. Of cycles
1	Denaturation 1	95C°	5min	1 cycle
2	Denaturation 2	94C°	30sec	35cycles
	Annealing	57C°	30sec	
	Extension 1	72C°	30sec	
3	Extension 2	72C°	7min	1 cycle
4	Holding	4 C°	-	1 cycle

Set B: Two primers were used (GSTM1 del. Do-F and GSTM1 del. Do-R). This set was corresponded to a downstream non-coding region.

Table 2-5: PCR program for mixture B.

No.	Steps	Temperature	Time	No. Of cycles
1	Denaturation 1	95C°	5min	1 cycle
2	Denaturation 2	94C°	30sec	35cycles
	Annealing	55C°	30sec	
	Extension 1	72C°	30sec	
3	Extension 2	72C°	7min	1 cycle
4	Holding	4 C°	-	1 cycle

2.3.11.4 Agarose Gel Electrophoresis

After DNA extraction and PCR amplification, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA or amplification. PCR was completely dependable on the extracted DNA criteria[(111)]

❖ **Solutions**

1 X TBE buffer.

Loading dye.

DNA ladder marker.

Ethidium bromide (10mg / mL)

Procedure :

1- **Preparation of Agarose Gel**:

The gel with a final concentration of 1% of agarose was prepared by dissolved 1 g of agarose in 100 mL 1X TBE then heated until all gel particles were dissolved .then added 1µL of Ethidium Bromide and stirred to get mixed and then allowed to cool down at 20-25 ^{0}C.

2- Casting of the Horizontal Agarose Gel:

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to gel at room temperature for 30 minutes. The tray was filled with 1X TBE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

3- DNA Loading

Two µL of loading buffer was applied to each 5µl DNA sample, and samples were added carefully to the individual wells. PCR products were loaded directly. Electrical power was turned on at 7v/cm for 2 h. DNA moves from Cathode to plus Anode poles. The Ethidium bromide stained bands in gel were visualized using Gel imaging system.

2.4 Statistical Analysis

All statistical analysis in studies were performed using SPSS [112].Descriptive analysis was used to show the mean and standard deviation of variables. The significance of difference between mean values was estimated by Student T-Test. The probability $p \leq 0.05$ = significant, $p > 0.05$ = non-significant. Correlation analysis was used to test the linear relationship between parameters.

3.1 Patient Descriptive Data:

3.1.1 Distribution of Groups :

The present study included 84 subjects divided into three groups; two of atherosclerosis volunteers patients, first one is the cardiovascular volunteers patients (28 subjects), the second one is the hypertensive volunteers patients (28 subjects), and 28 apparently healthy individuals as control group, all shown in figure (3-1) below with their percentage

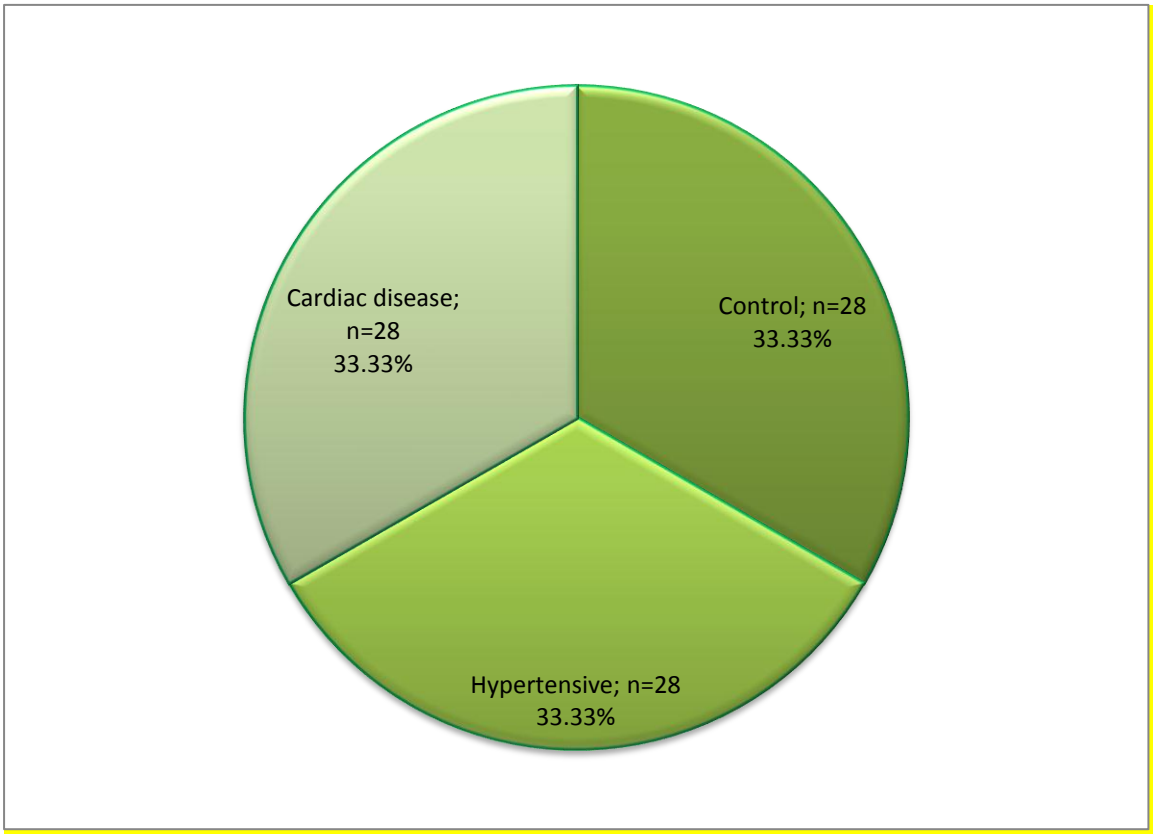


Figure (3-1): Distribution of studying group.

3.1.2 Clinical Feature of groups:

The clinical feature of the group in the underlying study (shown in table 3-1) which include; ages , body mass index, systolic and diastolic blood pressure, hemoglobin and family history of diseases.

Table (3-1): Clinical Feature of Studying Group (mean ± SD):

Characteristic	Cardiovascular group n=28	Control group n=28	Hypertension group n=28
Age (year)	52.3±9.3	43.4±9.3	46.8±8.2
BMI (Kg/ m^2)	32.59 ± 1.24*	27.27 ±0.97	31.48 ± 0.95*
Systolic blood pressure(mmHg)	136.94±5.46	119.5±3.96	151.62±17.9*
Diastolic blood pressure (mmHg)	86.96±9.98	81.59±8.01	94.18±12.16
Hb g/dL	13.15±1.18	12.00±1.36	12.5±1.6
Menopausal no. (%)	17 (60.72%) *	10(35.71%)	16 (57.14%) *
Menstruating no. (%)	11(39.28%)*	18(64.29%)	12(42.86%)*
Family history of disease no (%)	14(50%)	12(32.14%) (For both diseases)	12(42.85%)

* significance difference relative to control group with $p< 0.05$.

The significant difference in BMI between CVD patients and control group may be interpret the incident with that disease ($p<0.05$). This result was agree with (Ngoungou B. *et al*) [113] who recorded this relation as a risk factor for cardiovascular incident. For coronary heart disease and mortality the risk increases with increasing body mass indices BMIs >25 kg m^{-2} [114], obesity increases total blood volume and cardiac output. Also cardiac workload is greater in obesity. Typically, obese patients have a higher cardiac output but a lower level of total peripheral resistance at any given level of arterial pressure. Most of the increase in cardiac output with obesity is caused by stroke volume[114].

The significant difference in BMI ($p<0.05$) between HT and control group reveal the positive correlation between obesity and disease, this finding was agree with (Ahmed A. *et al*) and (Shugar L *et al)* they found a significant increase in BMI in hypertensive patients [39,115]. Obese patients are more able to be hypertensive than lean patients, and weight gain is typically associated with increases in arterial pressure [116].

3.1.2 Groups According to Menopausal State :

From distributive data (table 3-1) there was a significant difference ($p<0.05$) in menopausal and menstruating subjects. Further, inside the groups (as shown in table 3-2), of cardiovascular group patients ; percent of menstruating subjects was 11 (39.28%) which is differ significantly ($p<0.05$) from menopausal subjects which is 17 (60.72%). Inside the hypertensive group subjects the menstruating was 12(42.86%) and the menopausal subjects 16 (57.14%) .These results was agreed with several studies like (Roberta L *et al)*, (Zanchetti *et al)* , (Megan *et al)*, and (Masoumeh *et al)* . But disagree with (Luoto *et al)*.[117-121].

Table (3-2): Groups According to Menopausal State:

Group	No. (%)		P-value
	menopausal	menstruating	
Cardiovascular patients	17 (60.72%)	11(%39.28)	0.0036**
Hyper tension	16 (57.14%)	12(%42.86)	0.0479*

* significant difference with $p<0.05$, **highly significant with $p<0.01$

This difference in percent in menopausal with respect to menstruating between both patients group may be due to increasing body iron stores that increased in menopausal women. This high iron status has been associated with an increased risk of age-related diseases, such as cardiovascular and hypertension diseases. Like other transition metals, iron is thought to contribute to the development of oxidative stress by catalyzing reactions that produce oxidants [(122)].

3.2 Lipid profile levels:

Lipid profile of the patients and control groups are summarized in table (3-3) and figure (3-2). A significant increase has been shown ($p <0.01$) in serum cholesterol, triglycerides, LDL-cholesterol and VLDL cholesterol in cardiovascular patients group compared to control group, while HDL-cholesterol shows a significant decrease ($p<0.01$) in patients group compered to control group. LDL/HDL ratio (atherogenic ratio) showed highly significant increase in patients group compared to control group.

Table (3-3): Values of Lipid Profile Levels in the Cardiovascular Diseases and Control Group (Mean ± SD)

Characteristic	Cardiovascular patient n=28	Control group n =28	Comparison of Significant p value
Cholesterol (mmol/L)	4.62 ± 0.25	4.23 ± 0.24	<0.01**
Triglyceride (mmol/L)	2.209 ± 0.17	1.31 ± 0.09	<0.01**
HDL-**cholesterol** (mmol/L)	1.15 ± 0.06	1.42 ± 0.09	<0.01**
LDL-**cholesterol** (mmol/L)	3.86 ± 0.29	3.53 ± 0.17	<0.05*
VLDL-**cholesterol** (mmol/L)	0.433 ± 0.03	0.284 ±0.02	<0.01**
LDL/HDL(mmol/L)	5.49 ± 0.94	2.83 ± 0.24	0.01**

* significant with p<0.05, **highly significant with p<0.01

There were also a significant difference in lipid profile of hypertensive patients compared with control group as shown in table (3-4).

Table (3-4): Values of the Lipid Profile in Hypertension and Control Group (mean ± SD)

Characteristic	HT patient group n=28	Control group n=28	P value
Cholesterol (mmol/L)	5.2 ± 0.9	4.23 ± 0.24	<0.01**
Tri glyceride (mmol/L)	2.4 ±1.03	1.31 ± 0.09	<0.05*
HDL (mmol/L)	1.1 ±0.4	1.42 ± 0.09	<0.01**
LDL (mmol/L)	4.13±1.2	3.53 ± 0.17	<0.01**
VLDL-**cholesterol** (mmol/L)	0.502 ± 0.03	0.284 ±0.02	<0.01**
LDL/HDL mmol/L	4.3±1.2	2.83 ± 0.24	<0.05*

* significant with p<0.05, **highly significant with p<0.01

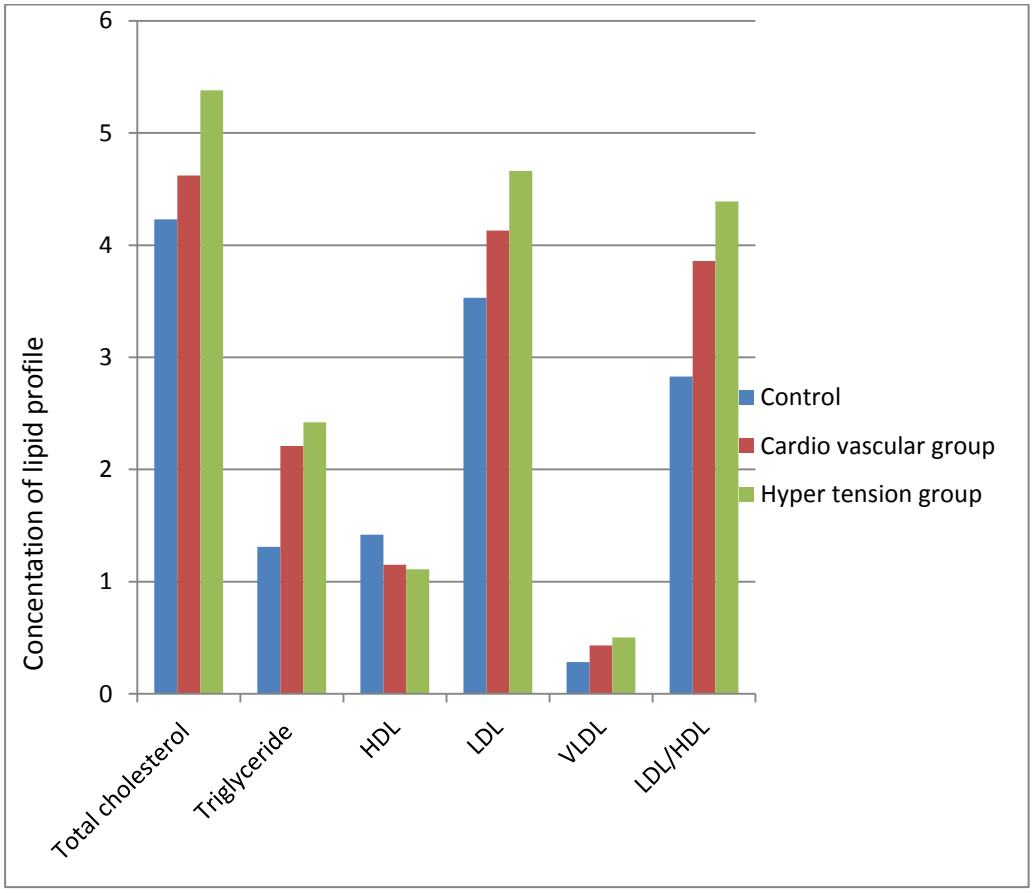
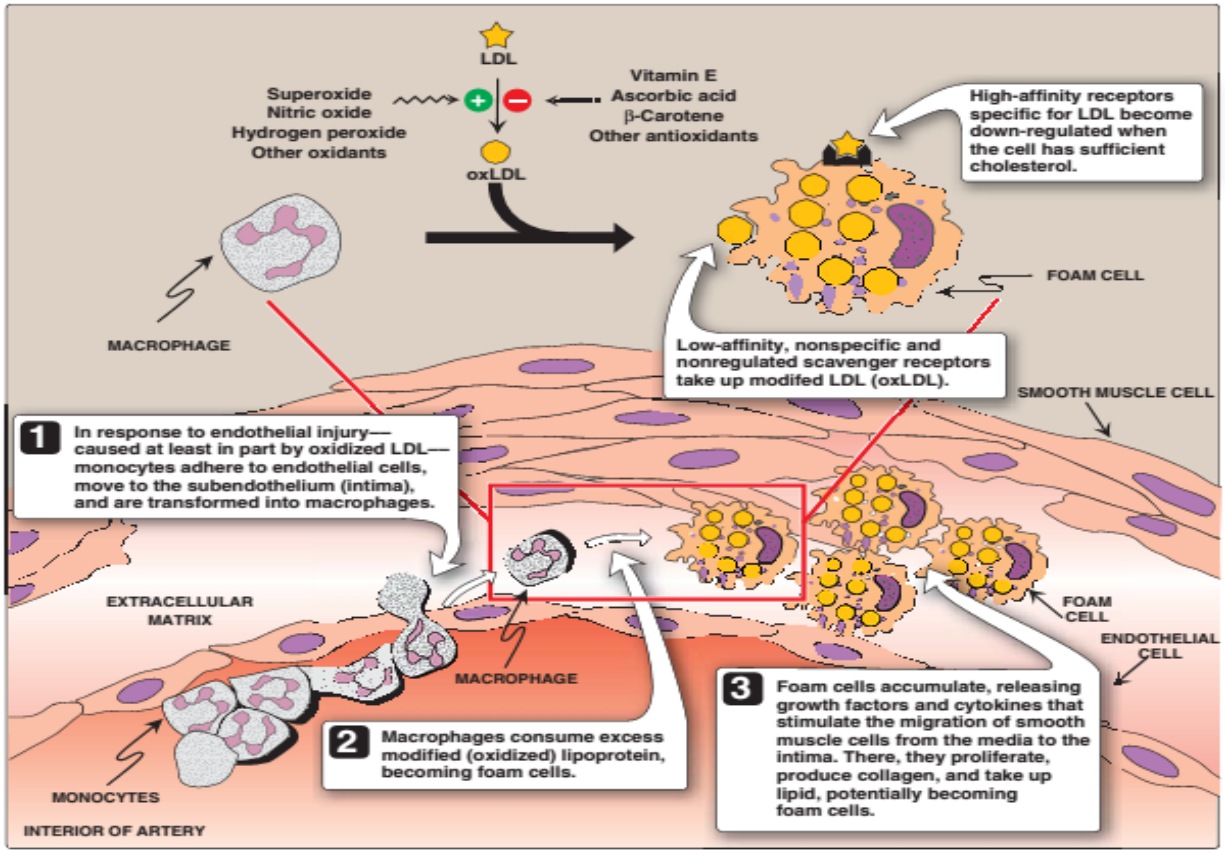


Figure (3-2): Values of lipid profile between groups

The significant increase in cholesterol in both groups ($p<0.05$) was agreed with (Mohsen M. *et al.*)[123] which may be due to that cholesterol plaque causes the muscle cells to enlarge and form a hard cover over the affected area. This hard cover is what causes a narrowing of the artery, blood flow will be reduced and increasing blood pressure. Plaques are covered with a fibrous cap and it creates a bump on the artery wall, as the process of atherosclerosis continues, the bump gets bigger. A big enough bump can create a blockage[2,4].

The significant increase in LDL ($p<0.01$) in cardiovascular patients is agrees with study of (Jasim M.) [23] which is may be due to that the LDL-cholesterol invasion, crosses damaged endothelium.

The cholesterol enters into the area of the damaged vessel and deposit there[124] Inflammatory cells, like macrophages, will also enter the damaged area, causing inflammation and engulfing the lipids. Once inside the vessel wall, LDL-cholesterol particles get stuck and their content becomes more prone to oxidation. The damage caused by the oxidized LDL-cholesterol molecules triggers a cascade of immune responses which over time can produce an atheroma. First, the immune system sends specialized white blood cells (macrophages and T-lymphocytes) to absorb the oxidized LDL-cholesterol, forming specialized foam cells. As a result, the macrophages will often have a foamy appearance. These white blood cells are not able to process the oxidized LDL-cholesterol [125]. The LDL lipoprotein increase atherogenicity and available data suggest that LDL is more atherogenic. The figure 3-3 below explains this effect.



Figure(3-3): Role of oxidized lipoproteins in plaque formation in arterial wall[93]

Decreased HDL-C in patients indicates decreased rate of reverse cholesterol transport and therefore accumulation of TG rich lipoproteins is associated with hypertension and CVD. This results was agreed with (Mohsen M. *et al*) [123] and (Khan *et al)* [126] .

The ratio of LDL/HDL cholesterol appears to be as useful as the total cholesterol/HDL cholesterol ratio. Their similarity can be explained by the fact that approximately two thirds of plasma cholesterol are found in LDL and, consequently, total and LDL cholesterol are closely related [125]. Our results show high significant difference in both diseased under study with respect to control and this agree with (Milan J *et al)* [127] who found the LDL/HDL cholesterol ratio was a powerful predictor of cardiovascular risk.

Epidemiologic data also suggest that hypercholesterolemia and perhaps coronary atherosclerosis itself are risk factors for ischemic stroke. Increasing

evidence also points to increased levels of plasma triglycerides, LDL-cholesterol and a decreased concentration of HDL- cholesterol as an important risk factor for peripheral vascular disease, stroke, and CAD [123]

3.3 Oxidative Stress Markers and Parameters

3.3.1 Malondildehyde (MDA) levels :

The mean levels of serum MDA showed a significant increase ($p < 0.01$) in cardiovascular patients group (5.49 ± 0.94) μmol/L and hypertensive group (6.35 ± 0.71) μmol/L when compared to control group (3.31 ± 0.26) μmol/L as shown in table (3-5) and figure (3-4).

Table (3-5): Values of MDA Between Groups (mean ± SD):

Characteristic	MDA μmol/L	Control group n=28	*p* value
Cardiovascular patient n=28	5.49 ± 0.94	3.31 ± 0.26	<0.01
Hypertenson group n=28	6.35 ± 0.71		<0.01

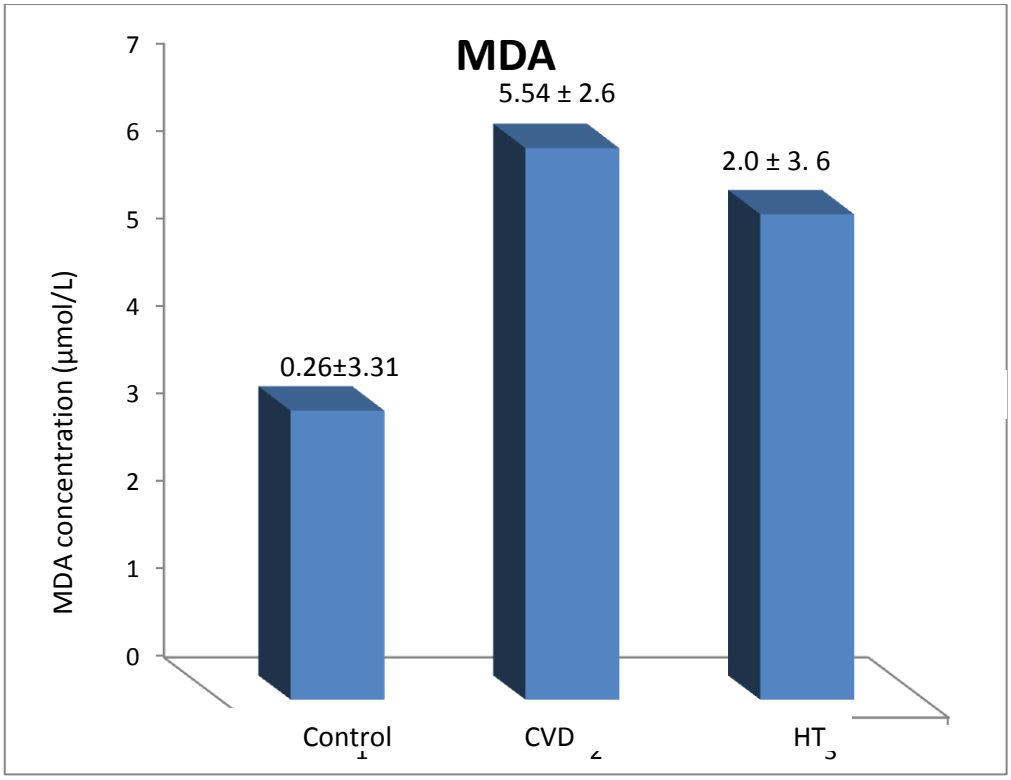


Figure (3-4): Mean Conc. (µ mol/L) of Oxidative Marker MDA for Studied Groups.

Our findings in cardiovascular patient is also found by (Jawalekar *et al*)[129] , and (Chopra *et al*)[130].

Also the significantly elevated ($p<0.01$) in diagnosed hypertensive patient and this agree with (Dhananjay)[131].

In addition, it has been demonstrated that increased intracellular generation of ROS plays an important role in chronic inflammatory responses to arterial diseases , so this causes damage to the membrane polyunsaturated fatty acids leading to the generation of MDA cause elevation in MDA in these patients[128].

A positive correlation observed between MDA and LDL- cholesterol in both diseases (as shown in figures(3-5, 3-6). this result also shown by (Venkata Rao *et al*)and (Ogunro P. *et al*) [132,133]. There was no correlation between BMI and MDA and this agree with (Ali P. *et al*) [134].

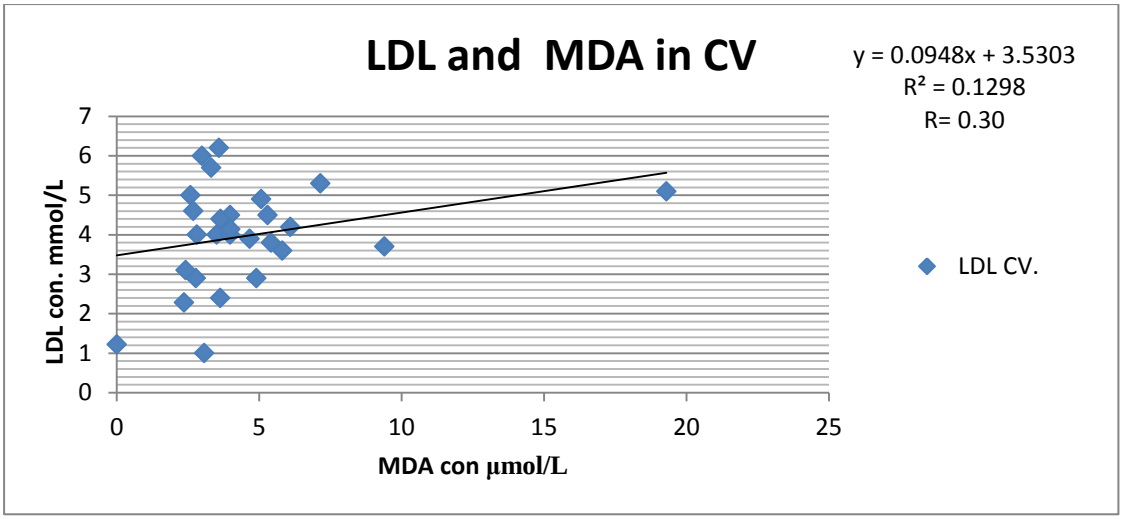


Figure (3-5): Correlation of MDA with LDL in CVD Patients.

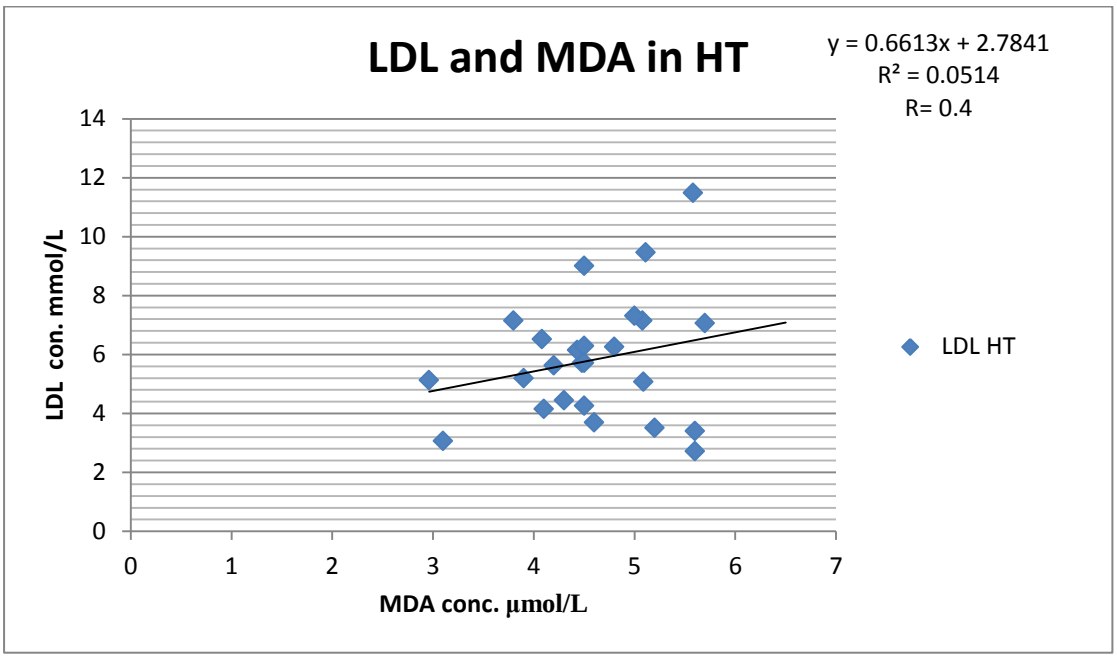


Figure (3-6): Correlation of MDA with LDL in Hypertensive Patients.

Lipid peroxidation produces reactive aldehydes such as MDA and MDA acetaldehyde that form immunogenic adducts on for example LDL-cholesterol particles. Hypercholesterolemia and hypertriglyceridemia are independent risk factors that alone or together can accelerate the development of atherosclerosis and progression of atherosclerotic lesions. One of the initial events in the development of arterial diseases is the accumulation of cells containing excess

lipids within the arterial wall so increase the probability for oxidation of more lipids and produced as MDA [52,53].

3.3.2 8-Hydroxydeoxyguanosine Levels:

The (8-OHdG) is the main DNA modification formed by (ROS). The (mean ±SD) of 8-OHdG for cardiovascular patients (104.67±24.94 ng/ml), and for hypertension patients was (105.43± 28.94 ng/ml) with respect to control group which is (82.62±19.13 ng/ml) as revealed by table (3-6) and figure (3-7)

Table 3-6: Values of 8-OHdG in Patients and Control Group (mean ±SD):

Patient group	8-OHdG [ng/ml]	Control group	*P* value
Cardiovascular group	104.67±24.94	82.62±19.13	<0.01
Hypertension	105.43±28.94		<0.01

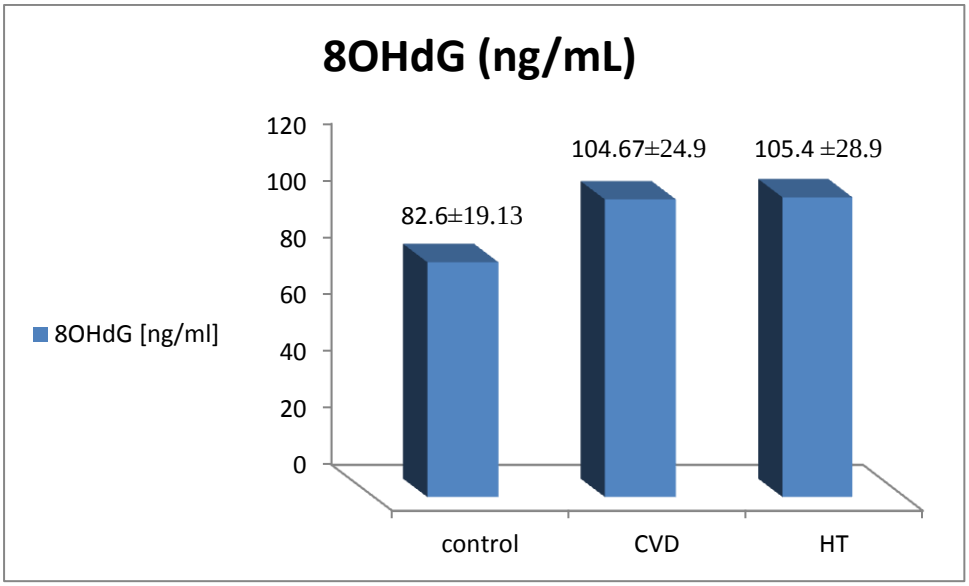


Figure (3-7): 8-OHdG Levels in Patients and Control Group

Our results show a significant difference between cardiovascular patient and control group ($p<0.01$). these results was agreed with Kaya y *et al*[64] who found a significant difference in 8-OHdG levels in hypertensive patient. Also (Fructaci *et al*) [135] found an elevation in 8-OHdG in cocaine related cardiomyopathy cases.

DNA damage has been related with the development of cardiovascular pathologies in the general population, which is supported for the monoclonal origin of cells from human atherosclerotic plaques [64].

The significantly increased in 8-OHdG in hypertensive subjects as compared to the control group which may be due to the increased generation of ROS in certain type of white blood cells which contribute in reduction bioavailability of nitric oxide and thus to the endothelial dysfunction, as some of the hypertension-induced organ damage, which occur due to hyperactivity of mechanisms that increase ROS production.

There was a positive correlation between age and 8-OHdG with R= 0.3, this result agree with other studies that found positive correlation in other disease, (Akihiko *et al),* (Al Wassiti E. *et al)* and (Teruaki)[135-138].

There was a significant correlation between 8OHdG and MDA in the study groups which related directly, as shown in figures (3-8) (3-9) and (3-10).

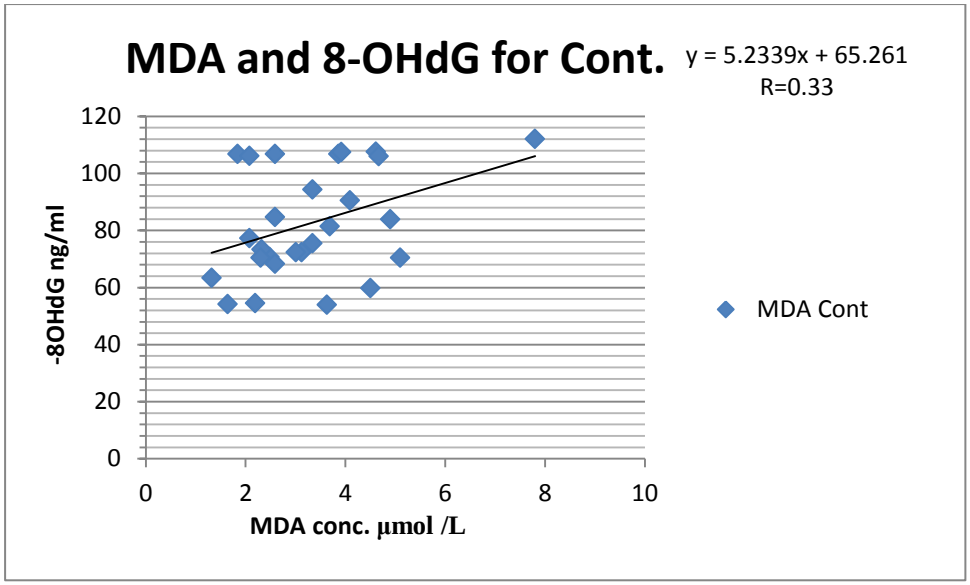


Figure (3-8): Correlation Directly Between MDA and 8-OHdG for Control group.

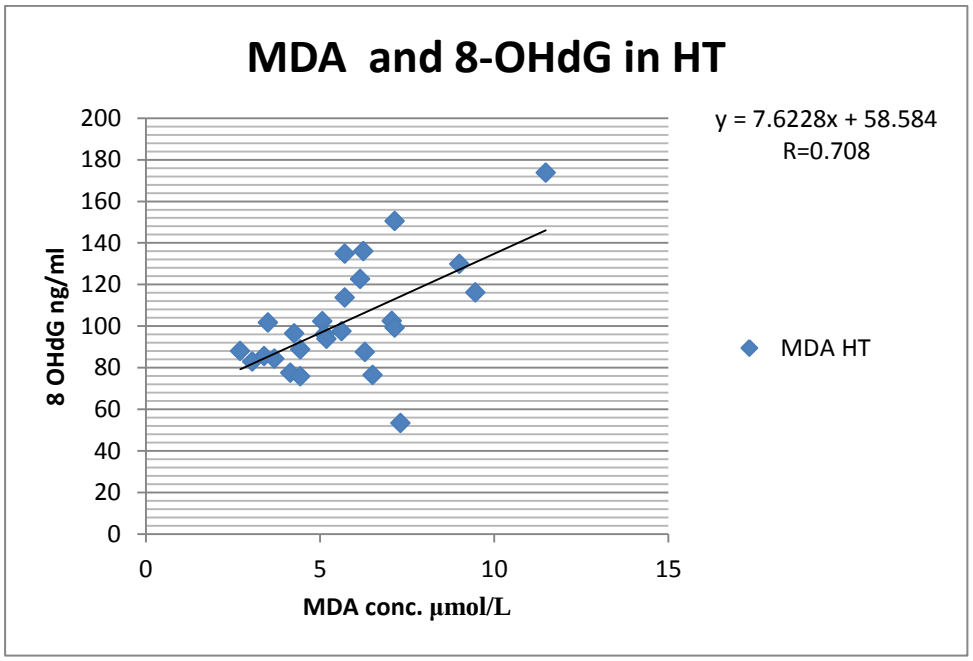


Figure (3-9): Correlation Directly Between MDA and 8-OHdG for Hypertensive Group.

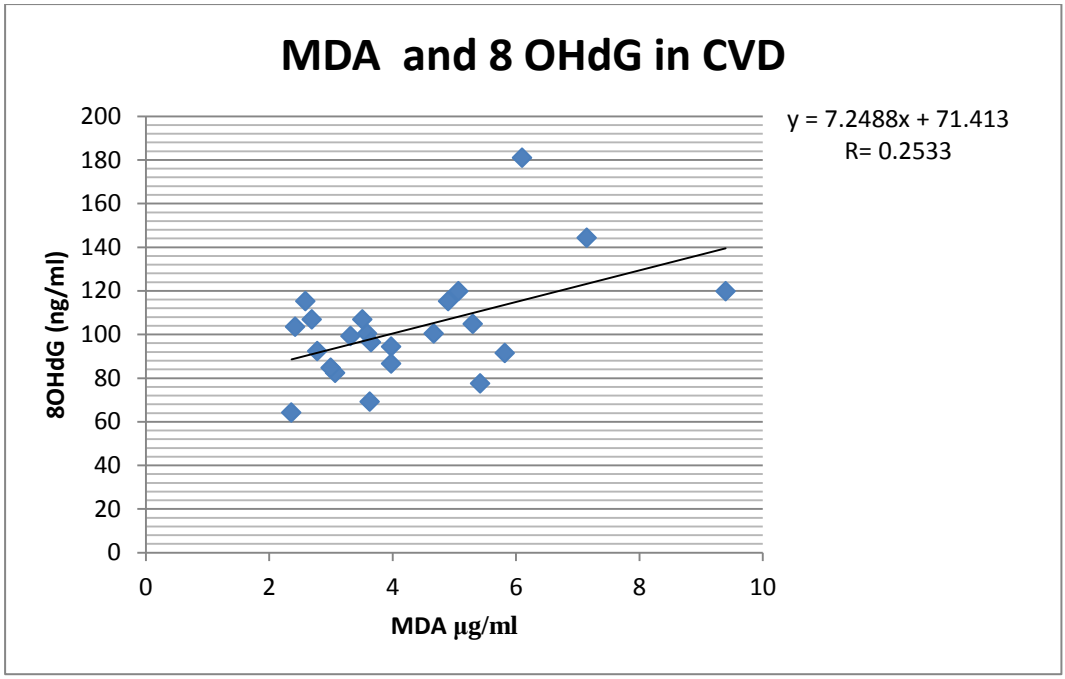


Figure (3-10): Correlation Directly Between MDA and 8-OHdG for CVD Group

The direct correlation between these two parameters shows increasing oxidative stress in all levels in the cell. The positive correlation between MDA and 8OHdG was also found by (Sakona *et al*) in healthy supplier by antioxidant nutrition[(139)].

3.3.3 Serum Albumin Levels:

The mean levels of total serum albumin showed a significant decrease in both patients group when compared to control group ($p<0.05$) table 3-7 and figure 3-11 show results :

Table (3-7): Values of Albumin Between Groups (mean ± SD):

Characteristic	Albumin g /L	Control group n =28	Comparison of Significant *p* value
Cardiovascular patient N=28	35.49 ± 1.47	44.18 ±2.20	<0.01
Hypertension group N=28	35.52 ± 1.84		<0.01

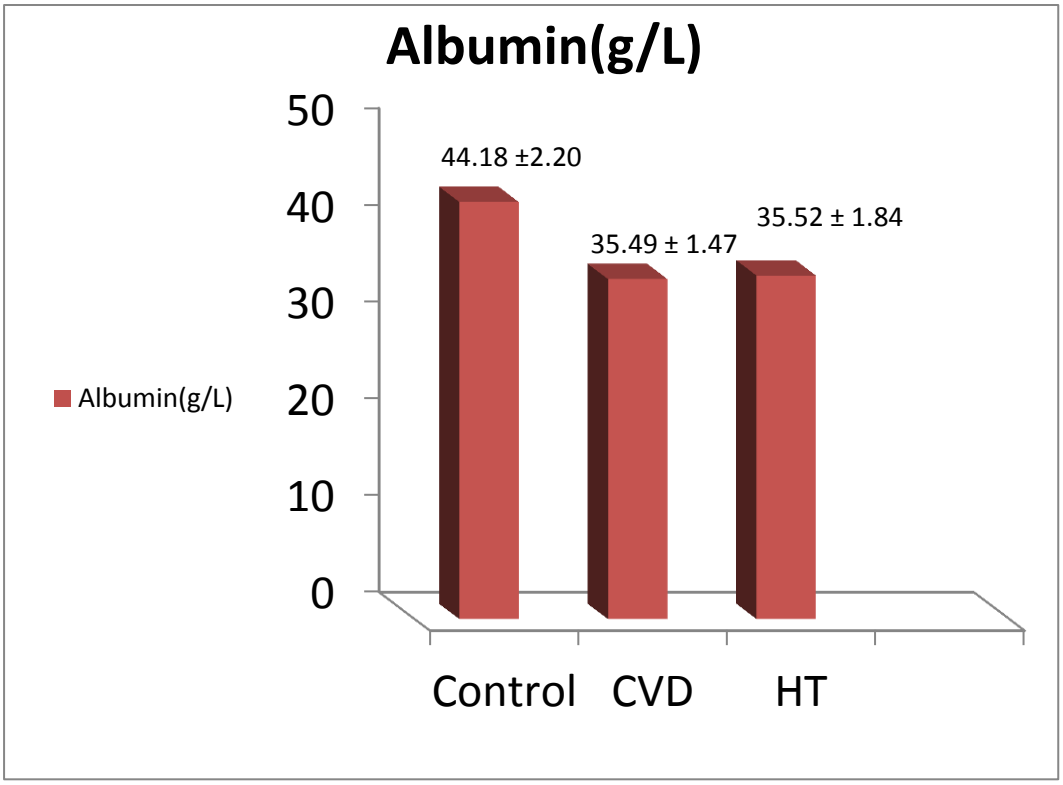


Figure (3-11): Values for Albumin [g/L] in Patients and Control Groups

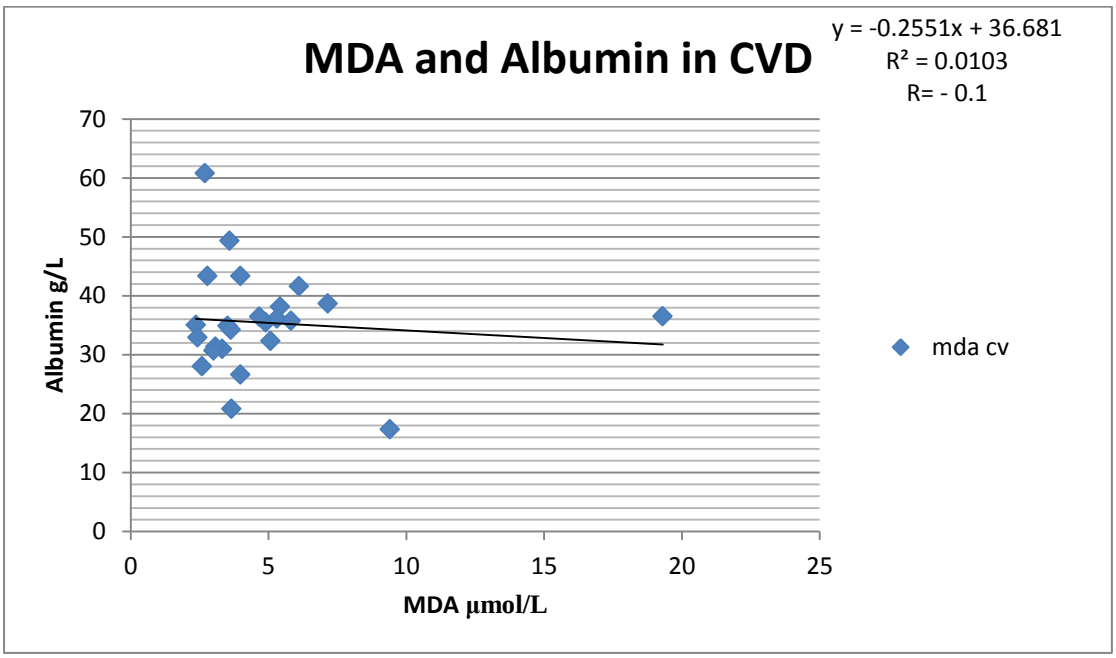
The results of the present study showed that the levels of albumin was significantly decrease (p<0.01) in diagnosed cardiovascular and hypertensive

patients which is agree with Liu *et al* in CVD and (Oda E) in hypertention[140,141]

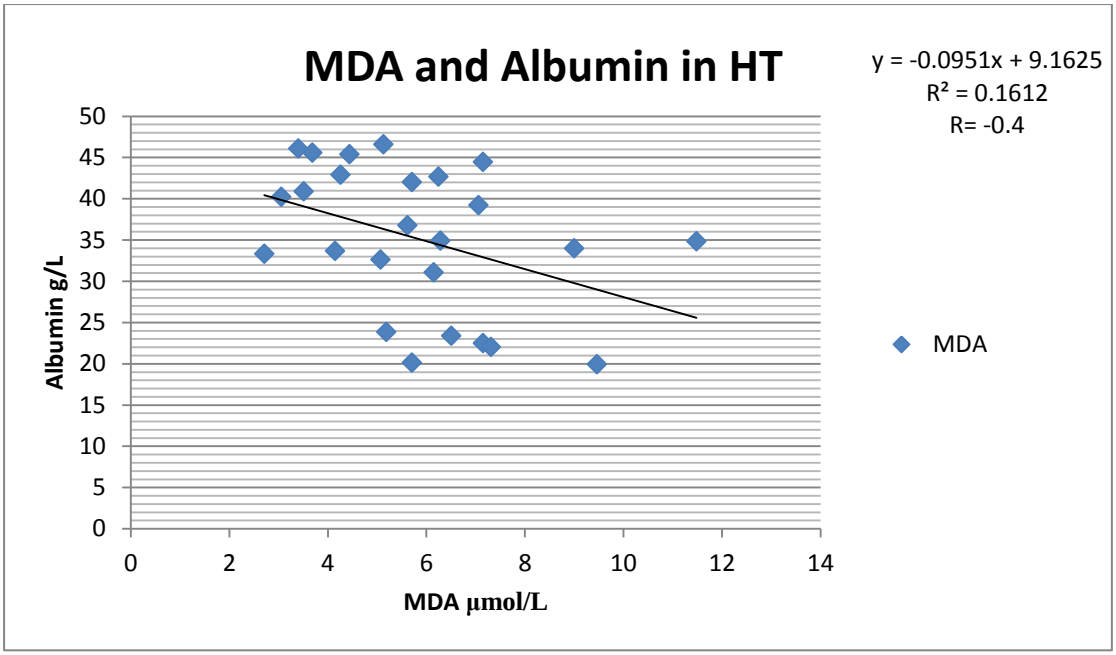
The decrease in albumin in patient is may be due to its function as antioxidant activity.

The atherosclerosis has been considered as an inflammatory and immunizing disease. It is widely accepted that inflammation represents a risk factor for atrial fibrillation and for prothrombotic conditions. Different molecules behave differently during an inflammatory phase; albumin synthesis decreases, while other inflammatory globulins rise[142]. In hypertension which is not inflammatory disease in basic, the decrease in albumin levels may be due to antioxidant activity more than its behavior as acute phase protein.

There was a negative correlation between albumin and MDA as shown in Figures (3-12),(3-13)



Figure(3-12): Correlation Between Albumin and MDA in CVD patients



Figure(3-13): Correlation Between Albumin and MDA in Hypertensive Patients

Weak negative correlation (r= -0.1) between MDA and albumin in cardiovascular disease patient and control group was observed in this study which agreed with (Al Fartosi k.*et al*) who found a negative correlation between MDA and Albumin [58].

3.3.4 Serum uric acid levels:

The mean levels of serum Uric acid showed a significant increase ($p<0.05$) in both patients groups in comparison to control group as shown in Table (3-8) and figure(3-13).

Table (3-8): Values of Uric Acid Between Groups (mean ± SD):

Group	Uric acid mg/dL	Control group n =28	Comparison of Significant *p* value
Cardiovascular patient n=28	4.89 ± 0.30	3.81±0.22	0.04
Hypertension group n=28	5.46 ± 0.36		0.021

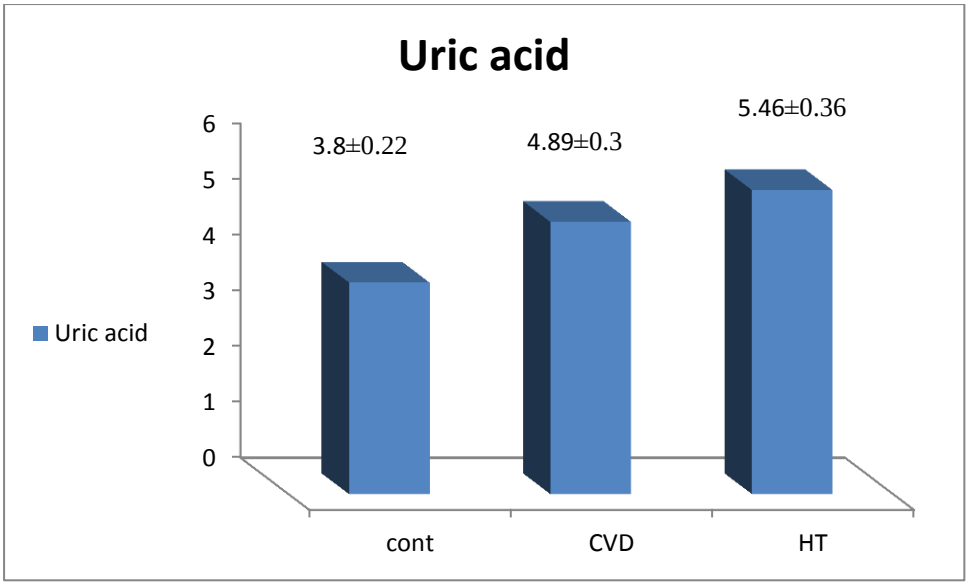


Figure (3-14): Values for Uric Acid [mg/dL] in Patients and Control Groups

The current study shows that there were elevated level of serum uric acid in patients groups when compared to control group and this results are agree with other studies, who's reporting an elevation of uric acid in serum of patients with CVD (Li Qin *et al),(* Cerezo C. *et al)*, (Takayama S)[143-145] and in hypertention disease (Ali Malek , Heinig M, Jin M.) [146-148]. Our findings reported the high results compared to control group which may be due to disease conditions that overwhelming antioxidant activity of uric acid.

Uric acid was considered an inert waste product that crystallizes at high concentrations to form renal stones and provoke gouty arthritis. Subsequently, uric acid was recognized to be a powerful antioxidant that scavenges singlet oxygen, oxygen radicals, and peroxynitrite and chelates transition metals, to reduce, for instance, iron ion–mediated ascorbic acid oxidation. Together, these antioxidant actions underlie the protective effects of uric acid action in cardiovascular diseases [146]

Several studies have demonstrated a strong relationship between serum uric acid levels and coronary heart disease (CHD) [143-145]. Some studies suggested that uric acid may be an independent related with for cardiovascular disease [149]. Increased serum uric acid was found to be associated with important risk factors for atherosclerosis like hypertension.[143,147]. Together,

the available information indicates that uric acid has complex chemical and biological effects and that its pro-oxidant or NO-reducing properties may explain the association among hyperuricemia, hypertension, the metabolic syndrome, and cardiovascular disease[150].

3.3.5 Serum Urea levels:

The mean levels of serum urea showed was higher significantly ($p<0.05$) in both patients groups compared to control group as shown in Table (3-9)

Table (3-9): Values of Urea in Studying Groups (mean±SD):

Group	Urea	Control group n =28	Comparison of Significant *p* value
Cardiovascular patient n=28	5.57 ± 1.76	4.27±1.90	0.04*
Hypertension group n=28	5.8 ± 2.6		0.021*

* significant difference with p<0.05

The present study showed high level of the urea in patients groups when compared to control group and this result agreed with (Jasim N.) [23] in CVD and with (Rakhee *et al)* in hypertension and disagree with (Pragna Patel. *et al).*[152-153)]

3.3.6 Serum Creatinine Level:

The mean levels of serum creatinine of both patients groups compared to control group are shown in table (3-10)

Table (3-10): Values of Creatinine in Studying Groups (mean±SD):

Group	Creatinine mg/L	Control group n =28	Comparison of Significant *p* value
Cardiovascular patient n=28	0.953 ± 0.16	0.701 ± 0.1	0.06
Hypertension group n=28	1.09 ± 0.16		0.21

The results showed non-significant in Creatinine levels in patients groups when compared to control group.

Urea is the major excretory product of protein metabolism. It is formed in the liver from amino groups ($-NH_2$) and free ammonia generated during protein catabolism[17]. In large cohorts of patients with acute and chronic heart disease, an elevated urea has been shown to be a strong predictor of morbidity and mortality[151].In the heart failure and hypertension the blood flow decrease so less blood is delivered to the kidney; consequently, less urea is filtered [93]. A reduction in renal blood flow leads to a decrease of glumular filtration, this is lead to a decrease distal tubular flow rate which lead to increase of urea reabsorption and decreased secretion which may be the reason for elevated serum urea concentration.[154].

There was no significant difference between patients ant control in creatinine concentration, Creatinine produced as a waste product of muscle creatine, about 1 % of the total muscle creatine pool is converted daily creatinine through the spontaneous, nonenzymatic loss of water. Since it is

released into the blood at a constant rate, and since its excretion is closely matched to glomerular filtration rate[153].

3.4 Molecular Study:

3.4.1. DNA Isolation

Blood samples from controls and patients were used for DNA isolation procedure at the same day blood sample were taken. Approximately, all samples yielded intact genomic DNA, as shown in
(Figure 3-15)

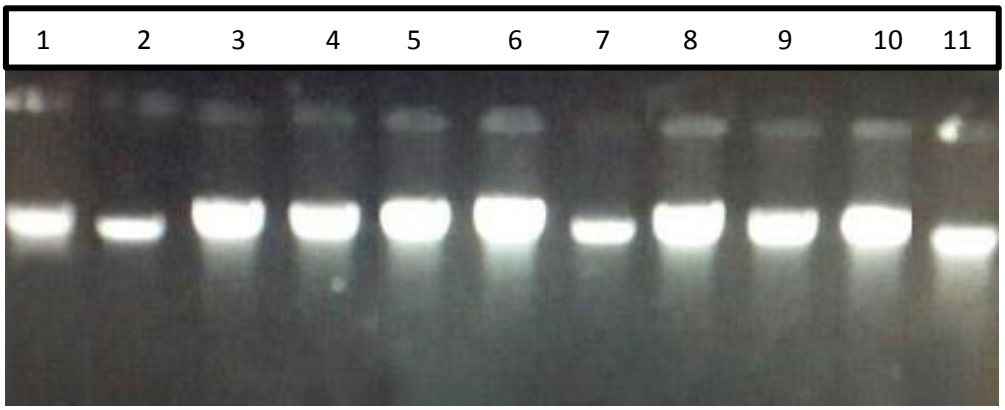


Figure (3-15): Genomic DNA Profile from Blood Sample of Subjects, Agarose gel (1%), 5 V/cm for 1 hr., Stained with Ethidium Bromide. Lane 1 placenta's DNA, lane 2-5 controls' DNA, lane 5-7 CVDs' group DNA, lane 8-11 HTs' group DNA.

The concentration and purity of DNA was determined by nano drop spectrophotometry and results are shown in table (3-11,3-12).
The 260/280 purity ratio of DNA purity with values for "pure" nucleic acid commonly in the range of (1.8-2.2)[155].

Table: (3-11) Concentrations of DNA of Study Groups (Mean± SD):

Group	DNA conc. ng/µL	DNA conc. of Control n=28	*p*-value
Cardiovascular patients n=28	88.05 ± 10.86	81.76 ±4.45	0.210
Hypertension n=28	89.27 ± 7.73		0.83

Table: (3-12) Purity of Extracted DNA of Study Groups (Mean± SD):

Characteristic	DNA purity	Control n=28	*p*-value
Cardiovascular patients n=28	1.80 ± 0.02	1.83 ± 0.02	0.240
Hypertension n=28	1.83 ± 0.07		0.852

3.4.2 Fenton Reaction in Vitro:

Active oxygen species such as superoxide anion radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) can damage almost all cell components, including DNA, membranes, and proteins [29]. Reduction of H_2O_2 by reduced transition metals results in the formation of $^{\bullet}OH$ and related oxidants via the Fenton reaction [29.33]:

$$Fe^{2+} + H_2O_2 + H^+ \longrightarrow Fe^{3+} + H_2O + {}^{\bullet}OH$$

The produced hydroxide radical has been already done in vitro test tube experiments under Fenton's reaction conditions. $^{\bullet}OH$ has damaging effect on

DNA, it may cause denaturation for double strands, mutation, as a means of inducing strand cleavage in DNA [156]

Placental DNA used as approximately unaffected DNA by oxidative stress. Using fenton reaction to show oxidative stress effect on DNA, damaged of placenta's and control's DNA was monitored by agarose gel electrophoresis and the result show this effect.

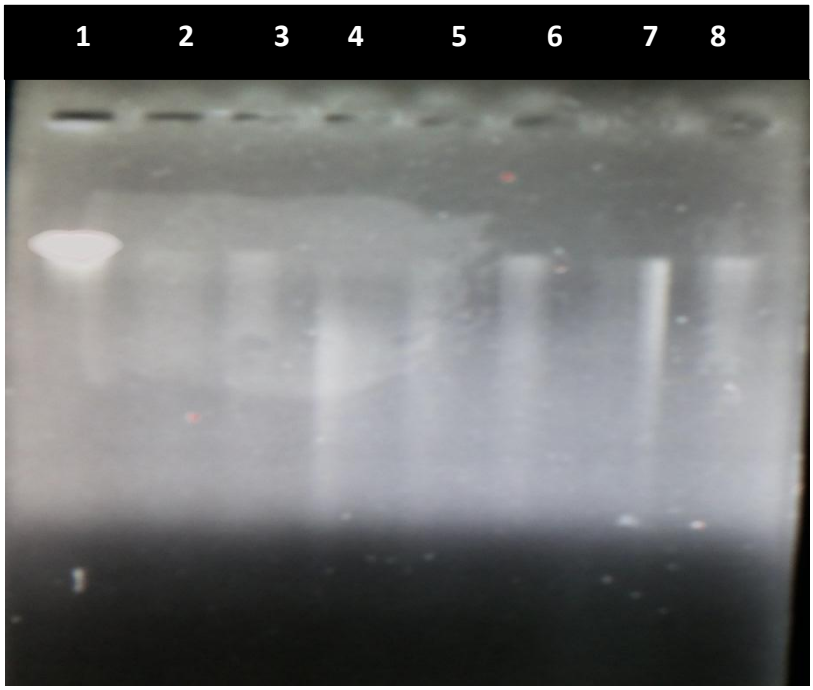


Figure (3-16): Fenton reaction on DNA, lane 1: placenta's DNA, lane 2-9 : DNA with Fenton's reaction 2- 14 min.

Placental DNA used as approximately unaffected DNA by oxidative stress using fenton reaction to show oxidative stress effect on DNA This result agree with (Plionical *et al*)[157] who suggested high concentration of H_2O_2 led to the degradation of all of major membrane proteins.

3.4.3 Evalution of Glutathion S transferase mu Gene (GSTM1 gene) deletion:

Glutathione S - transferase (GST) modulates the effects of various cytotoxic and genotoxic agents, GST ,along with other antioxidant enzymes,

such as Se-dependent GPx-1, provides the cell with protection against a range of harmful electrophiles produced during oxidative damage to membranes[158]. GSTs can work as endogenous antioxidants to protect cells from oxidative stress. The GSTs catalyze the conjugation of glutathione to a wide range of electrophiles and represent a protective mechanism against oxidative stress [73,158].

GSTM1 (glutathione S-transferase mu 1) is a protein-coding gene. *GSTM1* locus was amplified at exons 4 and 5 by polymerase chain reaction (PCR) technique as previously described to differentiate between the null polymorphism and the presence of one or more copies of the gene as in figure(1-9). And the exons that included in this study were 1 and 5 :

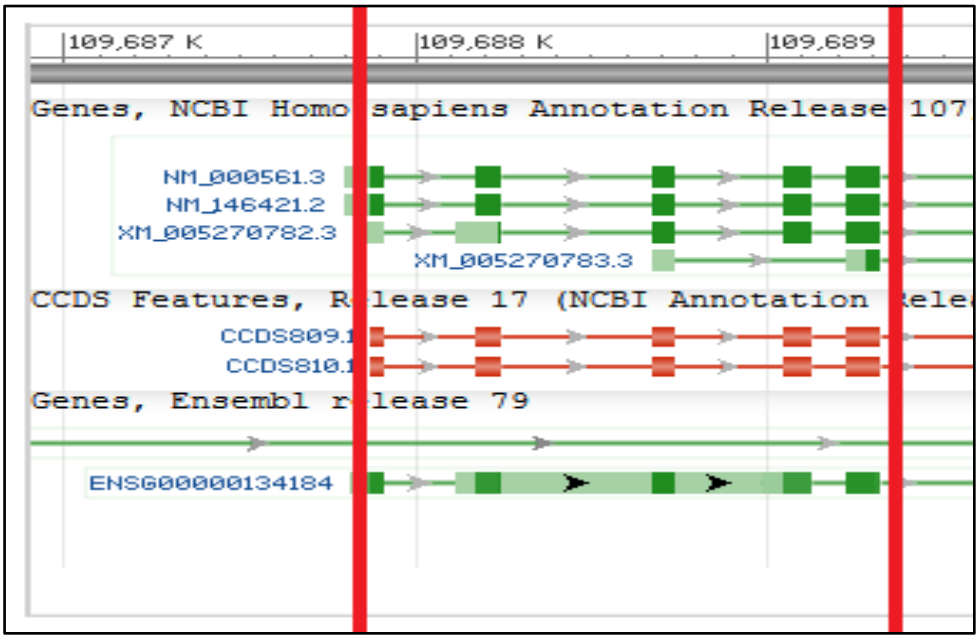


Figure (3-17): Studied Exons of GSTM1. [75]

The microdeletion of GSTM1 gene was detected by using the primers pairs the results were observed in several levels of deletion, some samples give complete deletion, these were pointed as (C), while the other show partial deletion , these were pointed as (P), both may lead to transcription of antioxidant enzyme. The sample that had been shown does have both deletion, pointed as (R). So according to study results there were three groups:

1- R group : presence of both primers which give positive results with both primers.

2- C group: which consider as a null genotype, give negative results with both pairs of primers.

3- P group: Give Positive results with one of side, and no with other side.

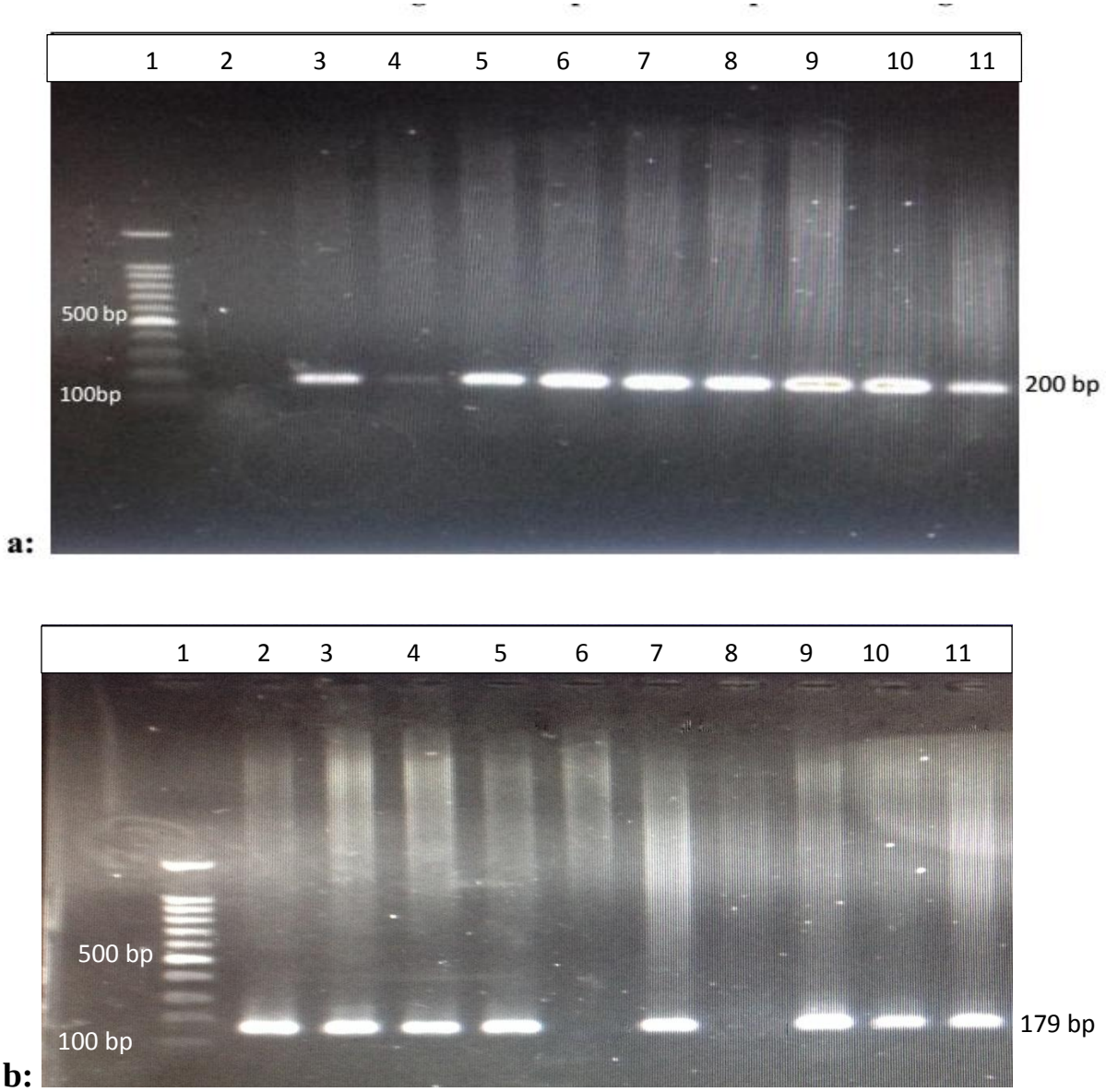


Figure (3-18) Gel electrophoresis for GSTM1 genotype. Ladder PCR products resolved by (2%) agarose gel electrophoresis (1h/70v). Lane1 DNA molecular weight marker,lane 2-6 controls' DNA, lane 6-11 samples DNA for patients.**a:** first two primer pair, **b:** second two primer pair

The distribution of GST genotype and alleles of the three study groups were given; the GSTM1 positive genotype occurs at higher frequencies in healthy controls (46.42%) and lower for Cardiovascular (33.33%) group and (37.04%) for HT group. Ramprasath *et al*.[159] who found the GSTT1 positive genotype occurs at higher frequencies in controls than in T2DM with cardiovascular and T2DM without cardiovascular patients. As for the GSTM1 positive genotype was occurs at higher frequencies in controls than in T2DM with CAD patients and T2DM without CAD patient. (Dadbinpour *et al*)[160] who observed that the absence or deletion of detoxification pathway of GSTT1 has no significant effect on the side effects of T2DM, but GSTM1–null had significant relationship with diabetes retinopathy, indicating the role of detoxification of this genes in this regards.

(Kolla K. *et al*.)[161] found that the frequency of both positive and null of GSTT1 and GSTM1 did not differ significantly between control and patients.

Table (3-13): Distribution of Microdeletion of GSTM1 Between Groups (n%)

Group	No. (%)			Chisquare-χ^2	P-value
	C (null)	R (complete)	P (partial)		
Control	2 (7.41%)	13 (46.42%)	13(48.15%)	9.327 **	0.0049
Cardiovascular	4 (14.28%)	9 (33.33%)	15(55.56%)	11.840 **	0.0012
Hyper tension	6 (21.42%)	10 (37.04%)	12 (44.44%)	9.072 **	0.0052
Chi-square-χ^2	5.139 *	4.924 *	5.081 *	---	---
P-value	0.0447	0.0529	0.0496	---	---
* significant difference with p<0.05, **highly significant with p<0.01					

3.4.3 GSTM1 Gene Variation and Biomarkers Influences

This study was designed to investigate the role of genetic polymorphisms of GSTM1 and biomarkers of oxidative stress in cardiovascular and hypertension patients and healthy controls.

The results have been showed that absence of GSTM1 gene that has significant influence for MDA concentration for all cases and controls. Furthermore, it has association with albumin concentration just for control group while for patient group it has influence in 8OHdG. Partial deletion has unique effect with cardio vesicular patient

Table (3-14): Effect of Gene in Study Parameters in Cardiovascular groups (Mean ± SD)

Characteristic	C	R	P	P value
MDA μmol/L	6.20 ±1.71	4.00 ± 0.45	5.35 ±1.38	0.025*
Albumin g/L	32.39 ±1.3	38.77 ±3.1	41.15±4.18	0.051
8OHdG ng/mL	106.3±33.3	97.5±17.3	93.90±28.57	0.042*
Uric acid .mg/dL	4.08 ± 0.25	5.49 ± 0.61	4.70 ±0.38	0.319
Urea mmol/L	7.14 ± 2.34	5.54 ± 0.48	5.27 ±0.32	0.251
Creatinine mg/dL	1.89 ± 0.94	0.955 ± 0.18	0.973±0.17	0.163

* significant difference with $p<0.05$.

The microdeletion of GSTM1 in hypertension group and its correlation with MDA is shown in table below:

Table(3-15): Effect of Gene in Study Parameters in Hypertension (Mean ±SD)

Characteristic	C	R	P	*P* value
MDA μmol/L	6.72 ± 1.65	4.15 ± 0.22	4.72 ± 0.15	0.0023**
Albumin g/L	35.27 ±4.09	41.78 ± 3.38	34.56 ±2.73	0.05*
8OHdG ng/mL	110.45±23.12	90.31±22.8	105.48±29.23	0.043*
Uric acid mg/dL	5.91 ±0.81	5.88 ± 0.65	4.94 ± 0.51	0.446
Urea mmol/L	7.32 ± 1.32	5.29 ± 0.90	5.75 ± 0.72	0.402
Creatinine mg/dL	0.655 ±0.08	1.07 ± 0.51	1.02 ± 0.14	0.616

* significant difference with p<0.05, **highly significant with p<0.01

The significant increase in MDA levels in GSTM1 null gene as also found by (Abhishek *et al*) who found this correlation in exposure to pollutants [162].

Albumin was decrease significantly in null genotype of GSTM1 that were lacking efficiency in antioxidant esponses due to the combination of the *GSTM1* deletion[159].

This agree with (Nochelas *et al*) [75] who found increase in ROS effects in null of this gene.

(Al- Ubeidy M.) found correlation between deletion of this gene and risk of cardiovascular disease[163]

The concentration of albumin is lower significantly in null and positive genotype in all groups and in partial deletion there no significant. That's may be means, in the form of gene deletions, yielding no transcription or translation of the respective proteins , Decreased functionality of detoxifying and antioxidant enzymes may pose a greater risk of toxic insult potentially

caused by lowered control of reactive oxygen species leading to chronic oxidative stress. the greatly reduced activity of detoxifying enzymes conferred by genetic polymorphisms (hereafter referred to as "null polymorphism") increases oxidative stress"[75, 78, 159].

There was no difference significantly between R and C and P in all groups in concentration of uric acid and urea and createnine concentration .

Conclusion

1. Oxidative stress in its markers and parameters is the main chemical manifestation of hypertension and cardiovascular diseases in the patients of underlying study. This conclusion was obvious in high sgnficant difference between serum malondialdehyde, 8-OHdG and albumin in patients of both hypertension and cardiovascular diseases. Uric acid, urea, creatinin field in achieve this results.
2. The association between items of lipid profile and malondialdehyde leads to concludes that high serum levels of lipid is very risky due to high probability of its oxidation which is exacerbate the diseases of underlying study. Also the association between malondialdehyde and 8-OHdG leads to conclude the deep effect of cells from membrane to the nucleus by oxidative species
3. The association between deletion of glutathione-S- transferase gene (in genome) with increasing serum malondialdehyde leading to believe that oxidation damage have adverse effect on defense system in addition to biomolecules.

Recommendation and Future Work

1- Estimation of glutathione -S- transferase enzyme in parallel with Glutathion -S- transferase mu gene (GSTM1 gene), so we can get full picture on the oxidative stress effect on the defense body system with regard to this gene.

2- Design the intervention study involve supplementing patient volunteer with natural antioxidant to follow up the decreasing oxidative stress in response to these antioxidant.

3- Detect the values of body Iron or Cupper as oxidant maker.

4- Study the effect of drugs supplements for these disease on oxidative stress

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الخلاصة

ارتفاع الضغط وامراض القلب الوعائية تتشارك في ان احد الاسباب الرئيسية المؤدية للاصابة بها هو تزايد الجهد التاكسدي والذي بدوره يزيد من شدة المرض ويفاقم اعراضه. الجزيئات الفعالة الناتجة من الجهد التاكسدي بالاضافة الى انها تسبب تلفا بالانسجة باكسدة الجزيئات الحيوية مثل ال شريط النووي والدهون والبروتينات والسكريات، فهي تؤدي الى توليد وسائط ذات تاثيرات التهابية كامنة. الهدف من هذه الدراسة هو لبحث بعض مؤشرات الجهد التاكسدي في مرضى ارتفاع ضغط الدم وامراض القلب الوعائية. بالاضافة الى بعض المؤشرات الكيموحيوية المتعلقة بهذه الامراض.

هذه الدراسة استلزمت 84 امرأة تتراوح اعمارهن من (30-65) قُسمتْ بشكل متساوي الى ثلاث مجاميع، الاولى والثانية هي لمرضى ارتفاع ضغط الدم ومراض القلب الوعائية على التوالي من مستشفى ابن النفيس، والثالثة فلنساء سليمات مثلتْ مجموعة سيطرة. لكل عينة في المجموعات الثلاث، تم قياس هذه المؤشرات: المالون داي الديهايد، 8-هيدروكسي ديوكسي كوانوسين ، الالبومين، حمض اليوريك، اليوريا، الكرياتنين. ايضا اجريت دراسة ترحيل كهربائي على الشريط النووي للمرضى والاصحاء مع الشريط النووي المشيمي لبحث تأثير الجهد التأكسدي بعد تعريضه لتفاعل فنتون مختبريا، ودراسة وجود جين الكلوتاثيون اس ترانسفيريز بواسطة تفاعل البوليميريز التتابعي PCR.

قورنت النتائج بمجموعة السيطرة فمستويات الدهون في الدم سجلت ارتفاعا معنويا ($p<0.01$) في كلا المرضين مقارنة بمجموعة السيطرة. وكانت ارتفاع معنوي ($p<0.01$) في مستويات المالون داي الديهايد وال 8- هايدروكس ديوكسي كوانوسين، في كلا ارتفاع الضغط وامراض القلب الوعائية. بينما سجل الالبومين انخفاضا معنويا ($p<0.01$) في مستوياته. حمض اليورك وبشكل غير متوقع باعتباره مضادا للأكسدة سجل ارتفاعا ($p<0.05$) في مستوياته مقارنة بمجموعة السيطرة. دراسة الترحيل الكهربائي اظهرتْ فرقا معنويا ($p<0.01$) في كل من المرضين ومجموعة السيطرة في وجود جين الكلوتاثايون اس ترانسفيريز ، وان هناك علاقة طردية بين غياب هذا الجين وارتفاع مستويات المالون داي الدهايد و8 هيدروكسي ديوكسي كوانوسين وعكسية مع الالبومين .

الارتفاع في مستويات مؤشرات الجهد التاكسدي ربما يعزى الى التلف التاكسدي في الانسجة مما يسبب هذه الامراض الالتهابية. استنتج من هذه الدراسة بان هناك علاقة ايجابية بين الاكسدة الناتجة من هذه الامراض وتفاقمها وان التلف الناتج من الاكسدة يشمل النظام الدفاعي والجزيئات الحيوية . وتقترح الحاجة لزيادة اخذ مضادات الاكسدة كاجراء وقائي من هذه الامراض.

Appendix

(Subjects Questioners)

التاريخ:			
تسلسل المريضة:			
رقم المريضة:			
الأسم:			
العمر:		سن اليأس؟:	
الوزن:		الطول:	
نوع المرض:			
مدة المرض:			
هل هناك أمراض ثانية؟			
هل المرض وراثي؟			
العلاج المتعاطي			
السكن:			
العمل:			
تدخين:			

جمـــــــــــــــــهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعـــــــــــــــة النهريـــــــن
كليـــــــــــــــة العـــــــلوم
قـــــــــــــــسم الكيميـــــــاء

دراسة مستويات الاجهاد التأكسدي عند مريضات الأوعية القلبية وضغط الدم

رسالة
مقدمة الى كلية العلوم- جامعة النهرين
كجزء من متطلبات نيل درجة الماجستير في علوم الكيمياء

من قبل
ياسمين محي الدين حسين
(بكالوريوس 2012)

باشراف
الاستاذ المساعد الدكتور علاء حسين جواد

جمادى الآخر
1436 هـ

نيسان
2015 م

Chapter One

Introduction
&
Literature Review

Chapter Two

Materials & Methods

Chapter Three

Results & Discussion

References

Conclusions & Recommendation